14 Extracellular Enzymes Associated with Microbial Flocs from Activated Sludge of Wastewater Treatment Systems

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CONTENTS

14.1 Introduction 295
14.2 Microbial Flocs in Wastewater Treatment 296
14.3 Extracellular Enzymes 297
14.3.1 Forms of Phosphorus in Wastewater 297
14.3.2 Phosphatase Enzymes 298
14.4 Quantifying Extracellular Enzyme Activity Associated with Microbial Flocs 298
14.5 Measurement of Phosphatase Activity in Activated Sludge using Fluorogenic Substrates 300
14.6 Localizing Extracellular Enzyme Activity Associated with Microbial Flocs 303
14.7 Identification of Populations of Cells Responsible for Extracellular PO_4ase Activities within Floc-Associated Microbial Communities 307
14.8 Conclusions 311
Acknowledgments 312
References 312

14.1 INTRODUCTION

The tendency to aggregate is a common trait among microbial cells in contrived experimental laboratory environments, controlled industrial processes, and in nature.[1–3] When aggregation takes place on a surface, it often leads to the formation of biofilms,[4,5] while aggregation of cells in aqueous suspensions results in the formation of flocs.[6] Flocs formed in the water column of the ocean have been termed "marine

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snow."[7] Regardless of what form the aggregation process takes, cells engaged in this behavior become physically associated with each other through extracellular polymeric substances (EPSs) secreted by at least a portion of the aggregated microbial community. Some of the cells secrete EPS that contributes to the structure of the floc or biofilm, which promotes the formation of close, stable, physical associations between cells of the same and different populations.[8] The physical associations lead to cooperation among cells of the populations present and the formation of consortia.[9,10] EPSs also form matrices that function as diffusion barriers between the cells and the external environment,[11,12] which in some instances, protect the cells from insults of the surrounding environment.[13] Extracellular enzymes secreted by floc-encapsulated microorganisms represent another class of EPS that participate in the transformation of toxic chemical species in the environment to nontoxic products,[14] as well as the transformation of nutritionally valuable chemical species in the environment for uptake and utilization by members of the floc-associated microbial community.[15]

In this chapter, we will describe the exoenzymes involved in the degradation of polymeric organic compounds in the activated sludge process of wastewater treatment. Specifically we will describe the distribution of phosphatase (PO_4ase) exoenzymes within flocs produced during the activated sludge process, and the floc-associated microbial populations that synthesize and secrete this important class of enzymes.

14.2 MICROBIAL FLOCS IN WASTEWATER TREATMENT

Floc formation and associated chemical and biological reactions are an important part of many industrial processes.[16–18] Floc formation provides a means of separating solids from the liquid phase in process streams. In wastewater treatment that utilizes the activated sludge process, the quality of the effluent is highly dependent on the efficiency of the solid–liquid separation processes. Flocs play a key role in this regard, and an understanding of floc properties and behavior has led to significant advances in biological C, N, and P removal and in the sludge dewatering process. Floc properties control the efficiency of phase separation.

Floc properties are controlled by the nature of the wastewater. The chemistry of the wastewater controls the types of microorganisms that grow in the system as well as the quality and quantity of EPS elaborated by the microbial community. EPS composition and form depend on process conditions. EPS in activated sludge can be found in two forms: directly associated with microbial cell biomass or physically separated from the biomass as free colloids or slimes.[19,20] Floc properties and behavior directly reflect EPS properties. Bulking and foaming reduce the efficiency of the separation process. Bulking occurs when aggregates do not form dense, compact flocs — a direct consequence of EPS production and composition by the microorganisms in the system. Recently, Sponza[21] provided evidence that EPS composition depends more on waste stream composition than on process operating conditions. Thus, an understanding of microbial EPS production offers new opportunities to control the flocculation process and overall efficiency of wastewater treatment. This chapter will focus on the fraction of EPS comprised of extracellular enzymes.

14.3 EXTRACELLULAR ENZYMES

Hydrolysis of polymeric substances is the first and rate-limiting step in the process of wastewater treatment.[22,23] Due to their large size, organic polymers such as proteins, polysaccharides, and lipids must be hydrolyzed to their subunit molecules before microorganisms can oxidize or recycle them back to inorganic C, N, S, and P. The microbial enzymes responsible for these extracellular hydrolysis reactions are referred to as extracellular enzymes or exoenzymes. Microbial metabolism of complex organic matter begins with reactions catalyzed by extracellular enzymes. Consequently, extracellular enzymes are essential components of many biochemical pathways utilized by microorganisms for growth. Microbial polysaccharases, esterases, lipases, and proteases are examples of extracellular enzymes that play an important role in the biodegradation of polysaccharides, lipids, and proteins, respectively.[15,24] Exoenzymes also liberate inorganic phosphate (P_i), N, and S from organic molecules. Phosphatases (PO_4ases) are a class of enzyme that liberates P_i from phosphate-containing organic compounds. PO_4ases occur as intracellular enzymes where they control intracellular P_i concentrations needed for energy yielding phosphorylation reactions. They also occur in the cell envelope and as extracellular enzymes where they scavenge P_i from polymeric phosphorylated organic compounds to meet their inorganic phosphate requirements.

14.3.1 FORMS OF PHOSPHORUS IN WASTEWATER

Phosphorus removal from wastewater has become an important part of the overall treatment process. Enhanced biological P removal can achieve 90% to 95% reduction.[25] Some of the steps in the biochemical pathway of enhanced P removal are known, but others remain to be elucidated,[26] and the microorganisms responsible for some of the steps are still not known.[27] Phosphorus entering the activated sludge process exists in both organic and inorganic forms (Table 14.1). These data suggest that one-third to one-half of the total P in the system exists as detrital organic-P. While much attention has been directed toward the microbial populations that accumulate poly-P_i, little attention has been directed to those populations in the activated sludge

TABLE 14.1
Forms of Phosphorus in Activated Sludge

Form	Percent of total *P*
Nucleotide-P, lipid-P, nucleic acid-P, protein-P	31[28]
Trichloroacetic acid-insoluble-P	13
poly-P (inorganic)[29]	
RNA and protein (organic)[30]	
Inorganic-P	
PO_4^{3-} (P_i)	14[28]
poly-phosphate (poly-P_i)	32–44[28]

that process detrital organic-P or those involved in P regeneration (production of P_i from organic-P outside the cell). Little is known, for example, of the processes that depolymerize high molecular weight detrital organic-P compounds in wastewater or those that hydrolyze the phosphomonoesters to the organic subunits and P_i. These processes, however, are likely to influence overall P removal from wastewater streams.

14.3.2 Phosphatase Enzymes

PO_4ase enzymes hydrolyze the P–O bond of phosphomonoesters. PO_4ases are subdivided into alkaline and acid PO_4ases, based on the pH at which optimum activity occurs. Alkaline PO_4 function in the neutral and basic pH range, are induced at low external P_i concentrations, are localized at or near the cell surface, and hydrolyze phosphomonoester-containing organic compounds to provide cells with an alternative source in P_i. Alkaline PO_4ases may also be induced under P_i-sufficient but carbon-limited conditions to meet metabolic requirements of bacterial cells. Acid PO_4ases display maximum activity over the pH range of 4 to 6, are typically intracellular, and are not repressed by P_i. Alkaline PO_4ase has been reported to hydrolyze inorganic poly-P_i, a P-containing storage product that accumulates in a variety of bacteria found in activated sludge.[26,28,31–33] Alkaline PO_4ases have been implicated in P_i release from intact bacterial cells containing poly-P storage products in anaerobic lake sediments.[34] Alkaline PO_4ases may participate in the degradation of cell-surface-associated poly-P, which represents a more transient but significant portion of the total poly-P_i accumulated by cells of *Acinetobacter Iwoffi* JW11.[35] Since poly-P_i formation and degradation exerts significant control over enhanced P removal from wastewater,[25,26] a better understanding of the role of extracellular bacterial PO_4ases in poly-P_i degradation and detrital organic-P regeneration should lead to better control over and more efficient P removal in the activated sludge process of wastewater treatment systems.

14.4 QUANTIFYING EXTRACELLULAR ENZYME ACTIVITY ASSOCIATED WITH MICROBIAL FLOCS

Goel et al.[36] used *p*-nitrophenolphosphate as the surrogate substrate for quantifying alkaline and acid PO_4ase activity, *p*-nitrophenol α-D-glucopyranoside as the surrogate substrate for α-glucosidase activity, and azocasein as the model substrate for protease activity in activated sludge. After an appropriate incubation period to allow enzymatic release of P_i, glucose, or peptide from the substrate, the activated sludge samples were centrifuged and the absorbance of the supernatant fraction was measured spectrophotometrically at 410 nm for *p*-nitrophenol (alkaline and acid PO_4ases, α-glucosidase), and at 440 nm, for trichloroacetic acid (TCA) soluble peptides (protease activity). Whiteley et al.[37] used the same substrates to measure exoprotease and extracellular alkaline PO_4ase activity in samples from two large-scale stirred tank laboratory reactors operated in series: the first seeded with a mixed culture of methanogenic bacteria from a standing digester at a sewage works, and the second seeded with a mixed culture of sulfate-reducing bacteria.

Cadoret et al.[12] assayed L-Leu-aminopeptidase, α-glucosidase, protease, and α-amylase activities in whole and dispersed activated sludges, as well as activities associated specifically with the floc EPS fraction. Enzyme activities were based on the rate of release of a chromogenic enzyme substrate following hydrolysis of synthetic substrates over an appropriate incubation period. L-Leu-*p*-nitroanilide, *p*-nitrophenyl-α-D-glucopyranoside, azocasein, and amylose azure were used as substrates for L-Leu-aminopeptidase, α-glucosidase, protease, and α-amylase, respectively. Nitroanaline and *p*-nitrophenol hydrolysis were quantified spectrophotometrically at 410 nm. Azocasein hydrolysis was determined by measuring the amount of cold TCA soluble material produced at 340 nm using a spectrophotometer. α-Amylase hydrolysis was determined by the amount of brilliant blue recovered in the cold TCA soluble material following release from amylose azure and treatment with cold TCA.

Pletschke et al.[38] monitored adenosine triphosphate sulfurylase (ATPS) in methanogenic and sulfidogenic stirred tank reactors operated in series using primary sludge from an anaerobic digester at a sewage works. ATPS catalyzes the first step in dissimilatory sulfate reduction: the formation of adenosine 5′-phosphosulfate (APS) and inorganic pyrophosphate (PP_i). ATPS activity was assayed by measuring ATP produced by reaction of APS with PP_i. The rate of ATP production was measured in the coupled spectrophotometric assay involving phosphorylation of glucose and the subsequent oxidation of glucose-6-phosphate to D-6-P-glucono-δ-lactone coupled to the reduction of NADP.

Boczar et al.[15] assayed esterase activity in the supernatant (cell free) fraction of filtered activated sludge. Esterase activity was determined by measuring the release of fluorescein from fluorescein diesters of acetate, butyrate, caproate, and caprylate, or the release of *p*-nitrophenol or α-napthol from *p*-nitrophenol or α-napthol esters of acetate, butyrate, capriate, caprylate caprate, laurate, myristate, palmitate, and stearate. The amount of *p*-nitrophenol and α-napthol produced was determined spectrophotometrically at 400 nm, and the amount of fluorescein produced was determined spectrophotometrically at 495 nm. Frolund et al.[39] monitored sludge bulk extracellular esterase activity using fluorescein diacetate as an enzyme substrate. A spectrofluorimeter was used to quantify the fluorescence resulting from the formation of fluorescein.

Whiteley et al.[40] monitored lipase activity in methanogenic and sulfidogenic stirred tank reactors operated in series using primary sludge from an anaerobic digester at a sewage works. The assay measured enzymatic cleavage of glycerol from the lipid triacetin. Sludge samples from the reactors were sonicated to release enzymes from the particulate fraction and then centrifuged to separate the enzymes from the particulate material. Triacetin was added to the supernatant fraction, and following an incubation period, the enzyme reaction was terminated by addition of sulfuric acid and sodium periodate. Following addition of $NaHSO_3$ and chromotropic acid reagent and heating, the reaction mixture was cooled and the glycerol released was quantified spectrophotometrically at 570 nm, Since particulate matter interfered with the lipase assay, the sample had to be sonicated and the particulate fraction removed by centrifugation prior to assaying for enzyme activity. As a result, it was not possible to determine whether the lipases were free in solution, bound to the floc matrix, or associated with the cells.

14.5 MEASUREMENT OF PHOSPHATASE ACTIVITY IN ACTIVATED SLUDGE USING FLUOROGENIC SUBSTRATES

Van Ommen Kloeke and Geesey[41] measured PO4ase activity in activated sludge samples using the soluble, artificial, fluorogenic enzyme substrate methylumbelliferyl phosphate (MUFP) (Figure 14.1). MUFP has been used to monitor phosphomonoesterase activity in lakes.[42,43] MUF substrates are likely hydrolyzed by PO4ases either at or near the surface of intact cells or by PO4ases released into the environment by cells.[31] After an appropriate incubation period, the reaction mixture was centrifuged to remove interfering particulate material and the supernatant fraction assayed for fluorescence intensity using a spectrofluorometer at an excitation wavelength of 360 nm and emission wavelength at 430 nm. This substrate yielded quantitative PO4ase activity in various activated sludge fractions at relatively high sensitivity. Using freshly collected activated sludge samples from the wastewater treatment plant in Bozeman, MT, which contained 2.9 ± 0.3 g l^{-1} total solids and 7 to 14 mg l^{-1} P_i (Table 14.2), it was determined that the particulate fraction (operationally defined as that which sedimented by centrifugation at $27,000 \times g$ for 15 min) exhibited linear PO4ase activity at pH 7.0 of 7.9 μmol l^{-1} h^{-1}, or 91% of the total activity in "as collected" samples at MUFP final concentrations of 100 μM over a 3 to 4-h period (Figure 14.2). At a final MUFP concentration of 500 μM, the PO4ase activity was approximately twice that observed at the 100 μM final MUFP concentration, and the activity associated with the particulate fraction was slightly greater than that associated with the "as collected" sample. A Lineweaver Burke plot of reaction rates over a range of added

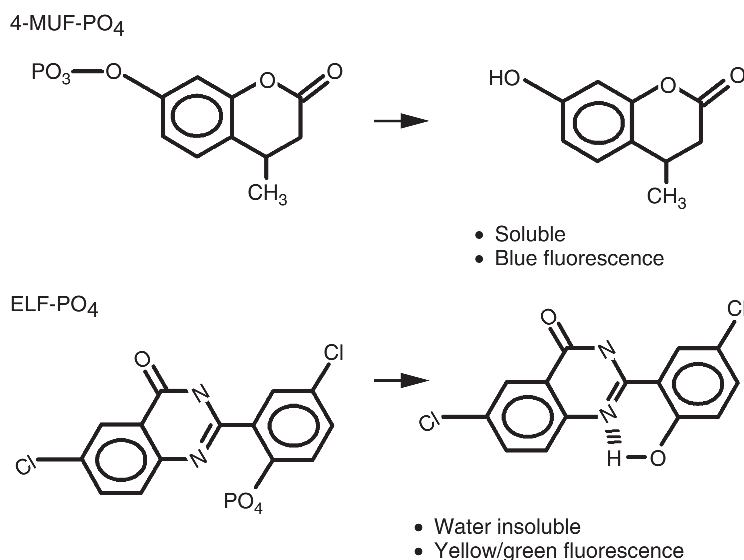

FIGURE 14.1 MUF-PO_4 and ELF-PO_4 fluorogenic substrates for measuring activity and localizing extracellular PO4ase

TABLE 14.2
Chemical Constituents of Activated Sludge Solids and Liquid Phases

Sample	Total Solids $g\,l^{-1}$	Volatile Solids ($g\,l^{-1}$)	Protein ($mg\,l^{-1}$)	Humics ($mg\,l^{-1}$)	Carbohydrate ($mg\,l^{-1}$)	Uronic Acids ($mg\,l^{-1}$)	pH
Solids	2.9 ± 0.03	1.9 ± 0.1	871 ± 21	324 ± 9	339 ± 2	11.7 ± 2	ND[a]
Liquid	ND[a]	ND[a]	2.0 ± 1.0	BD[b]	1.0 ± 1.0	BD[b]	7.2

[a] Not determined.
[b] Below detection.

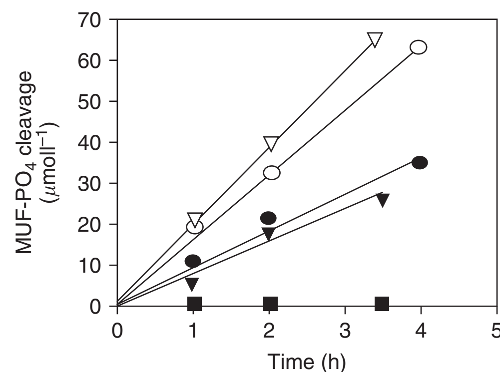

FIGURE 14.2 PO_4ase activity in activated sludge fractions. Samples assayed at 500 μM MUF-PO_4: (○) "as collected" activated sludge, 15.2 $\mu mol\,l^{-1}\,h^{-1}$; (▽) resuspended floc, 18.4 $\mu mol\,l^{-1}\,h^{-1}$; (■) activated sludge supernatant, 0.1 $\mu mol\,l^{-1}\,h^{-1}$; and samples assayed at 100 μM MUF-PO_4: (●)"as collected" activated sludge, 8.7 $\mu mol\,l^{-1}\,h^{-1}$; (▼) resuspended floc, 7.9 $\mu mol\,l^{-1}\,h^{-1}$

MUFP concentrations yielded a V_{max} of 2×10^4 $\mu mol\,l^{-1}\,h^{-1}$ and Km of 133 μM with respect to MUFP (Figure 14.3). Conversion of MUFP to MUF was not the result of abiotic reactions since autoclave sterilization of the activated sludge samples before addition of the MUFP yielded no fluorescent product.

Additional evidence for the enzymatic release of P_i from detrital organic-P in activated sludge was obtained by following the concentration of P_i in samples of activated sludge under different conditions. Autoclave-sterilized activated sludge showed no change in P_i concentration over a 24-h period (Figure 14.4). Treatment of activated sludge with benzene to arrest growth of microorganisms present, but to allow PO_4ases present to remain active, resulted in an increase in P_i from 13.6 to 18.8 $\mu g\,ml^{-1}$ over a 24-h period (Figure 14.4), suggesting the production of new P_i from polyphosphate (poly-P) or organic-P sources present in the activated sludge. That benzene arrested the growth of the microorganisms in the activated sludge was demonstrated by recovering the same number of colony-forming units (cfus) at the end of the

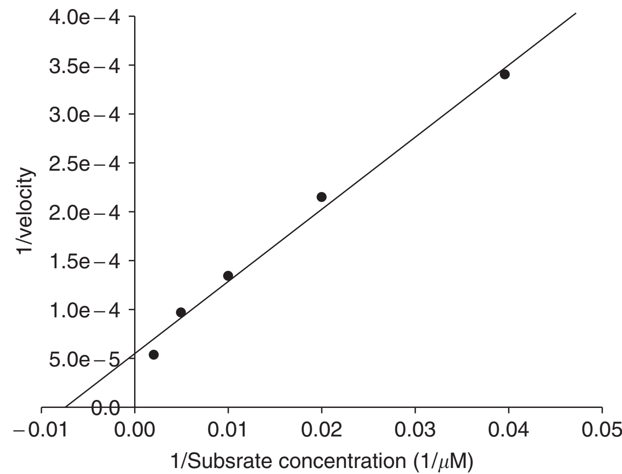

FIGURE 14.3 Lineweaver Burke plot of PO_4ase activity in "as collected" activated sludge using MUF-PO_4 as substrate

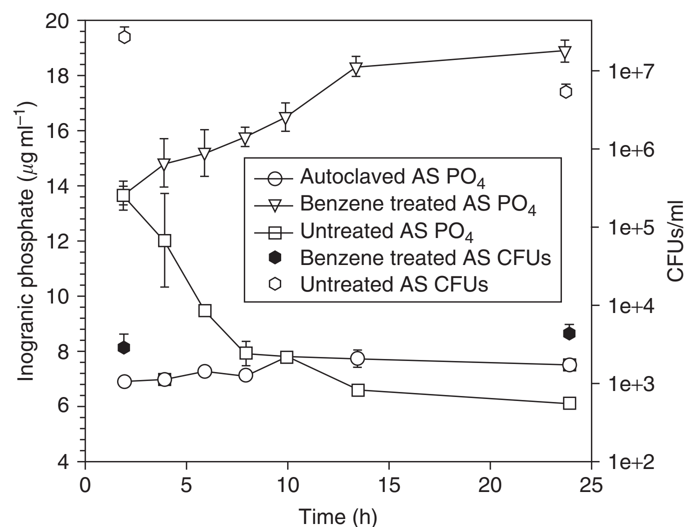

FIGURE 14.4 Effect of extracellular PO_4ase activity on the release of P_i from organic-P

24-h incubation period as recovered at the beginning of the incubation (Figure 14.4). In contrast, untreated samples showed a decrease in P_i concentration from 13.6 to 6.0 μg ml^{-1}. The data indicate that the majority of the P_i initially present in the activated sludge as well as that produced during the 24-h incubation is converted to poly-P or organic-P by the active microbial populations present in the activated sludge. These results suggest that more P_i is being incorporated into microbial biomass in the form of poly-P and organic-P than is accounted for by the net loss of P_i from the system.

The difference is likely due to the release of "new" P_i from the detrital organic-P pool by PO_4ase exoenzymes.

14.6 LOCALIZING EXTRACELLULAR ENZYME ACTIVITY ASSOCIATED WITH MICROBIAL FLOCS

A number of the studies, including those cited above suggest that the majority of exoenzyme activity detected in the activate sludge process of various wastewater treatment plants is associated with the organic particulate fraction, comprised primarily of flocs derived from the activated sludge process. Protease, PO_4ase, lipase, and esterase activities partition strongly with the particulate phase.[15,37,41] By separating activated sludge into different fractions using centrifugation, filtration, and sonication, Goel et al.[36] determined the activity for alkaline and acid PO_4ase, α-glucosidase, and protease that was associated with bacterial cells, free in the bulk solution, and loosely associated with cells or entrapped in the flocs. A major fraction of the total enzyme activity was found to be associated with the flocs. Richards et al.[44] and Frolund et al.[39] also found that exoenzymes were immobilized in the sludge floc matrix.

Cadoret et al.[12] measured extracellular enzyme activities in whole sludge, in dispersed activated sludges, as well as that associated specifically with the floc EPS. The activated sludge from a treatment plant was treated as follows before being assayed as "whole" sludge for enzyme activities: sludge was allowed to settle, then resuspended in deionized water, then homogenized, and the process repeated. Dispersed activated sludge was first prepared as "whole" sludge before subjecting to sonicated or cation exchange using Dowex-Na^+ 50×8 resin. EPS was recovered from the supernatant fraction of the centrifuged aqueous phase following removal of the cation exchange resin and associated adsorbed material. They found that 17% of the L-Leu-aminopeptidase, 5% of α-glucosidase, 23% of protease, and 44% of α-amylase activity of sludge dispersed by sonication or cation exchange resin were recovered in the EPS fraction. Most of the enzymes remained associated either to tightly bound EPS or directly bound to the bacterial cell surfaces.

Whiteley et al.[37] determined the PO_4ases and protease activity in the supernatant fraction of liquor recovered from methanogenic and sulfidogenic sewage sludge reactors following incubation with substrates selective for these enzymes, sonication, and centrifugation. The majority of the exoprotease and PO_4ase activity was found associated with the particulate fraction that was released upon sonication. Most enzyme activity was therefore found either associated with or immobilized within the particulate matter.

Pletschke et al.[38] determine ATPS activity in the supernatant fraction of liquor recovered from methanogenic and sulfidogenic sewage sludge reactors following sonication and centrifugation. Since >90% of the enzyme activity was released into the supernatant after sonication, it was suggested that the ATPS enzymes were located either intracellularly or immobilized in or on the floc material.

Boczar et al.[15] assayed esterase activity in the particulate fraction of activated sludge using substrates of different chain lengths. C_4-ester yielded the highest activity of the different substrates tested, with activity decreasing with increasing chain length above C_4. The *p*-nitrophenol esters were hydrolyzed at higher rates than fluorescein esters. Esterase activities determined after freeze–thaw treatment suggested that a significant portion of the fluorescein esters was hydrolyzed by enzymes located on the cell surface or within the cell envelope. In general, the bulk of the esterase activity was associated with the particulate flocs with no significant amount of activity detectable in the bulk solution. Similar results were reported by Teuber and Brodish[45] who found that 75% to 99% of the PO_4ase, glucosidase, and aminopeptidase activity was associated with sludge material that pelleted upon centrifugation at $3000 \times g$.

Van Ommen Kloeke and Geesey[41] used a precipitating fluorogenic enzyme substrate ELF-PO_4 (Figure 14.1) to localize PO_4ase activity in the activated sludge liquor. Since this substrate is converted to a water-insoluble, crystalline, yellow, fluorescent product at the site of organic-P hydrolysis, the position of the resulting fluorescent objects identifies the site of active PO_4ase enzymes when viewed by fluorescence microscopy (Figure 14.5). It is evident from the distribution of fluorescence that the PO_4ase activity is localized in discrete locations throughout the matrix of the floc particles, possibly reflecting the distribution of PO_4ase-producing microorganisms. Some areas are as large as 40 μm in diameter (Figure 14.5, inset), although most are on the order of 5 μm in diameter. The intensity of the fluorescence emanating from these local regions in the floc particles increased linearly over a 40-min period (Figure 14.6). Incubations of 60 min allowed the development of maximum density of ELF crystals before increasing crystal size, and fluorescence intensity caused the fluorescent image of adjacent crystals to merge into a single fluorescent object. Sixty-minute incubations were thus used to determine the density and distribution of PO_4ase activity in the flocs.

Frolund et al.[39] showed that a large portion of various classes of exoenzymes present in activated sludge were adsorbed to the EPS matrix. When the EPS was treated with a cation exchanger, large quantities of enzyme were released into the bulk aqueous solution. Since the activities of these extracellular enzymes strongly partition with the floc-containing fraction, these enzymes likely have evolved to remain associated with the cell surface or the floc matrix (Figure 14.7). Flocs provide a conditioned environment conducive for extracellular enzyme accumulation and activity outside the cell. Goel et al.[36] showed that the floc-bound nature of extracellular alkaline PO_4ase, acid PO_4ase, α-glucosidase, and protease enabled the enzymes to remain active under anaerobic as well as aerobic phases of operation. Recently, it has been shown that the EPS of activated sludge aggregates inhibited the diffusion of high molecular weight substrates of extracellular enzymes, limiting their availability to the enzymes and the rate of catalysis.[12] These results challenge the idea that hydrolysis of the organic polymers is the first and rate-limiting step in the activated sludge process, and support the suggestion by Guellil et al.[46] that sorption or uptake of the organic polymers by the exoenzyme-containing floc is a rate-limiting step that precedes hydrolysis (Figure 14.7). Thus, the benefits provided to exoenzymes by EPS

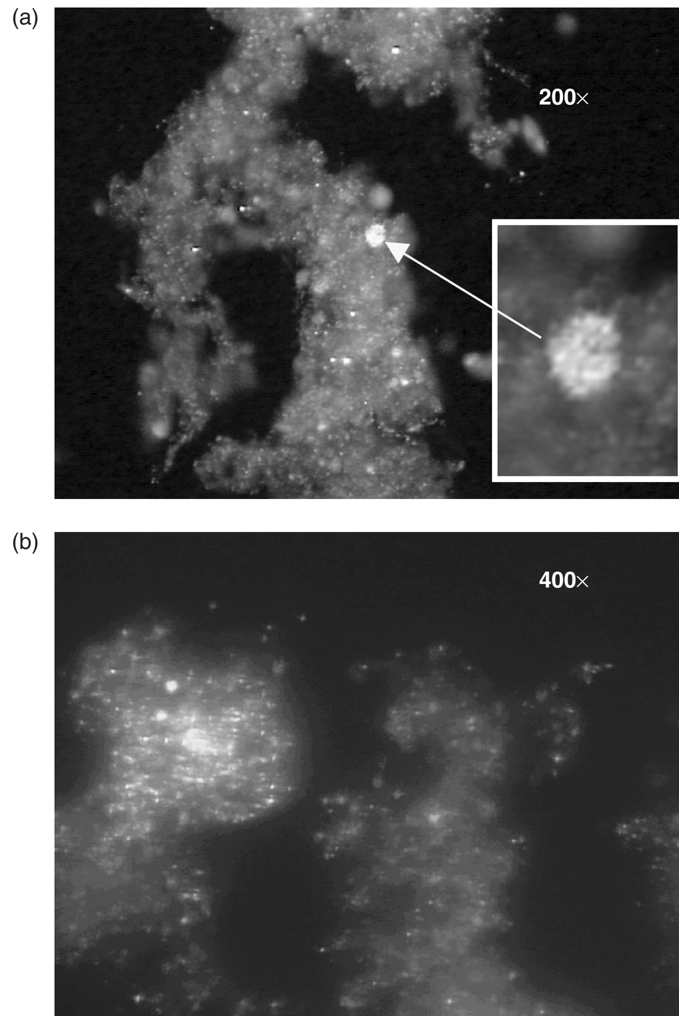

FIGURE 14.5 Epifluorescence microscopic image of ELF crystals precipitated in floc particles recovered from activated sludge and incubated with 100 μM ELF-P for 60 min. (a) Crystals distributed throughout floc matrix. Arrow shows crystal with diameter of approximately 40 μm. (b) Crystals concentrated in one floc particle at densities higher than in adjacent particles

are, to some extent, offset by the added diffusion resistance the EPS exerts on the enzyme substrates.

Floc formation requires expenditure of energy generated by the bacteria in the system. The main source of this energy is the polymeric organic material in the wastewater aqueous phase that can only be accessed by the microorganisms if first degraded into useable subunits by exoenzymes (Figure 14.8). Since even the smallest

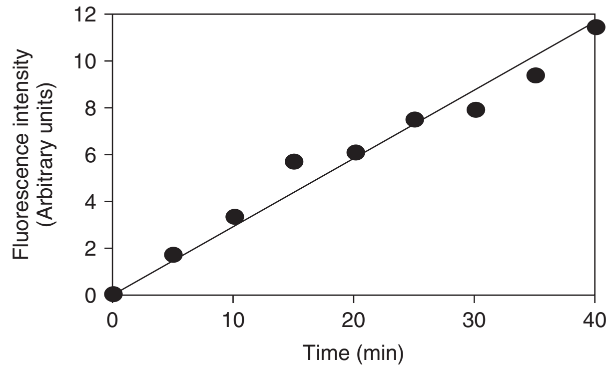

FIGURE 14.6 Increase in fluorescence intensity of area of floc particle as a function of time during which ELF-PO_4 is being hydrolyzed to ELF by extracellular PO_4ase enzymes

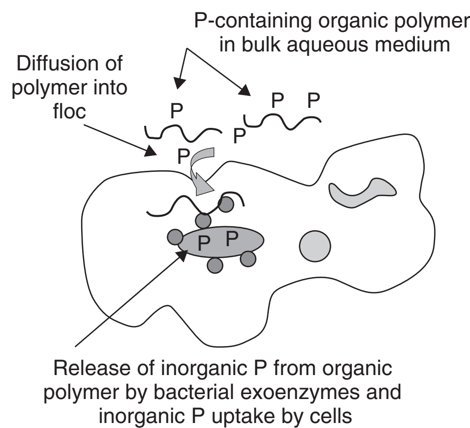

FIGURE 14.7 Schematic representation of detrital, polymeric, organic-PO_4 diffusion from bulk liquid phase into floc matrix containing extracellular PO_4ase-producing bacteria. P_i released at cell surface is taken up by the cells and transformed into biomass-P

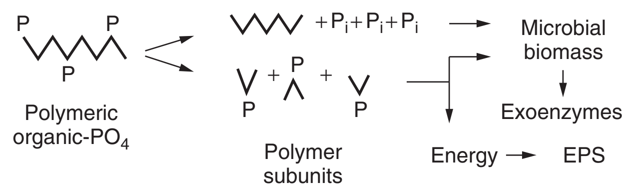

FIGURE 14.8 Schematic diagram of pathway of formation of microbial biomass, exoenzymes, and EPS. Polymer subunit intermediate products have different properties than original polymeric compounds. Production of intermediate products may affect P removal efficiency

floc particles resolved by light microscopy contained regions of ELF fluorescence, exoenzyme production and secretion likely occurs during or possibly even prior to floc formation in the system.

When floc material was homogenized in a tissue homogenizer to disperse bacterial cells, and the preparation incubated with ELF-P, approximately 9% of the cells that were visualized after staining with the nucleic acid stain acridine orange had ELF crystals precipitated in their immediate vicinity.[41] PO_4ase activity was thus contributed by a small fraction of the floc-associated bacterial community. Of the total area of fluorescence produced by all crystals of ELF evaluated in homogenized floc preparations, approximately 80% was contributed by crystals associated with cells that also reacted positively with SYTO9, a fluorochrome that is taken up by cells with a membrane potential, and thus considered viable.[41] The other ELF crystals could not be associated with any other objects in the floc. PO_4ase activity was therefore primarily associated with active bacterial cells in the floc.

14.7 IDENTIFICATION OF POPULATIONS OF CELLS RESPONSIBLE FOR EXTRACELLULAR PO_4ASE ACTIVITIES WITHIN FLOC-ASSOCIATED MICROBIAL COMMUNITIES

Considerable progress has been made in recent years in identifying the types of bacteria present in activated sludge systems. Besides the poly-P-accumulating bacteria of the genus *Acinetobacter*, those aligning with the cytophaga-flavobacteria group have also been widely reported in activated sludge systems in different parts of the world.[28,47–49] Snaidr et al.[50] reported that the cytophaga-flavobacteria group contributed 12% of the DAPI-stained bacterial community of an activated sludge system. Manz et al.[47] found a similar fraction of the bacteria present in activated sludge that hybridized with the *Bacteria*-specific probe EUB338 probed positive with a cytophaga-flavobacter-specific 16S rRNA oligonucleotide probe. The extent to which these groups of bacteria are responsible for extracellular enzymatic activities measured in activated sludge floc has only recently been assessed.[41,51]

The detection of the cultivable bacterial populations in homogenized activated sludge floc preparations that exhibit extracellular PO_4ase activity was facilitated by the development of a new screening technique.[51] Adding the precipitating fluorogenic substrate 2-(5′-chloro-2′-phosphoryloxyphenyl)-4-[3H]-quinazolinone ($CPQ\text{-}PO_4$) to a bacterial cultivation medium, after medium sterilization but prior to solidification, offered the opportunity to detect PO_4ase active colonies following inoculation and incubation of samples of homogenized activated sludge floc preparations plated on this medium (Figure 14.9). Upon enzymatic cleavage, this molecule yields the stable, highly fluorescent precipitate 2-(5′-chloro-2′-hydroxyphenyl)-4-[3H]-quinazolinone (CPQ)[52] (Figure 14.9). CPQ excites at 345 nm and fluoresces at 530 nm.[51] Phosphatase activity can then be monitored as the colonies grow by viewing the plates under UV light. Another benefit of

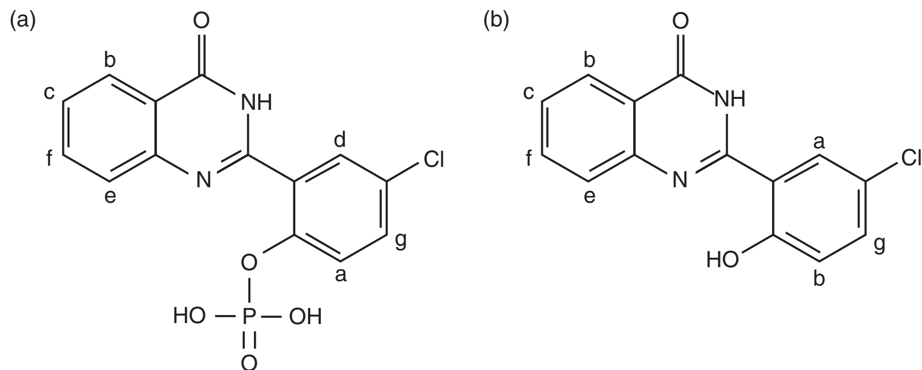

FIGURE 14.9 Fluorogenic substrate (a) 2-(5′-chloro-2′-phosphoryloxyphenyl)-4-[3H]-quinazolinone is hydrolyzed to (b) 2-(5′-chloro-2′-hydroxyphenyl)-4-[3H]-quinazolinone to produce a water-insoluble fluorescent precipitate

this approach is that it distinguishes between activity of secreted (bacteria-free) and cell-associated PO_4ase activity, due to the precipitating nature of the probe. Colonies actively secreting PO_4ase enzyme into their environment have a fluorescent halo, whereas those PO_4ase-positive colonies which have only cell-associated activity do not produce a halo.[51] Using this approach, these investigators found that 35% of the cfus that grew on LB/HEPES/CPQP agar medium yielded an intense yellow–green fluorescence, indicating the presence of phosphatase-positive clones (Figure 14.10).

The PO_4ase-positive colonies from the homogenized activated sludge preparations displayed two types of fluorescence when plated on the above medium. Some colonies displayed fluorescence that was limited to the area occupied by the colony, whereas, fluorescence displayed by other colonies extended into the surrounding medium producing halos around the colonies. Of all fluorescent colonies obtained on the plates, 36% displayed halos. Isolates displaying these different forms of PO_4ase activity on solid medium are shown in Figures 14.11 and Figure 14.12. Isolate A produced a fluorescent halo around its colony (Figure 14.11), whereas Isolate J produced fluorescence that was restricted to the colony (Figure 14.12). Isolates A and J were further evaluated for the distribution of PO_4ase activity in suspended batch cultures without added agar. Whereas 36% of the total PO_4ase activity produced by Isolate A was recovered with the cell fraction, with the remainder free in the culture menstruum, 99% of the total PO_4ase activity produced by Isolate J was recovered with the cell fraction.[51] PO_4ase activity was induced when cultures of both isolates entered the late exponential growth phase.

Selective identification of PO_4ase-positive colonies of bacterial populations in complex microbial communities makes it feasible to sequence the 16S rDNA gene to establish a phylogenetic affiliation for the population. When the DNA of cells from several of the fluorescent colonies recovered from plates inoculated with

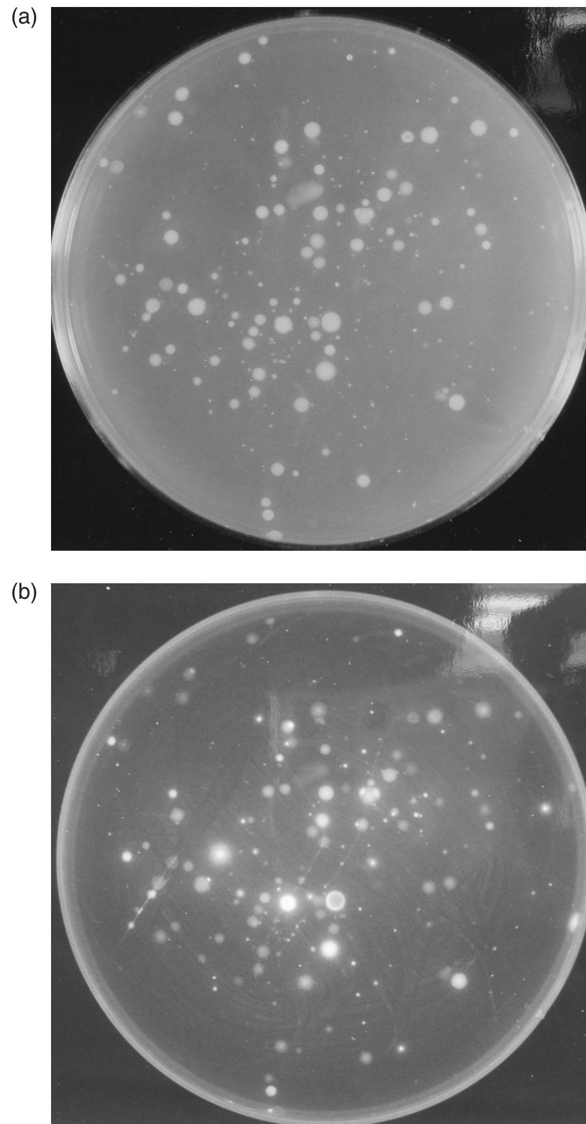

FIGURE 14.10 Colonies formed on LB/HEPES/CPQ-PO_4 solid medium after inoculation with activated sludge from a wastewater treatment plant. (a) Colonies under normal white light and (b) CPQ fluorescent, PO_4ase-producing colonies

homogenized activated sludge was extracted and a portion of their 16S rDNA amplified by polymerase chain reaction (PCR) using primer set 1056F/1392R, the resulting amplicons, when sequenced, grouped with the cytophaga-flavobacteria in the phylum cytophaga-flavobacter-bacteroides.[41] Three of the isolates reacted positively with the cytophaga-flavobacteria group-specific rRNA probe CF319a. The

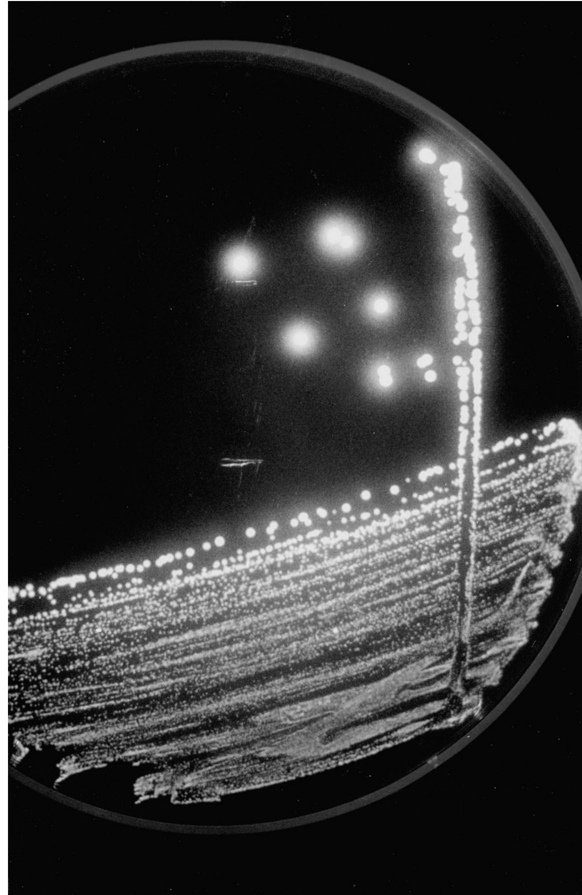

FIGURE 14.11 (**Color Figure 4.11 appears following page xx**)Colonies of Isolate A growing on LB/HEPES/CPQ-PO_4 solid medium. Note halo of fluorescent CPQ that has precipitated some distance away from the colonies reflecting diffusion of PO_4ase away from the cells and the colony

CF319a probe reacted positively with approximately 10% of the DAPI-stained bacteria and 17% to 20% of the ELF-precipitating bacteria associated with homogenized floc material from activated sludge samples.[41] These results suggest that although the cytophaga-flavobacteria group resists efforts to cultivate in the laboratory, it is an important contributor of PO_4ase activity in activated sludge. Using a combination of molecular approaches, it is possible to establish structure–function relationships among uncultivated members of complex microbial communities such as those established in flocs of the activate sludge process of wastewater treatment.

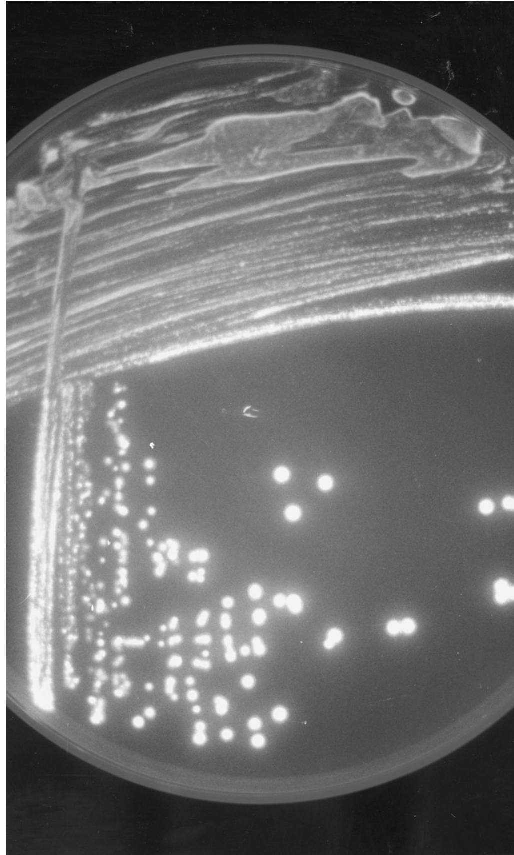

FIGURE 14.12 (**Color Figure 4.11 appears following page xx**) Colonies of Isolate J growing on LB/HEPES/CPQ-PO_4 solid medium. Note the fluorescent CPQ that has precipitated remains close to the perimeter of the colonies indicating that the PO_4ase enzymes remain closely associated with the cells in the colonies

14.8 CONCLUSIONS

Floc formation and associated microbial populations play a key role in the degradation of complex detrital organic matter in the activated sludge process of wastewater treatment. Extracellular enzymes represent an important class of EPS associated with the activated sludge floc particles. The extracellular proteases, lipases, and glucosidases hydrolyze large organic molecules too large to be taken up by bacterial cells to smaller subunits that are readily transported into the cell and metabolized. The floc matrix offers a conditioned environment outside the bacterial cells for these enzymes to carry out their function. Other extracellular enzymes such as PO_4ase regenerate the inorganic forms of non-conservative elements such as phosphorus. This represents the

initial step in removal of detrital polymeric organic-P from wastewater streams. Since one-third to one-half of the total P in wastewater exists as detrital polymeric organic P, the bacterial populations that produce active PO_4ase play a key role in P removal from wastewater in the activated sludge process. Members of the cytophaga-flavobacteria group appear to be particularly important in this regard since they account for 17% to 20% of the total PO_4ase-active cells in the system.

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