

Review

Enzyme technology and biological remediation

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Abstract

The heterogeneous complexity of sludges and wastewaters has created gross uncertainty and deviations in predictions of suitable models for their measurement. At the same time, it is becoming increasingly obvious that the current paradigms and ideologies are wrought with problems and limitations suggesting the need to move to a more consolidated analytical objective evaluation. Rapid developments in understanding activated sludge processes and wastewater remediation warrants exploitation of different strategies for studying their degradation.

It is time to replace subjective terms like sludge volume index (SVI), zone settling velocity (ZSV), filament index (F_1), fractal dimension (D), flocculating ability, surface charge (ζ), degree of hydrophobicity, chemical oxygen demand (COD) with a mathematical one that can provide an absolute quantitative relationship for the properties of wastewater and/or a sludge floc. There are no current objective values that can be introduced to represent the plethora of biological remediation terminologies such as bioleaching, biosorption, bioaugmentation, biostimulation, biopulping, biodeterioration, biobleaching, bioaccumulation, biotransformation and bioattenuation.

Enzyme technology has been receiving increased attention and this review focuses on the latest developments on the enzymology of biological remediation. It discusses the present pitfalls with current strategies and suggests that sludge–floc parameters, such as internal structure and composition, sludge retention time, microbial ecology, nutrient concentration, dissolved oxygen and type of industrial wastewater, whether from an aerobic or anaerobic system, are replaced by quantitative kinetic terms (K_m , V_{max} , K_{cat}) associated with the enzymology of the sludge floc and/or wastewater.

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1. Introduction

With a necessity of improvement in biological remediation techniques, enzyme technology has been receiving increased attention. Previous researchers have used enzymes in activated sludge systems as indicators of specific microbial populations [1], measure of active biomass [2,3] and as indicators of processes such as chemical oxygen demand and phosphorous removal [4]. According to Aitken [5], enzymes were first proposed for the treatment of waste in the 1930s, but it was not until the 1970s that enzymes were used to target specific pollutants in waste. The rapid developments and associated increase in complexity of understanding activated sludge processes and wastewater remediation warrants exploitation of different strategies for their degradation. The heterogeneous complexity of sludge has created gross uncertainty and deviation in predictions of suitable models for its measurement. Even the predictive powers of mathematical models or generic algorithms are limited or handicapped by the input of subjective information. Throughout the following discussion, the emphasis will be on moving away from the current ideologies and accepted paradigms on sludge–floc measurements towards a more unified consolidated analytical objective system. Enzymology may be regarded as being at the interface between, on the one hand, biological discovery and protein engineering and environmental biotechnology on the other. Microorganisms can be genetically ‘engineered’ to express specific xenobiotic metabolising enzymes that would degrade even the most recalcitrant pollutants. The role of microorganisms, however, is wrought with problems. The accumulation in the environment of highly toxic pollutants only emphasises the fact that micro-organisms, by themselves, are insufficient to protect the biosphere from anthropogenic pollution. Furthermore, although microorganisms may enhance the transformation of the pollutants making them more effective agents of bioremediation and biodegradation it leads to the generation of a considerable amount of biomass. Any biostimulation approach has limited potential since individual bacteria, that are capable of remediating a given pollutant, maybe inhibited by the presence of other pollutants. A limiting factor in the bioremediation of polluted contaminated sites is the very slow rate of degradation that limits, further, the practicality of using bacteria during these processes.

Characteristics of primary and secondary sludge are quite different in terms of nutrients and pollutants. While secondary sludge has higher nitrogen and phosphorus contents that are important pre-requisites for agricultural use, primary sludge tends to be more ‘polluted’ with heavy metals and organic chem-

icals. The latter, which include polychlorinated hydrocarbons (PCBs), polycyclic aromatic hydrocarbons (PAHs), benzene, toluene, ethylbenzene, xylene (BTEX), petrochemicals, agricultural chemicals (biopesticides) and dyes, tend to have low water solubility and lipophilic properties and consequently are associated with the sludge organic particulate matter. This poses further problems if the dried sludge is applied to farmlands as these recalcitrant pollutants may enter the food chain and water table. Wastewater sludge can be viewed as a two-phase system—a solid network of hydrophilic polymeric materials enclosing a liquid (water) within. It is possible to enzymatically attack this complex bioreactor in order to recover valuable resources, remove toxic materials and recover the water. Bioprocesses that convert one material into another using biological agents (e.g., living microbes or enzymes) involve biological remediation techniques that generally fall under the umbrellas of either aerobic or anaerobic digestion. Furthermore, within these two systems the activated wastewater sludge processes may be categorized into several divisions (Fig. 1). Intrinsic bioremediation is the removal, transformation or detoxification of any contaminating pollutant from the environment to a less toxic form by any natural process. Alternatively, any attempt to manipulate contaminated environmental areas by the addition of stimulants or additives (biostimulation) or the addition of special specific biochemicals or microorganisms (bioaugmentation) can be classified as accelerated bioremediation. Biostimulation and bioaugmentation are often used in conjunction with one another—one to supply the nutrients to enhance the microbial growth and the other to enhance environmental hazard waste.

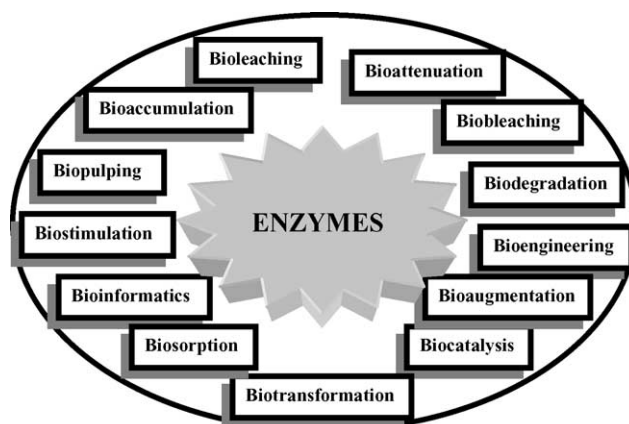


Fig. 1. Overview of the enzymology of biological remediation.

Biodegradation, mineralization, bioremediation, biodeterioration, biotransformation, biosorption and bioaccumulation are overlapping terms with minor subtle differences. Biodegradation is the general term used for all biological breakdown of chemical compounds and complete biodegradation leads to mineralization. Biotransformation is a step in the biochemical pathway which leads to the conversion of a molecule into a less toxic product. Biodeterioration is the breakdown of economically useful compounds but often the term has been used to refer to the degradation of normally resistant substances such as plastics, cosmetics, paint, wood products and metals [6]. Biosorption is the process by which metals are removed from aqueous solution by complexing to either living or dead biomass through functional sites that include: carboxyl, imidazole, sulphhydryl, amino, phosphate, sulphate, thioether, phenol, carbonyl, amide and hydroxyl moieties [7,8]. Bioaccumulation includes all processes responsible for the uptake of metal ions by living cells and includes enzymatic degradation, biosorptive mechanisms, together with intracellular accumulation and bioprecipitation [9].

It is an intention of this review to propose a strategy for the specific enzymology of all of the biological remediation processes, whether in aerobic or anaerobic systems, and to suggest a novel enzyme–floc model that will describe these mechanisms. The paper is divided into three. The first part critically evaluates the current strategies for sludge floc measurements; the second part describes the fundamental principles of enzymology while part three identifies significant roles that particular enzymes play in bioremediation.

2. Current strategies

The overall performance of any wastewater treatment, whether in activated aerobic sludge processes or anaerobic digesters, is controlled by the internal structure, composition and microbial ecology of the floc. Furthermore, the nature of activated sludge is dependent on many process variables, such as nutrient concentration, sludge retention time (SRT), reactor configuration, dissolved oxygen, type of industrial wastewater and consequently its study and its management is a complex and sometimes conflicting task [10]. Sludge flocs are characterized by being fragile, easily compressible, porous and permeable to fluids, irregular in shape with a networked multi-level structure [11,12]. It is safe to say that they are microreactors, capable of absorbing and decomposing pollutants yet grossly complex and differentiated by their own morphology and both physical and chemical attributes. It is not surprising, when it comes to any indepth study of the activated waste flocs, to be faced with conflicting answers to the numerous questions. In addition, the current accepted parameters and properties used to measure and analyse activated sludge flocs and/or biofilms are redundant and wrought with problems and limitations. It is time to replace subjective terms like sludge volume index (SVI), zone settling velocity (ZSV), filament index (F_1), fractal dimension (D_f), flocculating ability, degree of hydrophobicity, surface charge, volatile suspended solids (VSS), oxygen and nutrient utilization rates and chemical oxygen demand (COD) with an objective,

finite, mathematical term that can provide an absolute quantitative relationship for a sludge floc.

Settleability, compressibility and dewaterability of activated sludge are crucial parameters from which to monitor overall performances and efficiency of the treatment process. There are grave difficulties, however, in determining SVI and ZSV as well as giving support that they are non-specific measures of activated sludge characteristics [13]. Even attempts to correlate the SVI with the ZSV is questionable despite support from the literature to the contrary [14]. Further complications arise when it comes to comparing sludges from different sources since different factors (floc size, composition, presence of filaments) cause variations in settling properties. How is it possible, or even remotely feasible, to compare the SVI and the ZSV from a small measuring cylinder with that of a full-scale settling tank?

Fractal index is often used to describe the geometric characteristics of the multilevelled floc structure [15,16]. For linear, planar and three-dimensionally compact objects the exponent fractal dimension (D), determined from the slope of the double log plot (Eqs. (1) and (2)), will take on values of 1, 2 and 3 respectively.

$$M \propto R^D \quad (1)$$

$$\log M = D \log R \quad (2)$$

M is the mass of the floc, D the fractal number and R is the floc radius. [17]. Since floc aggregates are not circular or spherical the longest dimension, l , may be used in place of R and [Area] (as floc area) in place of M ; D_2 would then represent the fractal dimension in two dimensions. Filament index (F_1) measures the amount of filamentous bacteria and is usually rated on a scale of 1–5, with an index of 1 that corresponds to no filaments and an index of 5 to excess filaments. The variation in both these parameters with the textural variety in sludge wastes make it difficult, if not impossible, to calculate finite values. The issue is exacerbated further by researchers attempting to assign definitive structures to complex aggregates of activated sludge that possess a multi-level conglomerate of primary particles, microflocs and porous flocs [16]. The problem with filament index and its overall use in a floc dimensional study is that it is extremely difficult to quantify accurately for not only would its length be important but its morphology and surface properties as well. How is it possible to compare the characteristics of two, or more, different activated waste flocs from two different sites when one has a very low, and the other a high filament index? In one case, the flocs make a particle-to-particle contact resulting in a compacted, small size, high density “floc–floc” aggregate with excellent compressibility and settleability. In the other, a filament-to-filament or rather floc-to-filament, large, loose, low density aggregate forms with large voids between them and consequently poor compressibility and settleability.

The method for determination of flocculating ability of activated sludge flocs is based upon the ability for flocs to re-flocculate after they have been subjected to fracture. Depending on the differences in physico-chemical properties of sludge flocs will reflect the different abilities of different flocs to both deflocculate and re-flocculate. Can one really compare different sludges

on a quantitative basis? Are there elements in the bulk water that, once released during deflocculation, prevent any kind of reflocculation? In simple terms, it is obviously clear that problems, in sludge floc studies are manifested when using flocculating ability.

Sludge floc surface charge, zeta potentials (ζ), hydrophobicity and viscosity have also been measured in their capacity for monitoring sludge characteristic properties. The extent of interaction between particles depends on both the thickness of the electrical layers and the surface potential. If any polyvalent counterions are present the electrostatic repulsion is reduced and the floc constituents more easily adhere to each other. Once again, however, the findings and suggestions are tantamount to speculation and should be approached with caution [18]. The importance of surface charge in sludge flocs can only be relevant in filamentous free sludges – a scenario that is hardly practical or feasible – otherwise the filaments themselves would act as a physical bridge between the flocs [19]. Surface charge is affected by surface area of the floc and with the ubiquitous presence of filaments there would be an increase in surface area leading to false measurements. Furthermore, as the sludge flocs settle the assumed high negative surface charge on the flocs would create a micro-repulsion leading to a more expanded state and consequently a fuzzy area in the correlation of surface charge to activated sludge properties. Even the rate of floc settling would be slower leading to erroneous results. All of the literature reports pertaining to the role of hydrophobicity on floc properties originate from homogeneous suspensions and not from heterogeneous aggregates. Consequently, it is unwise to use this parameter for any meaningful determination of sludge characteristic. Finally, inconclusive findings and a lack of literature for the role of viscosity on the rheological properties of activated sludge and its compressibility and settleability excuse any further discussion.

Extracellular polymeric substances (EPS), produced from bacterial cellular metabolism, cell autolysis and the wastewater itself, cement sludge components together and consequently any hydrolysis of the EPS would lead to significant changes in sludge structure [20]. EPS play a crucial role in the flocculation, settling and dewatering of activated sludge [21–24] and since the quantity and composition of EPS for a given sludge are strongly dependent on the extraction methods it suggests, without any standard extraction protocol in place, that any comparative interpretation of published results would be extremely difficult. EPS are highly charged and so absorb water to reduce any differences in osmotic pressure between the aggregates and surrounding liquid [18,25] and as a result the EPS would tend to become more gel-like rather than rigid particles. This makes current evaluations of sludge characteristics difficult to resolve. The precise role of polymers with respect to sludge properties is, therefore, very complicated and it would be grossly in error to use any standard protocol for EPS as a finite quantitative measurement.

The development of any mathematical model with a differential algebraic equation to describe a biological process related to wastewater activated sludge originates as a numerical simulation aimed at predicting the system behaviour or functional optimization. Nevertheless, in order to derive these algorithms various

component quantities and their respective interactions must be established, model parameters calibrated, initial and final conditions understood and a final validation of the model undertaken using actual experimental data. The key to any model is to select relevant quantities and processes and then to describe them by means of logical equations, rules, probabilities, transitions. Fluid dynamics, convective and diffusive transport, mechanical loads, biochemical reactions, metabolic entities, thermodynamics, competitive co-existence of species reflect a vast array of variable components. This myriad of challenging protocols required for an activated sludge floc and/or biofilm puts the viability of using such models near the bottom of the success ladder. It would be a gross improbability to design a single computer algorithm that would take into account all of the parameters and scenarios mentioned above and treat them, not as individual components but, as an integrated factorial matrix. It is ‘easy’ to design a model for any process or processes but it is the reality and viability of the process to hold true against actual experimental data that makes it acceptable.

It is time to move back a step and examine, at a cellular and genomic level, the biochemical and biotechnological properties, structure, formation and decomposition of activated sludge flocs. All of the parameters in the foregoing discussion, whether they be statistically correlated or not, must be consolidated into a single objective, finite, mathematical term that can provide an absolute quantitative relationship for a sludge floc. Even novel analytical techniques such as confocal laser scanning microscopy (CLSM), scanning and transmission electron microscopy (SEM, TEM), atomic force microscopy (AFM) and small angle laser light scattering (SALS) suffer from a similar fate as their ‘modus operandi’ is based upon parameters already discussed.

3. Principles of enzymology

3.1. Assays

To analyse the properties of an enzyme in sludge biomass a valid assay needs to be developed with a specific substrate and using conditions that produce maximum activity. So questions to ask are: (1) How much substrate? (2) How much sludge biomass (enzyme)? (3) What are the optimal pH and temperature? (4) How is enzyme activity measured?

3.1.1. Substrate

To find the amount of substrate necessary for an enzyme assay one must measure either the disappearance in substrate during the enzyme catalyzed reaction or the appearance of product. Though the assay may be more sensitive if appearance of product is measured, since one would start the assay with no product at all, it would be necessary to ensure that, in a biologically active environment the product is not used in any way by any alternative systems. Also, it would be necessary to establish that, in the absence of the enzyme under investigation, the substrate(s) do not react to produce any kind of product that may interfere with the assay protocol. Whichever way the evaluation method must be quantitative and give results in amounts of micromole

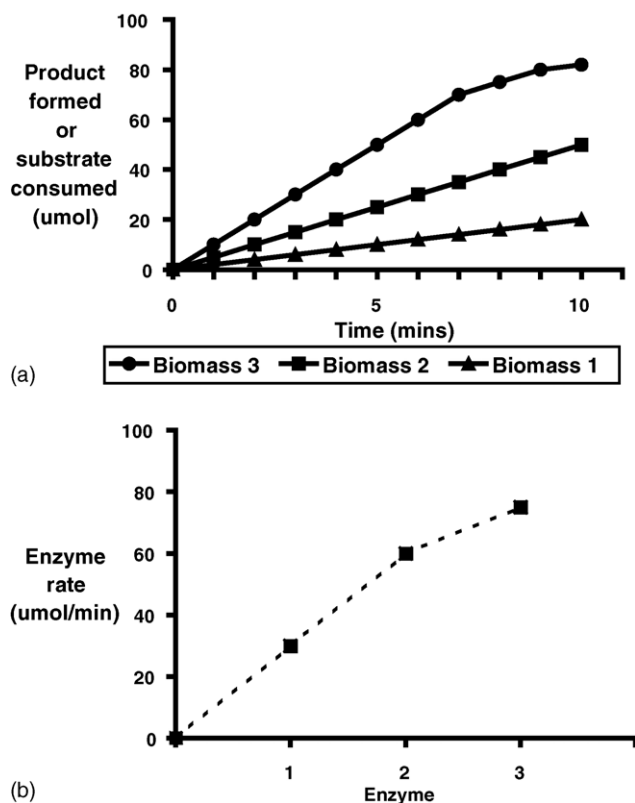


Fig. 2. Enzymatic progress curves: (a) amount of product per unit time per enzyme concentration and (b) rate of enzyme reaction with respect to enzyme concentration.

product formed or substrate consumed during a measured period of reaction time, usually minutes. This is called the enzyme catalyzed rate or enzyme activity.

3.1.2. Sludge biomass

In order to establish the minimum amount of activated sludge/biomass that will be necessary for the particular enzyme assay under study it is necessary to set up, what are called, progress curves. This is in order to find conditions from which there is a linear response to an enzyme assay with respect to its substrate (or product) and time.

Sludge biomasses 1 and 2 (Fig. 2a and b) produce linear responses for 10 min of reaction time used, while at sludge biomass 3, the amount of substrate (or product) falls off after 7 min and consequently using either 1 or 2 sludge biomass amounts would give a reliable valid assay. With sludge biomass 3 some other influence is present to consume substrate or limit the formation of product. When the amount of product produced (or substrate consumed) in 10 min is used to calculate the enzyme rate, a plot can be made to show what minimum amount of sludge biomass is valid for the assay.

3.1.3. Optimum pH and temperature

After the valid amount of sludge biomass (enzyme) to use is found, it is necessary to optimize the pH and temperature. This is done by resuspending the sludge biomass in buffers of different

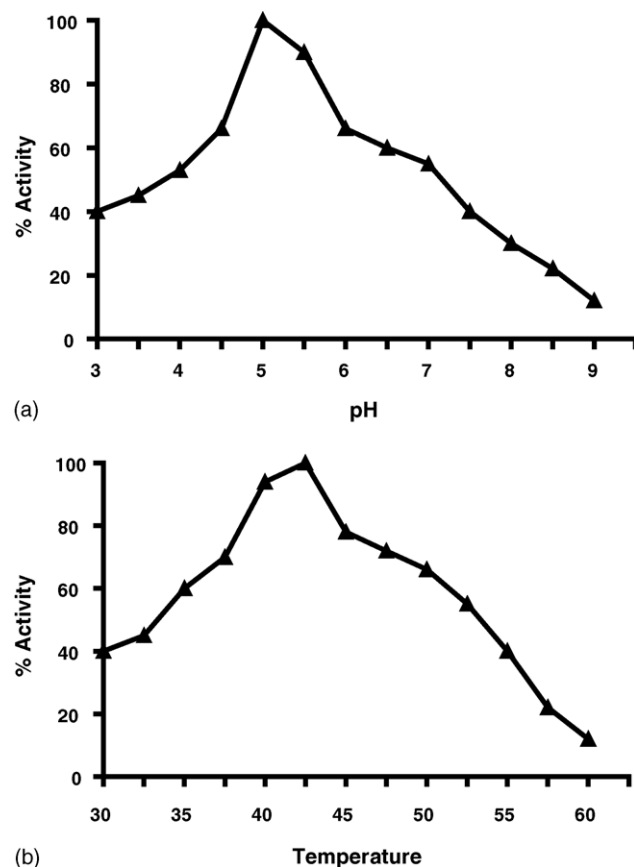


Fig. 3. Enzyme activity with respect to (a) pH and (b) temperature.

pH (or temperature), calculating the enzyme activity rates at each pH (or temperature) and then plotting the respective curves (Fig. 3a and b).

Amino acids constitute the active catalytic region of every enzyme and the various side chains of the basic and/or acidic amino acids can exist in either of two forms—ionized or protonated. Optimum pH for an enzyme lies somewhere between the pK_a values of the two (or more) catalytic amino acids. Consider the catalytic mechanism of a particular protease to be controlled by two glutamic acid residues—one ionized and the other protonated. Any pH lower than the optimum would protonate the ionized residue while any pH higher than the optimum would ionize the protonated residue leading to decreased activity.

Most enzymes have an optimum temperature, which may be related to the type of organism from which the enzyme was isolated. Some organisms grow well near room temperature and so their enzymes are most active at a temperature around 30–40 °C. At low temperatures, all the molecules in the solution slow down and so does the enzyme catalyzed reaction while at high temperatures, the thermal movement of the molecules become too great for the enzyme to hold its shape or conformation and so it begins to denature and lose its activity.

3.1.4. Enzyme activity measurements

According to the Beer Law (Eq. (3)) the absorbance, or fluorescence or whatever optical parameter is being used, is directly

Table 1
Reagents, procedures and sensitivities of different protein assays

Assay; Ref.; Sensitivity; Accuracy	Interference; Principle	Reagents	Procedure
Biuret [26] 1–10 mg Good	Ammonium salts; Colour between Cu ⁺ and peptide bond	Sodium potassium tartrate [2.25 g] CuSO ₄ [0.75 g] KI [1.25 g] in 100 ml 0.2 M NaOH and diluted to 250 ml	Biuret reagent (9.0 ml) + sample (1.0 ml) mixed, allowed to stand (20 min); absorbance read at 550 nm
Folin-Lowry [26,160] 20–300 µg Good	Strong acids, Ammonium salts; Colour between Cu ⁺ and aromatic amino acids and phosphomolybdate	Na ₂ CO ₃ (2%) in 0.1 M NaOH (49 ml); CuSO ₄ (1%); KOOCCH(OH)CH(OH)COONa (2%, 0.5 ml)	Lowry reagent (1.0 ml); sample (100 µl) mixed, allowed to stand (30 min), Folin reagent (100 µl, 1 M) added, incubated (30 min), absorbance read at 595 nm
Bradford [26,161] 1–100 µg Good	Absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465 to 595 nm when binding to protein occurs	Bradford reagent: Dissolve 100 mg Coomassie Brilliant Blue G-250 in 50 ml 95% ethanol, add 100 ml 85% (w/v) phosphoric acid. Dilute to 1 l, filter	Sample (5 µl) and Bradford reagent (250 µl) incubated and absorbance read at 595 nm
Bicinchoninic [BCA] [26] 0.2–50 µg Good	Strong acids, Ammonium salts; BCA reduces divalent copper to the mono-valent ion under alkali conditions. A molybdenum/tungsten blue product is produced	Reagent A: 1 gm sodium bicinchoninate (BCA), 2 gm Na ₂ CO ₃ , 0.16 gm sodium tartrate, 0.4 gm NaOH, and 0.95 gm NaHCO ₃ , in 100 ml distilled water. Adjust the pH to 11.25 with 10 M NaOH Reagent B: 0.4 gm cupric sulfate (5 × hydrated) in 10 ml water. Standard working solution (SWR): Mix 100 volumes reagent A with 2 volumes reagent B	Prepare samples containing 0.2–50 µg protein in 20 µl. Add 1 ml SWR to each sample and mix. Incubate 30 min at 60° Cool the samples and read at 562 nm

proportional to the concentration of the reagent.

$$A = \epsilon lc \quad (3)$$

where A is the absorbance, ϵ the extinction coefficient, l the path length and c is the concentration usually in $\mu\text{mol ml}^{-1}$. In the case of enzymes, this concentration is per unit time and is the same as activity, i.e. $\mu\text{mol ml}^{-1} \text{min}^{-1}$. Consequently, if one knows the extinction coefficient of a substance at a particular wavelength and one is measuring the change in optical parameter over time it is possible to determine the activity (v_1) of the enzyme (Eq. (4)):

$$v_1 = \Delta A \frac{V}{\epsilon tv} \quad (4)$$

where ΔA is the change in absorbance, V the total volume in the assay mixture, ϵ the extinction coefficient in $\text{ml } \mu\text{mol}^{-1}$, t the time in min and v is the volume of the sample.

The extinction coefficient for the substance under investigation can be found from the slope of a linear plot, usually by linear regression, between the absorbance and several concentrations of pure substance. For substances that do not absorb strongly they are usually reacted with a dye to produce a colour that is measured at some specific wavelength in the visible spectrum. The units of enzyme activity per mass of protein is referred to

as the specific activity and the amount of protein in the biomass may be determined in several ways depending on the sensitivity range of the protein. (Table 1) [26].

The kinetics of simple enzyme catalysed reactions was first characterised in 1912 by two biochemists Michaelis and Menten as they derived the hyperbolic equation:

$$v_1 = \frac{V_{\max}[S]}{K_m + [S]} \quad (5)$$

where v_1 is the rate of the enzyme catalysed reaction, $[S]$ the concentration of substrate, K_m the Michaelis–Menten constant and V_{\max} is the maximum reaction rate. It can quickly be established that K_m is also equal to the substrate concentration that would give 50% V_{\max} . At low substrate concentrations, the rate of an enzymatic reaction is of first-order and is directly proportional to substrate concentration ($v = k[S]$) (Fig. 4, point a) while at high substrate concentration the rate becomes independent of substrate concentration, is represented by zero order kinetics ($v = V_{\max}$) (Fig. 4, point b) and almost all of the enzyme molecules are bound to substrate. To avoid using this curvilinear plot Lineweaver and Burk [27] introduced an analysis of enzyme kinetics by a straight line double reciprocal plot of $1/v_1$ against $1/[S]$ (Fig. 4, inset) with a slope of K_m/V_{\max} and an intercept on

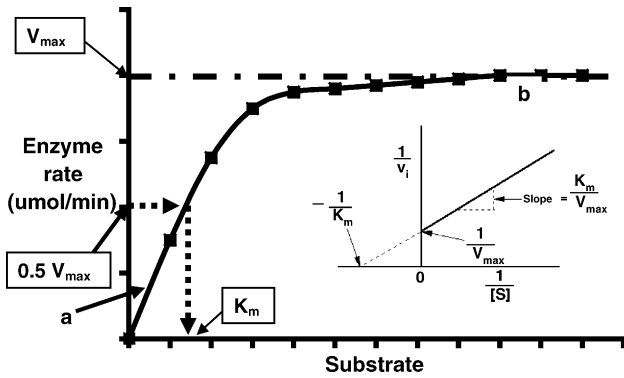


Fig. 4. A typical Michaelis–Menten curve representing change in velocity of an enzyme catalysed reaction with respect to substrate concentration (Inset: Lineweaver–Burke linear relationship).

the ordinate at $1/V_{\max}$, from the following relationship.

$$\frac{1}{v_1} = \left\{ \left[\frac{K_m}{V_{\max}} \right] \left[\frac{1}{S} \right] \right\} + \frac{1}{V_{\max}} \quad (6)$$

Another term that is often used is K_{cat} . This is the catalytic formation of product by an enzyme and is the time required for an enzyme to ‘turnover’ a substrate molecule.

3.2. Enzyme classification

Enzymes are grouped into six functional classes by the Enzyme Commission of the International Union of Biochemists [28,29] assigning each enzyme a unique four-digit number. The first describes the enzyme class, the second to the class bond of the substrate, the third to a sub-class or functional group of the substrate and the fourth to the actual molecule. It is the intention of this review not to present a detailed account of all of these enzymes but to report on those that are associated with biological remediation (Table 2).

3.3. Energy of reaction

Enzymes are biological catalysts responsible for supporting almost every type of chemical reaction. They are physiologically

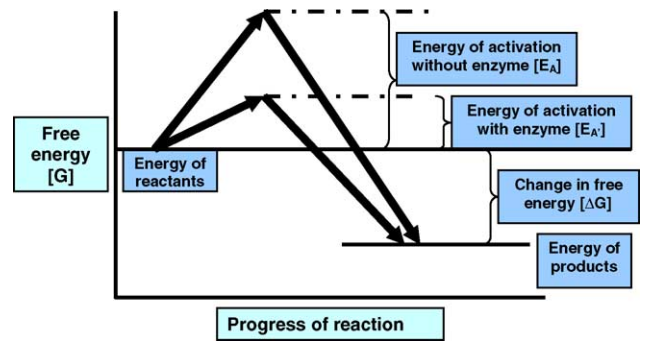
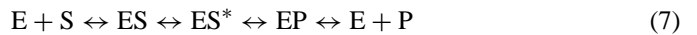


Fig. 5. Energy of reactants and products and activation energy with, and without, an enzyme.

important because they speed up, by at least 1000-fold, the rates of reactions by decreasing the amount of energy required to form a complex of reactants, known as the transition state complex, that is competent to produce reaction products. The free energy required to form an activated complex is much lower in the catalyzed reaction and consequently at any instant a greater proportion of the molecules in the population can achieve the transition state. The result is that the reaction rate is increased (Fig. 5).

3.4. Enzyme–substrate interactions

Enzymes interact with their specific substrate to form an enzyme–substrate complex [ES] by either a ‘Lock-and-Key’ or ‘Induced Fit’ model (Fig. 6) which then passes to a transition state [ES*] and eventually to an EP complex which dissociates into product and free enzyme.



In the ‘Lock-and-Key’ model, the active site of the enzyme is complementary in shape to that of the substrate. With the, more favoured, ‘Induced Fit’ model, however, an initial weak interaction between enzyme and substrate rapidly induces conformational changes in the enzyme thereby strengthening the binding and bringing catalytic sites and scissile substrate bonds close together. Such catalysis takes place at the active site, within

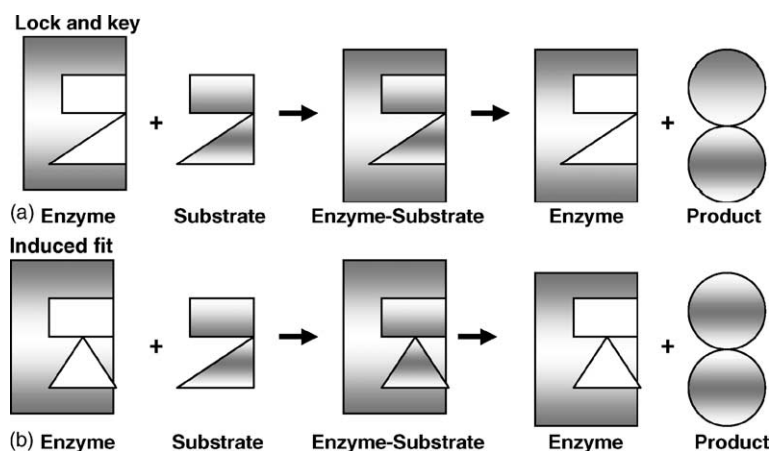
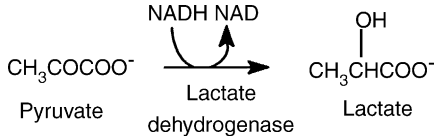
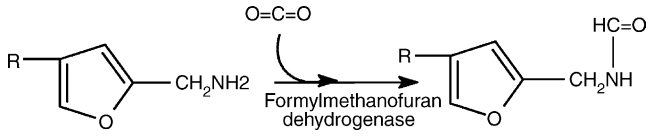
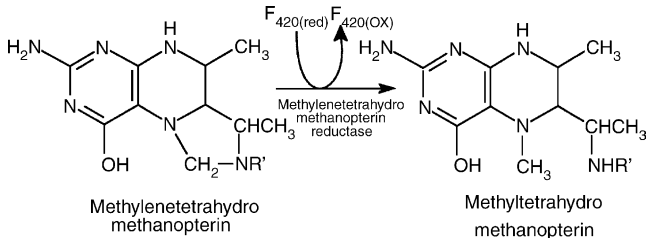
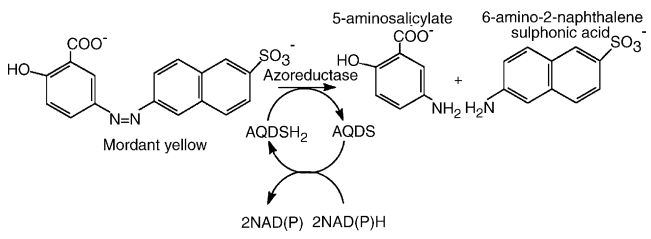
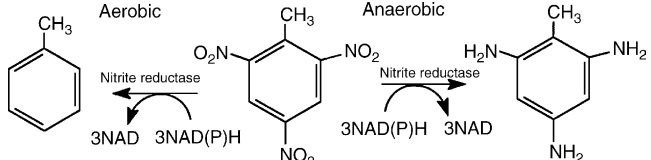


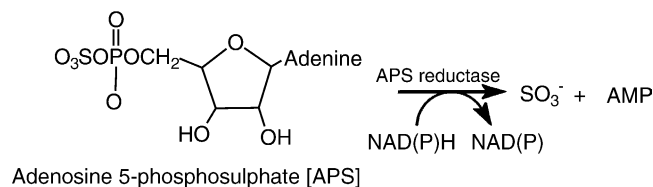
Fig. 6. Enzyme–substrate complex with (a) Lock-and-Key and (b) Induced Fit model.

Table 2
Reagents, reactions and classification of enzymes associated with biological remediation

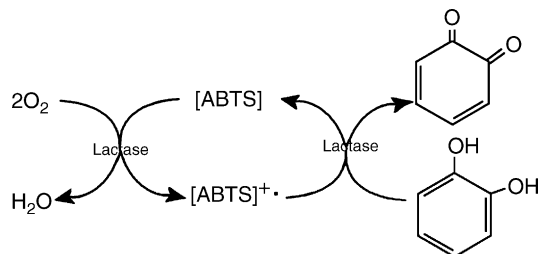
No. [Ref]	Classification; Properties	Reaction	Reagents; Assay
1	Oxidoreductases	Adds or removes H ₂	
1.1.1	Dehydrogenases	 <p>Pyruvate $\xrightarrow{\text{Lactate dehydrogenase}}$ Lactate</p> <p>Donors: Oxo groups Acceptors: NAD(P)H</p>	Assay vol: 6.25 ml; triethanolamine buffer, 0.1 M, pH 7.6; Mg Cl ₂ , 0.1 M; substrate, 10 mg ml ⁻¹ , 12 μl; NADP Na salt, 10 mg ml ⁻¹ , 16 μl; enzyme 20 μl
1.2.99 [73]	Dehydrogenase	 <p>Formylmethanofuran $\xrightarrow{\text{Formylmethanofuran dehydrogenase}}$ Methanofuran</p> <p>Donors: Oxo groups Acceptors: Other</p>	Assay vol: 1.0 ml; Tris buffer, pH 8.0, 50 mM; methyl viologen, 5 mM; HCOONa, 20 mM; enzyme 100 μl; sodium dithionite, 50 mM
1.5.99 [76]	Reductase	 <p>Methyltetrahydro methanopterin $\xrightarrow{\text{Methyltetrahydro methanopterin reductase}}$ Methyltetrahydro methanopterin</p> <p>Donors: CH–NH groups Acceptors: Other</p>	Assay vol: 1 ml; phosphate buffer, pH 6.8, 300 mM; Na ₂ SO ₄ , 2.2 M; DTT, 1 mM; coenzyme F ₄₂₀ 14 μM; Na ₂ S ₂ O ₄ 0.7 mM; incubate 4 min; HCHO, 15 mM added; incubate 1 min; H ₄ -MPT 16 μM added to form methylene- H ₄ -MPT; enzyme 50 μl
1.7.1.6 [114–116,168]	Azo reductase	 <p>Mordant yellow $\xrightarrow{\text{Azoreductase}}$ 5-amino-2-naphthalene sulphonyl acid</p> <p>Donors: Azo compounds Acceptors: NAD(P)H.</p>	Assay vol: 2.0 ml; Tris–HCl, pH 7.4, 25 mM; NADH 0.21 mM; FMN 20 μM; Mordant yellow, 25 μM; enzyme 50 μl. [AQDSH ₂ 25 μM]
1.7.2.2	Nitrite reductase	 <p>Nitrobenzene $\xrightarrow{\text{Nitrite reductase}}$ Aniline</p> <p>Donors: Nitro compounds Acceptors: cytochrome or copper</p>	Assay vol: 1.0 ml; phosphate buffer, pH 7.0, 50 mM; NADPH 0.2 mM; TNT 0.1 mM; enzyme 50 μl. Nitrite monitored at 540 nm. Sample 600 μl; PMS 10 mM, 1.5 μl; sulphanilamide, 1% in 0.68 M HCl, 200 μl; N-(naphthyl) ethylenediamine, 1%, 40 μl
1.7.99.3 [148–152]			1 U activity = consumption of 1 μmol NADPH per minute

1.8.99 [51]

Sulphur reductases

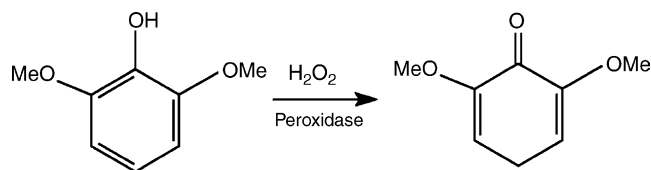
Donors: sulphur groups
Acceptors: NAD(P)HAssay vol: 1 ml; Tris-HCl, pH 8.0, 50 mM; AMP 2 mM;
K₃Fe(CN)₆ 5 mM; Na₂SO₃ 30 mM; enzyme 100 μlActivity measured in the direction of APS formation and monitored
by decrease at 420 nm due to ferri-cyanide reduction1.10.3.2
[102]

Oxidase

Donors: Diphenols
Acceptors: O₂Assay vol: 2.0 ml; 2,2'-azinobis-3-ethyl
benz-thiazoline-6-sulphonic acid [ABTS], 0.5 mM; CH₃COONa,
pH 4, 50 mM; diphenol 0.5 mM; enzyme 100 μl1 U enzyme = 1 μmol product per minute
Monitoring oxidation of ABTS at 420 nm

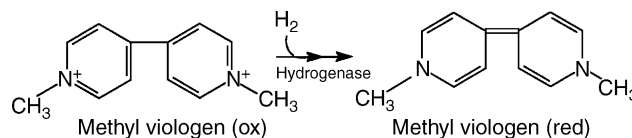
1.11.1

Peroxidase

Donors: Phenols
Acceptors: PeroxideAssay vol: 3 ml; malonate buffer, pH 4.5, 50 mM;
2,6-dimethoxyphenol, 0.1 mM, H₂O₂ 0.2 mM; MnSO₄ 1.0 mM;
enzyme 100 μlActivity monitored by oxidation of 2,6-dimethoxy-phenol at
470 nm

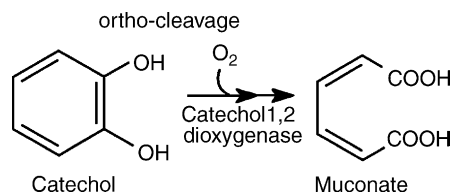
1.12 [169]

Hydrogenases

Donor: H₂
Acceptor: Fe-S proteinAssay vol: 3.15 ml; methyl viologen, 1 mM in Tris-HCl, 20 mM,
pH 7.8; sodium dithionite, 100 mM; enzyme 100 μl1 U enzyme = reduction of 1 μmol of methyl viologen per minute
under H₂ at 604 nm

1.13.11.1

Dioxygenase

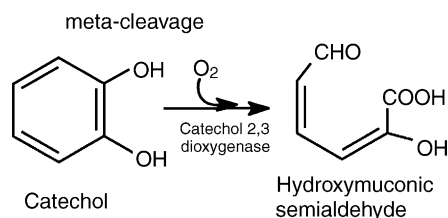
Assay vol: 1.0 ml; Tris-HCl buffer, pH 8.0, 50 mM; catechol 1 mM;
enzyme 100 μl

Activity monitored at 260 nm

1 U enzyme = μmole of catechol converted per minute

1.13.11.2

Dioxygenase

Assay vol: 1.0 ml; Tris-HCl buffer, pH 8.0, 50 mM; catechol 1 mM;
enzyme 100 μl

Activity monitored at 375 nm

1 U enzyme = μmole of catechol converted per minute

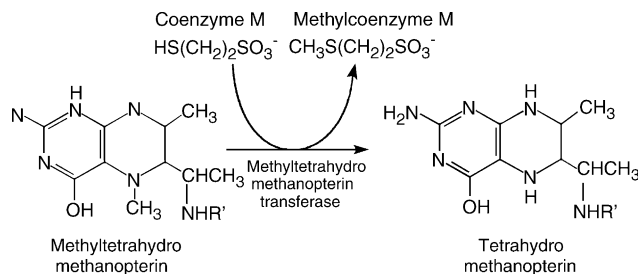
Table 2 (Continued)

No. [Ref]	Classification; Properties	Reaction	Reagents; Assay
1.13.11.18. [154–156]	Sulphur dioxygenase		Assay vol: 1.0 ml; Tris–acetate buffer, pH 7.4, 70 mM; sulphur 2%; enzyme 200 μ l; sulphite + thio-sulphate or sulphide were determined colorimetrically
1.14.12 [82]	Dioxygenases Incorporation of both atoms of O ₂ into the product.		Assay vol: 1.0 ml; Tris–HCl, pH 7.5, 50 mM, indole, 0.1 mM, NADH, 0.3 mM, ferrous ammonium sulphate, 0.1 mM, enzyme 100 μ l Assayed by decrease in A ₃₄₀ due to decrease of NADH
1.14 [83–85]	Monoxygenases Incorporation of one atom of O ₂ into the product and the other reduced to H ₂ O		Assay vol: 1.0 ml; Tris–HCl, pH 7.5, 50 mM, salicylate, 0.1 mM, NADH, 0.3 mM, ferrous ammonium sulphate, 0.1 mM, enzyme 100 μ l Assayed by decrease in A ₃₄₀ due to decrease of NADH
1.16.1.1	Reductase		Assay vol. 1.2 ml; enzyme 100 μ l; phosphate buffer, pH 7.5, 50 mM; NADPH, 100 μ M; Mg (OAc) ₂ , 200 μ M; EDTA, 500 μ M; mercaptoethanol, 0.1%; HgCl ₂ , 200 μ M; 60 min in dark; phosphate buffer, 50 mM, pH 8.0 with 10 mg nitroblue tetrazolium (NBT) and 1.5 mg phenazine methosulphate (PMS)
Mercuric reductase [124,125]	Donors: Metal ions Acceptors: NAD(P)H		Enzyme activity determined at 590 nm from formazan standard curve
Cupric reductase [119–121]			Assay vol: 400 μ l; sodium citrate pH 7.5, 200 mM; CuCl ₂ 20 μ M; bathocuproine disulphonate 100 μ M; FAD 40 μ M; NADH 100 μ M; enzyme 100 μ l
1.97.1 [127–129]	Chromate reductase		Assay volume, 1.0 ml; H ₂ SO ₄ , 0.1 M; 1,5-diphenyl-carbazide, 0.01%, Tris–HCl buffer, 50 mM, pH 7, K ₂ CrO ₄ , 0.05 mM, NADH, 0.1 mM; enzyme 100 μ l
	Electrons supplied by NAD(P)H or cytochrome c ₃		Assayed by decrease in A ₅₄₀ due to decrease in chromate

Transferases

2.1.1.86
[79]Transfer C₁ methyl group

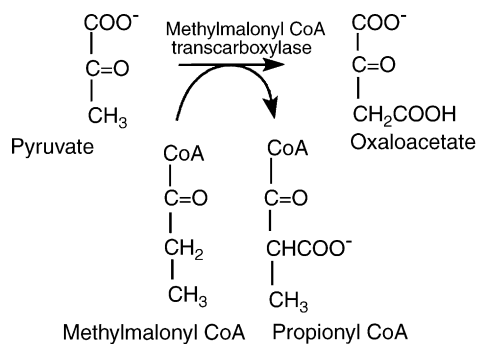
Transfers groups from a donor to acceptor.

Assay vol: 100 μl ; coenzyme M 6 mM; ATP 0.5 mM; $\text{CH}_3\text{-H}_4\text{MPT}$ 3 mM; MgSO_4 1 mM; Ti(III)citrate 0.7 mM; DTT 1 mM; Phosphate buffer pH 7, 40 mM

Analysed by release of thiol with dithiobisnitro-benzene

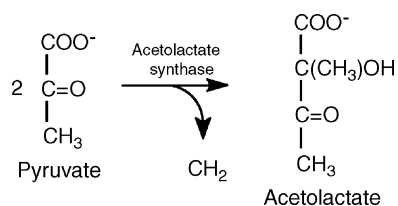
1 U enzyme = μmole of coenzyme M methylated per minute by $\text{CH}_3\text{-H}_4\text{MPT}$

2.1.3.1.

Transfer C₁ carboxy groupAssay vol: 600 μl ; Tris-HCl buffer, pH 7.0, 15 μM ; glutathione 3 μM ; NADH 0.15 μM ; pyruvate 6 μM ; methylmalonyl CoA 0.5 μM ; malic dehydrogenase 0.2 UAssay monitored by change in A_{340} by coupling with the reduction of oxaloacetate to malate and NAD

2.2.1.6 [63]

Transketolase or transaldolase

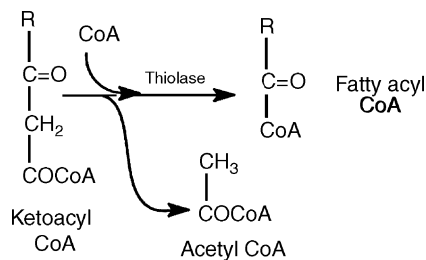
Assay vol: 1.0 ml; phosphate buffer, pH 7.8, 50 mM; pyruvate 100 mM; thiamine diphosphate 1 mM; MgCl_2 10 mM; FAD 10 μM

Activity monitored at 333 nm due to disappearance of pyruvate

1 U activity = 1 μmol acetolactate formed per minute

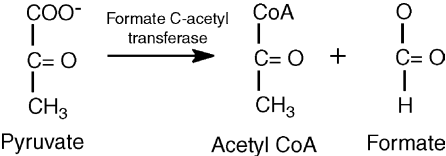
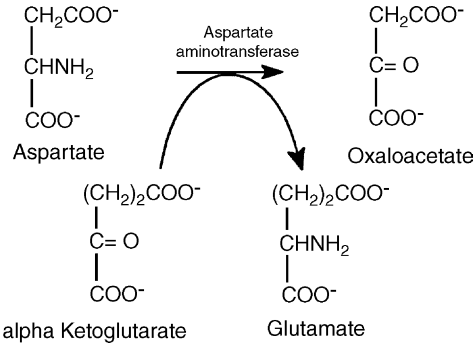
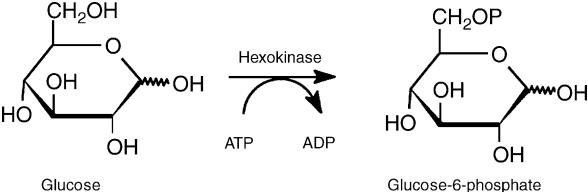
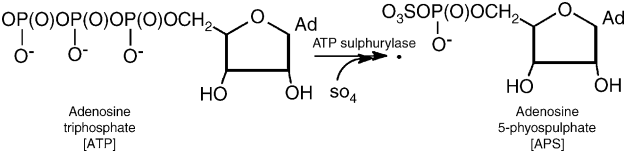
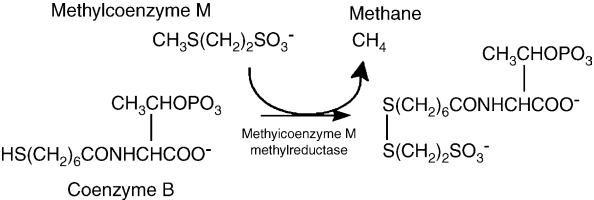
2.3.1.16

Transfers acyl group

Assay vol: 1.0 ml; Tris-HCl, pH 8.8, 50 mM; NADH 0.1 mM; 3-hydroxyacyl-CoA dehydrogenase 0.5 U; CH_3COCOA 1.5 mM; Formation of CoA measured with 5,5'-dithiobis(2-nitrobenzoic acid) at 412 nm

Activity measured in the direction of ketoacylCoA formation by coupling with its reduction to 3-hydroxyacyl CoA and NAD

Table 2 (Continued)

No. [Ref]	Classification; Properties	Reaction	Reagents; Assay
2.3.1.54 [61,62]	Transfers other than amino-acyl	 <p>Pyruvate $\xrightarrow{\text{Formate C-acetyl transferase}}$ Acetyl CoA + Formate</p>	<p>Assay vol: 1.0 ml; phosphate buffer, pH 7.6, 100 mM; pyruvate 20 mM; CoA 80 μM; NAD 1 mM; malate 6 mM; DTT 2 mM; citrate synthase 1 U; malate dehydrogenase 22 U; enzyme 100 μl</p> <p>Assay monitored by change in A_{340}</p>
2.6.1. [70,71]	Transaminases Transfers ketoacids to amino acids	 <p>Aspartate $\xrightarrow{\text{Aspartate aminotransferase}}$ Oxaloacetate</p> <p>alpha Ketoglutarate $\xrightarrow{\text{Aspartate aminotransferase}}$ Glutamate</p>	<p>Assay vol: 3.0 ml; phosphate buffer, pH 7.5, 100 mM; aspartate 120 mM; NADH 3 mM; malate dehydrogenase 200 U; α-ketoglutarate 100 mM; enzyme 100 μl</p> <p>Assay monitored by change in A_{340} by coupling with the reduction of oxaloacetate to malate and NAD</p>
2.7.1	Transfers phosphate to OH Donor: ATP Acceptor: OH	 <p>Glucose $\xrightarrow{\text{Hexokinase}}$ Glucose-6-phosphate</p>	<p>Assay vol: 1.0 ml; Tris-HCl, pH 8.0, 100 mM; glucose 20 mM; ATP 1 mM; NADP⁺ 0.3 mM; glucose-phosphate dehydrogenase 2 U; enzyme 100 μl</p> <p>Activity monitored at 340 nm by coupling with oxidation of glucose-6-phosphate to 6-phospho-gluconate and NADPH</p>
2.7.7. [49,50]	Transfers nucleotidyl phosphoryl groups.	 <p>Adenosine triphosphate [ATP] $\xrightarrow{\text{ATP sulphurylase}}$ Adenosine 5-phosphosulphate [APS]</p>	<p>Assay vol: 1.0 ml; Tris-HCl, pH 8.0, 100 mM; APS 1 mM; MgCl₂ 1 mM; PPi 1 mM; enzyme 20 μl; reaction terminated with NaOH, 6M, 100 μl. Centrifuged and aliquot used to measure ATP formation with standard hexokinase glucose-6-phosphate dehydrogenase coupled system</p> <p>Activity measured in the direction of ATP formation</p>
2.8.4 [80]	Transfers alkylthio groups	 <p>Methylcoenzyme M $\xrightarrow{\text{Methylcoenzyme M methylreductase}}$ Methane</p> <p>Coenzyme B $\xrightarrow{\text{Methylcoenzyme M methylreductase}}$ S(CH₂)₆CONHCHCOO-S(CH₂)₂SO₃⁻</p>	<p>Assay vol: 8.0 ml; MOPS NaOH buffer, pH 7.2, 500 mM; methyl coenzyme M 10 mM; coenzyme B 1 mM; aquacobalamin 0.3 mM; titanium citrate 30 mM; enzyme 100 μl; atmosphere 92% N₂/8% H₂. CH₄ conc. determined by gas chromatography</p> <p>1 U activity = produces 1 μmol methane per minute</p>

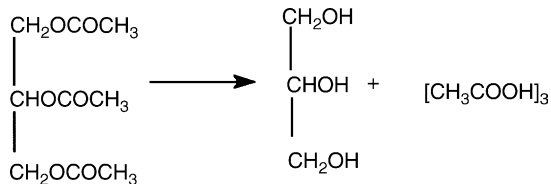
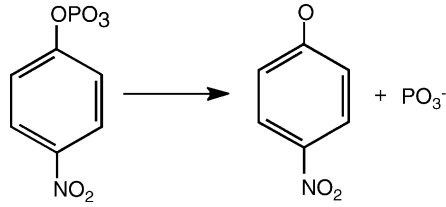
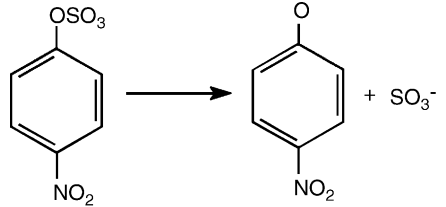
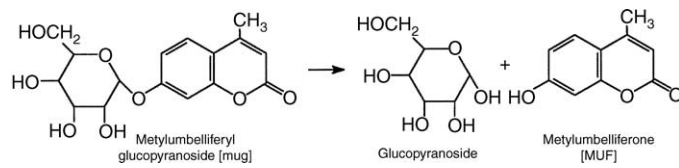
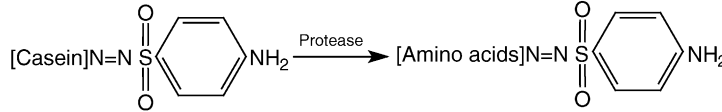
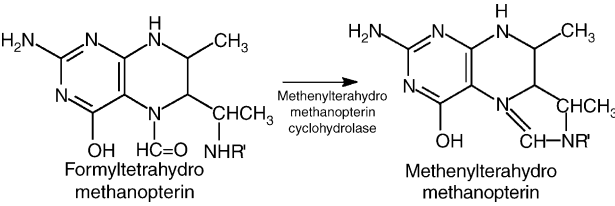
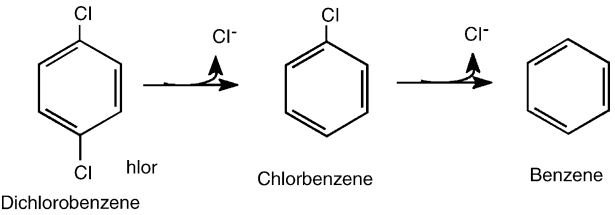
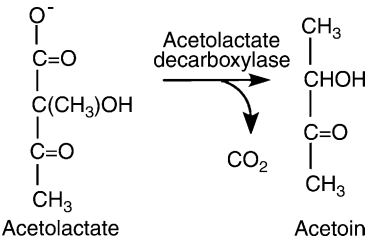
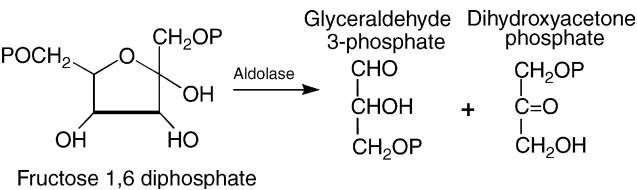
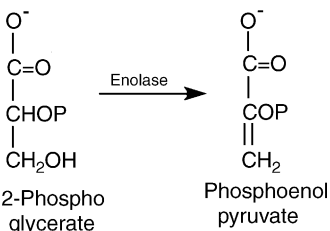
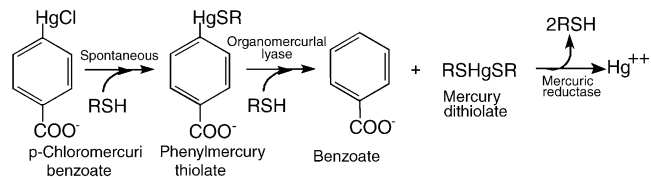
3	Hydrolases	Adds or removes H ₂ O	
3.1.1 [163]	Lipases Enzymatic cleavage of glycerol from triacetin and reaction monitored at 570 nm. Glycerol then determined by standard curve		Assay vol: 5.0 ml; phosphate buffer, 0.1 M, pH 7.5; triacetin, 1% w/v; enzyme 1.0 ml; H ₂ SO ₄ , 5 M; NaIO ₄ , 0.1 M; NaHSO ₃ 50 mM; chromotropic acid, 2.5 ml 1 U activity = forms 1 μmol glycerol per minute
3.1.3	Phosphatases Enzymatic cleavage of ρ-nitrophenolphosphate (ρ-NPP) to ρ-nitrophenol (ρ-NP) to generate ion, measured at 405 nm, in alkaline solution		Assay vol: 5.0 ml; enzyme 1.0 ml; ρ-NPP, 60 μM; acetate buffer, 10 mM, pH 8, 25 °C, 20 min; NaOH, 0.5 M added 1 U of activity = conversion 1 μmol ρ-NPP in 1 min
3.1.6	Sulphatases Enzymatic cleavage of ρ-nitrophenolsulphate (ρ-NPS) to ρ-nitrophenol (ρ-NP) to generate ion, measured at 405 nm, in alkaline solution		Assay vol: 5.0 ml; enzyme 1.0 ml; ρ-NPS, 60 μM; acetate buffer, 10 mM, pH 8, 25 °C, 20 min; NaOH, 0.5 M added 1 U of activity = conversion 1 μmol ρ-NPS in 1 min
3.2.1 [164]	Glucosidases Methylumbelliferylglucopyranoside [MUG] is hydrolysed to methyl umbelliferone [MUF]		Assay vol: 5.5 ml; glycine buffer, 0.4 M, pH 10.8; MUG, 1.5 mM; enzyme 1.0 ml; 30 °C, 5 min; EtOH, 95%, 0 °C Fluorescence measured at 365 nm [excite] and 455 nm [emission]
3.4 [165]	Proteases A ₄₄₀ of TCA soluble peptides from enzymatic cleavage of azocasein		Assay vol: 5.5 ml; enzyme 1.0 ml; phosphate buffer, 0.1 M pH 7.5; azocasein, 2% w/v, 37 °C, 60 min; trichloroacetic acid (TCA), 10% w/v, 0 °C added; centrifuged, 4000 × g, 10 min 1 U activity = μmol product that would increase A ₄₄₀ by 1 in 60 min

Table 2 (Continued)

No. [Ref]	Classification; Properties	Reaction	Reagents; Assay
3.5.4 [78]	Acting on carbon nitrogen bonds of cyclic amidines	 <p>Formyltetrahydro-methanopterin</p> <p>Methenyltetrahydro-methanopterin</p>	<p>Assay vol: 700 μl; Tricine-KOH, pH 8.0, 50 mM; K_2HPO_4 pH 8.0, 1.5 M; methenyl-H_4-MPT⁺, 30 μM; enzyme 50 μl.</p> <p>Activity measured in the direction of formyl H_4 MPT and monitored by decrease at 335 nm due to disappearance of methenyl H_4-MPT⁺</p> <p>1 U of activity = 1 μmol methenyl H_4-MPT⁺ hydrolysed to formyl H_4 MPT per minute</p>
3.8.1	Acting on C-halide bonds Dehalogenase	 <p>Dichlorobenzene</p> <p>Chlorobenzene</p> <p>Benzene</p>	<p>Assay vol: 1.0 ml; chlorobenzene, 0.3 pmol; phosphate buffer, pH 7.4, 300 pmol; enzyme 200 μl; reduced glutathione, 13 pmol, 0 °C, in phosphate buffer, 0.1 ml</p> <p>Enzyme activity monitored as ΔA_{260} per unit time</p>
4	Lyases	Adds or removes a group other than H_2O .	
4.1.1.5	Carboxy lyase	 <p>Acetolactate</p> <p>Acetoin</p>	<p>Assay vol: 1.0 ml; phosphate buffer, pH 6.0, 50 mM; acetolactate 10 mM; enzyme 100 μl. After 5 min reaction quenched with NaOH, 0.5 mM</p> <p>Acetoin measured at 522 nm with naphthol, 1% + creatine 0.5% in 1 M NaOH. Aliquot, 400 μl + reagent 4.6 ml</p> <p>1 U of activity = 1 μmol acetoin produced per minute</p>
4.1.2	Aldehyde lyase	 <p>Fructose 1,6 diphosphate</p> <p>Glyceraldehyde 3-phosphate</p> <p>Dihydroxyacetone phosphate</p>	<p>Assay vol: 3.1 ml; fructose-1,6-biphosphate 12 mM; hydrazine sulphate 3.5 mM; EDTA, pH 7.5, 10 μM; enzyme 100 μl</p> <p>Activity monitored at 240 nm due to formation of hydrazone with 3-phosphoglyceraldehyde</p>
4.2.1	Carbon oxygen lyase	 <p>2-Phosphoglycerate</p> <p>Phosphoenolpyruvate</p>	<p>Assay vol: 1.0 ml; Tris-HCl, pH 8.0, 100 mM; $MgSO_4$ 2 mM; 2-phosphoglycerate 5 mM; enzyme 100 μl</p> <p>Activity monitored from formation of phosphoenol-pyruvate at 240 nm</p>

4.99.1.2
[124,125,
162,166]

p-Chloromercuribenzoate
(pCMB) absorbs at 250 nm
which shifts as it is cleaved
by organo-mercurial lyase
into Hg (II) and benzoate



Assay vol: 1.0 ml; Tris-HCl, pH 7.5, 50 mM; p-CMB, 100 μ M;
L-cysteine, 1.0 mM; enzyme 100 μ l

Enzyme activity is measured as ΔA_{250} with respect to time

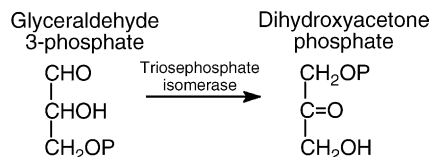
5

Isomerases/mutases

Catalyse geometric/structural changes within a molecule

5.3.1

Intramolecular
oxidoreductases



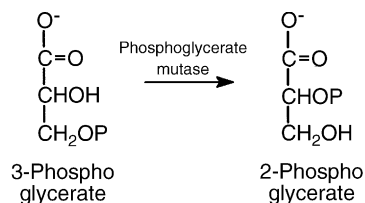
Assay vol: 1.0 ml; HEPES buffer, pH 7.3, 100 mM; NADH 0.5 mM;
glyceraldehydes-3-phosphate 4 mM; glycerol-phosphate
dehydrogenase 4 U, enzyme 50 μ l

Interconverts aldoses and
ketoses

Assay monitored by change in A_{340}

5.4.2

Intramolecular transferases



Assay vol: 1.0 ml; HEPES buffer, pH 7.4, 25 mM; NADH 0.25 mM;
3-phosphoglycerate 5 mM; MgSO₄ 1 mM; ADP 1 mM; enolase 1 U;
pyruvate kinase 1 U; lactate dehydrogenase 1 U; enzyme 100 μ l

1 U activity = converts 1 μ mol 3-phosphoglycerate to
2-phosphoglycerate per minute

Assay monitored at 340 nm

6

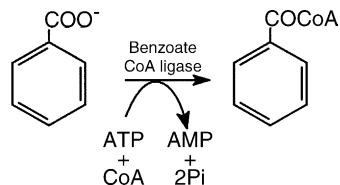
Ligases

Joins groups together

6.2.1
[88,167]

Forming acid-thiol bonds

Formation of AMP is coupled
enzymatically to myokinase
(1) pyruvate kinase (2) and
lactate dehydrogenase (3)



Assay vol: 1.0 ml; MgCl₂, 2.5 mM; ATP 0.5 mM; CoASH, 0.25 mM;
KCl, 10 mM; PEP, 10 mM; NADH, 0.35 mM; 2 U of myokinase,
pyruvate kinase, lactate dehydrogenase

Activity monitored by decrease A_{340}

AMP + ATP \rightarrow 2ADP (1)

2ADP + 2PEP \rightarrow 2 pyruvate + ATP (2)

2 pyruvate + 2NADH \rightarrow 2 lactate + NAD (3)

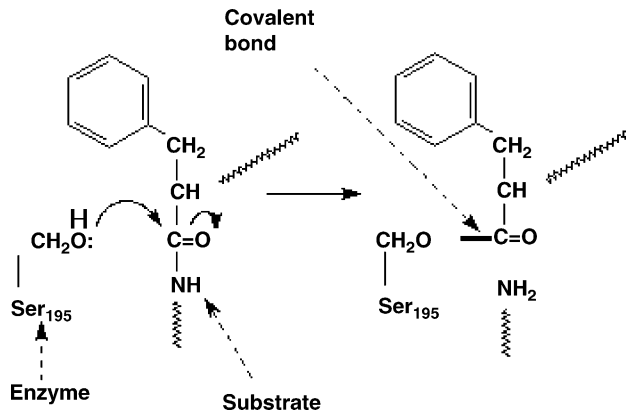


Fig. 7. Acyl covalent bond formation between an enzyme-serine and typical substrate.

the enzyme, which provides specific, high affinity binding of substrates and provides a favourable environment for catalysis.

After enzyme–substrate binding one, or more mechanisms of catalysis generates transition-state complexes and reaction products. (a) Destabilisation of ES complex may take place either by strain, distortion or desolvation. The zone of solvation around the substrate, while it is in solution, is lost as the substrate binds to the enzyme. The induced structural rearrangements that take place with the binding of substrate and enzyme ultimately produce strained substrate bonds, which more easily attain the transition state. (b) The substrate and enzyme when separate in solution are free to undergo translational motion leading to a disorientated high entropy situation while in the form of the ES complex they are both restricted and possess low entropy. (c) The substrate is orientated within the active site on the enzyme forming a covalent enzyme–substrate intermediate (Fig. 7) [30]. (d) General acid/base catalysis may occur where there is a transfer of a proton in the transition state either specifically by an acid or generally by some acidic/basic amino acid. (e) Some enzymes require metal ions to act as electrophilic catalysts that stabilise the increased electron density. (f) Enzyme substrate interactions induce strain in the substrate and orientate reactive groups into proximity with one another.

3.5. Cofactors/coenzymes

In many cases, a second component (cofactor, coenzyme or metal-ion activator) needs to be present on the active enzyme

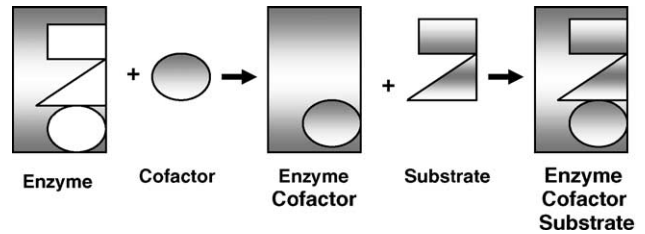


Fig. 8. Binding of substrate and cofactor to an enzyme.

before catalysis can occur. The apoenzyme is a term given to the protein portion of the inactive enzyme that initially binds to a prosthetic group, coenzyme or metal ion to produce the active holoenzyme. Even if the substrate is present at the active region of the enzyme catalysis does not occur until the second component is present (Fig. 8) [31].

4. Enzymology of biological remediation

4.1. Aerobic digestion

In the process of activated sludge flocc-forming microbes degrade wastewater sludge, pollutants or any organic material, under aerobic conditions, to effect a biosolid–liquid separation. While a fraction of the organic material is used for the synthesis of new microorganisms, resulting in an increase in biomass, the remaining material is channeled into metabolic energy and oxidized to carbon dioxide, water, nitrates, sulphates and phosphates to provide energy for both synthesis and cellular functions (Fig. 9). The settled biosolids are subsequently recycled to aeration tanks in order to maintain the biomass concentration and the supernatant is discharged. Once the organic waste material becomes exhausted then the organisms will begin endogenous respiration to oxidize cellular material. Unfortunately, a disadvantage of an aerobic treatment is the production of large amounts of biosolids (sludge) which contains volatiles, organic solids, nutrients, pathogens, heavy metals, inorganic ions, toxic organic chemicals and the original problem of dissolved organic waste is now transformed into a problem of particulate waste. Aerobic respiration is defined as the aerobic catabolism of nutrients to carbon dioxide and water involving glycolysis, the tricarboxylic cycle, an electron transport system and molecular oxygen as the final electron acceptor: this type of aerobic digestion is notable in organisms that require molecular oxygen,

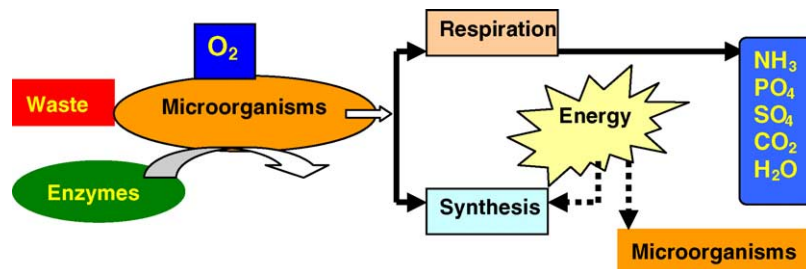


Fig. 9. Aerobic digestion of waste involving enzymes and microorganisms.

and facultative anaerobes that are capable of aerobic respiration but can switch to fermentation if oxygen is unavailable. Typical assays for enzymes associated with this aerobic digestion (dehydrogenase EC. 1.1.1; kinase EC. 2.7.1; aldolase EC. 4.1.2; enolase EC. 4.2.1; isomerase EC. 5.3.1; mutase EC. 5.4.2, are listed; Table 2).

4.2. Anaerobic digestion

By definition anaerobic digestion is the breakdown of organic material into biogas (CH_4 , CO_2) and in which a molecule other than oxygen is the final electron acceptor. For example, sulphate-reducing bacteria transfer electrons to sulphate (SO_4^{2-}) reducing it to H_2S , while others (nitrate reducers) transfer the electrons to nitrate (NO_3^-) reducing it to nitrite (NO_2^-), nitrous oxide (NO) or nitrogen gas (N_2). Alternative electron acceptors are Fe^{III} and Mn^{IV} . It is critical that an understanding of the conditions, under which anaerobic digestion is taking place, be made before there can be any assessment of bioremediation potential. Amongst the many treatment technologies available, anaerobic treatment process has proved to be unique and the most beneficial stabilisation technique as it optimizes cost effectiveness, it is environmentally sound, minimises the amount of final sludge disposal and has an ability to produce a net energy gain in the form of methane gas [32]. The anaerobic treatment technology currently available, however, is only capable of partially treating waste in a conventional wastewater treatment system with high levels of degradation requiring longer retention times and/or further treatment methods [33].

It is commonly accepted that there are four distinct steps: (a) complex organic matter is decomposed into simple soluble organic molecules using water (hydrolysis) and hydrolyase enzymes (glucosidases, lipases, proteases, sulphatases, phosphatases); (b) chemical decomposition of these single

monomeric unit molecules (monosaccharides, amino acids, fatty acids, glycerol) into volatile fatty acids by a process termed acidogenesis; (c) acetogenic fermentation into acetate, H_2 and CO_2 ; (d) methanogenic conversion into CH_4 and CO_2 (Fig. 10).

4.2.1. Hydrolysis

Wastewater treatment bioreactors are complex ecosystems that contain a wide variety of organic substances and a mixed culture of heterogeneous microbial populations that effect sequential substrate removal when complex substrates are degraded. In such mixed cultures, sulphate-reducing bacteria (SRB) are able to compete, in the presence of sulphate, with methanogenic bacteria (MB) and acetogenic bacteria (AB) for the available substrates [34–36]. Biological anaerobic wastewater treatment systems, in which complex organic matter is completely degraded by SRB are a promising alternative for the methanogenic wastewater treatment systems [37] and the complex physico-chemical sulphate removal methods [38]. In conventional methanogenic anaerobic digestion systems, solubilisation rates for primary sewage sludge (PSS) are reported at about 10 days in yields of 5–10% [39–43] while enhanced degradation of 52% has resulted under sulphidogenesis [44]. There is still no consensus, however, on the mechanism of enhanced hydrolysis though the key might be the activation of the hydrolytic enzymes through a sulphide gradient generated in situ during sulphate reduction [45].

Under anaerobic conditions the simple organic electron donor molecules, e.g. lactic acid, are used by the SRBs such as *Desulfovibrio* and *Desulfuromonas* to reduce sulphate to hydrogen sulphide and bicarbonate with a resultant increase in pH (Eq. (8)). Soluble metal salts react with H_2S in situ to produce insoluble metal sulphides, thereby reducing the concentration of metals and salts to acceptable levels (Eq. (9)). Bicarbonate ions react with protons to form CO_2 and water, thus removing the ‘acidity’

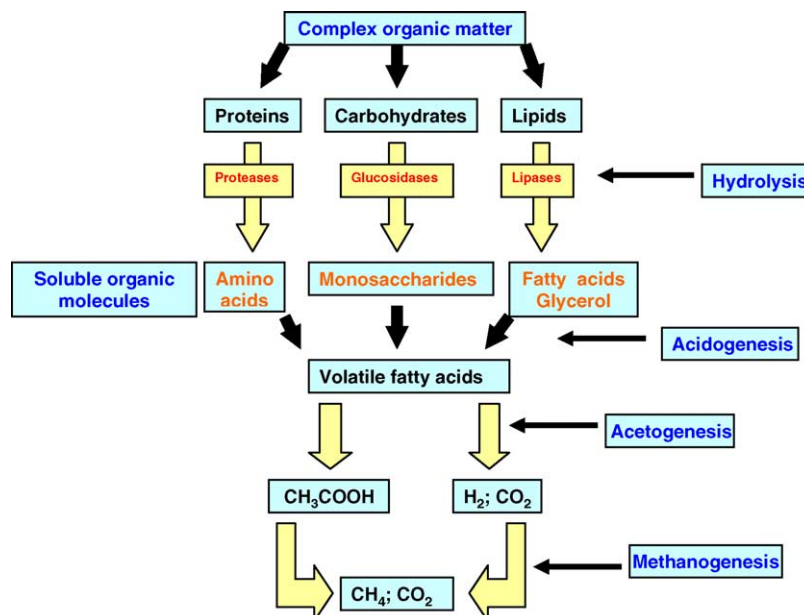
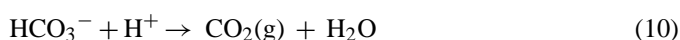
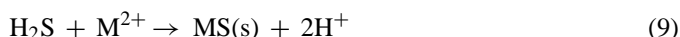
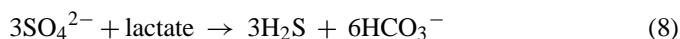


Fig. 10. Anaerobic digestion of complex organic matter into methane and carbon dioxide.

from solution as CO₂ gas (Eq. (10)).



Sulphate-rich wastewater is produced by many industries such as sulphuric acid in food processes, thiosulphate in the photographic industry, sulphite in tanneries, the sea-food processing industry, the leaching of sulphur rich soils in land fills and mines and by power-plant flue gases from the combustion of sulphur containing fuel [46].

Several enzymes in anaerobic sulphate reduction are worthy of mention. Adenosine 5'-phosphosulphate (APS), synthesized from sulphate and ATP with the enzyme ATP sulphurylase (EC. 2.7.7, Table 2), serves as the nucleoside sulphate donor in this process, and therefore plays a crucial role in sulphate activation, the key step for sulphate utilisation [47–50]. APS is then broken down into sulphite and adenosine monophosphate (AMP) by APS reductase (EC. 1.8.99, Table 2) followed by a further reduction to sulphide by sulphite reductase (EC. 1.8.99, Table 2) [51].

In an extensive study on the enzymology within an anaerobic biosulphidogenic reactor [49,52–55], it was established that the enzymatic activities associated with sludge solubilisation and enhanced hydrolysis of complex polymeric organic carbon are found predominantly associated with the organic particulate network. Disruption of this network increases the susceptibility of the macromolecules, entrapped within the floc and hence protected from enzymatic degradation, to be attacked by the hydrolytic enzymes that would ultimately lead to enhanced solubilisation of sludge. The rate at which enzymatic hydrolysis proceeds is best described by first order kinetics as it is rate-limiting and is strongly influenced by environmental and operational parameters such as pH, temperature, biomass, particle/floc size, type and concentration of particulate substrate and production concentration and by the reaction between the enzyme and its substrate [56]. Hydrolysis of these particulate organics is also enhanced in the presence of sulphide. The latter, apart from being a strong reducing agent and capable of reducing disulphide linkages that are essential for maintaining the three dimensional conformational structure, is also shown to increase the specific activity of all hydrolytic enzymes by nearly 5–10-fold [52–55]. Since the biopolymers (proteins, carbohydrates and lipids) are the major particulate organic fractions in sludge [57–59] the activities of the ubiquitous enzymes like lipases (EC. 3.1.1, Table 2), phosphatases (EC. 3.1.3, Table 2), sulphatases (EC. 3.1.6, Table 2), α -, β -glucosidases and cellulases (EC. 3.2.1, Table 2), and proteases/peptidases (EC. 3.4, Table 2), are equally important in the hydrolysis process. It is proposed that the products of biological sulphate reduction both directly and indirectly facilitate the contact between enzyme and substrate thereby enhancing overall enzyme activity. This is due to the neutralisation of the ions on the floc surface by sulphide, sulphite and associated bicarbonate and hydroxide ions, destroying the overall integrity of the floc structure and exposing

more substrate for enhanced enzyme activity [60]. Alternatively, the sulphite and sulphide species liberated during the sulphate reduction process, interact directly with the enzymes on the floc surface thereby enhancing their activity.

The initial development of anaerobic treatment processes, over a century ago, was for the treatment of domestic wastewaters, using anaerobic filters and hybrid processes that are still of interest today. Its applications then expanded to include separate sludge digestion and then to industrial wastewater. Biological stabilisation is widely considered to be one of the most attractive methods of reducing the major portion of the organic fraction in sludge, and anaerobic processes are favoured over aerobic digestion owing to the cost of aeration, the ability of anaerobic systems to maintain their temperature and the value of methane as a renewable energy source.

4.2.2. Acidogenesis

During this phase simple monosaccharides are converted via a series of acidogenic enzymes and the glycolytic pathway to ATP, NADH and pyruvate which is regarded as a key metabolite in anaerobic digestion. It is metabolized into acetyl CoA and formate through formate C-acetyltransferase (pyruvate formate lyase) (EC. 2.3.1.54, Table 2) [61,62] or into lactate via reduction with lactate dehydrogenase (EC. 1.1.1, Table 2). In butanediol fermentation, two pyruvates are condensed with acetolactate synthase (EC. 2.2.1.6, Table 2) [63] to yield CO₂ and acetolactate which is decarboxylated to acetoin (EC. 4.1.1.5, Table 2) [64] and eventually to 2,3-butanediol via a dehydrogenase [65]. Formate is converted by formate hydrogen lyase [66] to H₂ plus CO₂ while acetyl CoA is either reduced to acetaldehyde via acetaldehyde dehydrogenase and then to ethanol via alcohol dehydrogenase or converted to acetate via the high energy acetyl phosphate. Alternatively, two acetyl CoA molecules may condense producing acetoacetyl-CoA that can lose coenzyme A and CO₂ to form acetone [67]. An initial transcarboxylase (EC. 2.1.3.1, Table 2) reaction with methylmalonyl SCoA results in a conversion of pyruvate into oxaloacetate with a concomitant release of propionyl CoA. The final step is the release of propionic acid when CoASH is transferred to succinate [68]. Succinate can also be converted to succinyl-SCoA, and then back to methylmalonyl-SCoA via methylmalonyl-SCoA mutase, a Vitamin B₁₂ containing enzyme [69].

After an initial activation as their coenzyme A derivatives the long chain fatty acids are enzymatically degraded by sequential removal of two-carbon units liberating, after each cycle, acetyl CoA, which enters the metabolic pool and a fatty acid with two carbons less (EC. 2.3.1.16, Table 2). Propionyl CoA is the ultimate product in the case of a fatty acid with odd-numbered carbon atoms.

It is not the intention within the current article to give an in-depth analysis of anaerobic amino acid degradation and readers are directed to two excellent reviews [70,71]. All twenty amino acids are first enzymatically deaminated, via respective transaminases (EC. 2.6.1, Table 2), before their carbon skeletons are subsequently degraded into seven different metabolites—pyruvate, acetyl CoA, acetoacetyl-CoA, α -ketoglutarate, propionyl CoA, fumarate and oxaloacetate.

4.2.3. Acetogenesis

Acetogenic enzymes convert the products of the acidogenic step into acetate, H₂ and CO₂ that ultimately are the substrates for the methanogenic breakdown.

4.2.4. Methanogenesis

This constitutes the enzymatic production of methane from acetate or from a reduction of CO₂ (Eqs. (11) and (12)). Molecular hydrogen or reduced coenzyme F₄₂₀ are the electron donors with mixed disulphides (coenzymes M and B) as electron acceptors. Eight enzymes and a formyl, methenyl and methyl tetrahydromethanopterin are involved in the reduction of CO₂ [72].



Initially, methanofuran interacts with CO₂ with assistance of formylmethanofuran dehydrogenase (EC. 1.2.99, Table 2) [73] to produce formylmethanofuran. Coupled with tetrahydromethanopterin and a series of enzymes involving a transferase, a *cyclo*-hydrolase (EC.3.5.4, Table 2) a dehydrogenase and a reductase (EC.1.5.99, Table 2) [74–78] the C₁ unit is carried, via coenzyme F₄₂₀ into methyltetrahydromethanopterin. In the final step, the C₁ unit is associated with coenzyme M and a transferase enzyme (EC. 2.1.1.86, Table 2) before being reduced to methane [79]. Coenzyme B (7-mercaptoheptanoylthreoninephosphate) reacts with methyl coenzyme M liberating methane and coenzyme-M-S-S-heptanoylthreonine phosphate under the influence of methyl reductase (EC. 2.8.4.1, Table 2) [80].

A slight variation is reported [81] for the anaerobic enzymatic conversion of acetate to methane (Eq. (12)). The acetate, that is first activated as its coenzyme A derivative, is oxidised to CO₂ with carbon monoxide dehydrogenase while its methyl group is transferred via tetrahydrosarcinapterin to coenzyme M. This methylcoenzyme M is reductively demethylated with coenzyme B to methane and the resulting heterodisulphides converted back to their sulphhydryl forms.

4.3. Bioremediation/biodegradation

Polycyclic aromatic hydrocarbons (PAHs), widely distributed in the environment mainly from anthropogenic activities, are organic chemicals that are cytotoxic, mutagenic and carcinogenic. Aerobic degradation of PAHs is a benign process that involves the oxidation of the aromatic ring by specific dioxygenases and a complete biotransformation into carbon dioxide and water. Naphthalene, one of the simplest PAHs, serves as model for understanding their biodegradation. Naphthalene-1,2-dioxygenase (EC. 1.14.12, Table 2) catalyses the oxidation, with molecular oxygen, of naphthalene to *cis*-1,2-dihydroxy 1,2-dihydronaphthalene [82] while salicylate hydroxylase (monooxygenase) (EC.1.14.13, Table 2) oxidatively decarboxylates salicylate to catechol and carbon dioxide [83–85]. Both of these enzymes are assayed by measuring the decrease in absorbance at 340 nm due to the consumption of NADH in the presence of substrates.

All of the BTEX compounds (benzene, toluene, ethylbenzene and xylene) as well as the polychlorobenzenes (PCBs) have at least one aerobic pathway that includes degradation to a substituted catechol. Benzene is degraded to catechol; toluene has many separate biodegradative pathways, some of which include 3-methylcatechol as an intermediate product; ethylbenzene is degraded to 3-ethylcatechol; xylenes are all metabolized to mono-methylated catechols and the chlorobenzenes to chlorocatechol. In each of these cases, the aromatic ring of the substituted catechol is later cleaved by either an intradiol (ortho-cleaving) (EC.1.13.11.1, Table 2) catechol 1,2 dioxygenase or extradiol (metacleaving) catechol 2,3-dioxygenase enzyme (EC.1.13.11.2, Table 2). Subsequent metabolic pathways lead to acetaldehyde, acetate, succinate, pyruvate and eventually CO₂ and H₂O.

It has recently been reported [86] that sulphate reducers are capable of degrading a wide variety of PAHs by modification of the aromatic ring—in particular demethylation, dehalogenation, reduction, removal of amino, hydroxyl and methoxyl groups as these processes detoxify potential xenobiotics [87–92]. In all of these enzymatic degradative pathways, the intermediate is an aromatic carboxylate coenzyme A thioester, formed through a benzoate CoA ligase (EC. 6.2.1.25, Table 2), leading to ring reduction, hydration, dehydrogenation, ring cleavage to 3-hydroxypimelyl CoA and eventually to acetyl CoA [93]. Naphthalene, toluene, ethylbenzene and xylene follow similar pathways initially involving a carboxylation of the aromatic ring while with the biodegradation of halobenzenes a series of dehalogenations with dehalogenases (EC. 3.8.1, Table 2) take place.

To date (2004), there are over 1000 enzymes reported to be involved in the biodegradation of aromatic systems, whether they be organic pollutants or not and it is beyond the scope of this paper to explore this any further. A description of all of these alternate pathways has appeared [94].

4.4. Biopulping/biobleaching

Biopulping is the treatment of lignocellulosic materials with oxidative lignin-degrading fungal enzymes prior to thermomechanical pulping. This biological process is reputed to increase paper-strength and reduce both chemical energy consumption and environmental impact. These enzymes, which include lignin and manganese peroxidase and laccase, are responsible for the oxidative biodegradation of PAHs (anthracene, benzo[a]pyrene) [95–101] into CO₂ and H₂O. Lignin peroxidase (LiP) (EC.1.11.1, Table 2) use hydrogen peroxide to catalyze one-electron oxidations of phenolic and non-phenolic compounds leading to alkyl aryl cleavage, aromatic ring cleavage, demethylation, hydroxylation and polymerization while manganese peroxidase (MnP) (EC.1.11.1, Table 2) catalyze the Mn-mediated oxidation of lignin and phenolic compounds.

Laccases [*p*-diphenol-dioxygen oxidoreductase] (EC.1.10.3.2, Table 2) belong to the group of enzymes called blue copper oxidases that catalytically oxidise phenols [102] or chlorinated biphenyls with a four-electron reduction of O₂ to H₂O. Artificial

substrates such as ABTS (2,2'-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid), HBT (hydroxyl benzotriazole) or violuric acid can act as mediators enabling the oxidation of non-phenolic compounds which cannot be oxidized by laccases on their own, thereby expanding the range of applications of these enzymes [102].

It has been realized that thermostable xylanases can be implemented in biobleaching. [103] The process of lignin removal from chemical pulps to produce bright or completely white finished pulp is called 'bleaching.' Present-day bleaching of kraft pulp uses large amounts of chlorine-based chemicals and sodium hydrosulfite that produce toxic, mutagenic, persistent chemicals that bioaccumulate in biological systems. The main enzyme needed to enhance the delignification of kraft pulp is endo-xylanase, but enrichment with other enzymes such as mannanase, galactosidase, arabinosidase, glucosidase and xylosidase have been shown to improve the effect of enzymatic treatment of kraft pulp [104–107]. The enzyme does not bleach pulp, but rather changes the pulp structure thereby opening it up for further degradation. The cleavage of the carbohydrate portion of lignin-carbohydrate complex to produce smaller residual lignin molecules, which are easier to remove, is also a possible mechanism of xylanase prebleaching.

The decolourisation of textile dyes may also be regarded as a biobleaching process and the biological enzymatic treatment of industrial wastewater dyes remains one of the most challenging. Extensive studies are forthcoming on dye decolouration by non-specific extracellular oxidative enzymes (lignin peroxidase, manganese peroxidase, copper-containing phenol oxidase) from white-rot fungi [108–113] though these all have limited potential in both efficiency and reliability. Aromatic azo-dyes, in particular, are environmentally toxic and mutagenic and their enzymatic degradation involves the anaerobic reduction of the azo bond ($-N=N-$), with azo-reductases followed by an aerobic biotransformation of the generated aromatic amines into CO_2 , NH_3 and H_2O . Mechanistically azo reductases (EC.1.7.1.6, Table 2) which may be classified as flavoenzymes [114], may either transfer reducing equivalents such as NAD(P)H to the azo dye directly or through redox mediators (RM) like methyl viologen, menadione, neutral red, janus green [115], anthroquinone-2-sulphonate (AQS) or anthroquinone-2,6-disulphonate (AQD) (EC.1.7.1.6, Table 2) [116].

4.5. Biobleaching

This is described as the extraction of metals from their respective ores by biotechnological processes and enzyme based technologies for metal removal present an economic alternative for today's mining, mineral and waste water treatment industries.

The role of enzymes in a biobleaching process may be associated with either: (i) active transport ATPases, e.g. cadmium, copper, arsenate or (ii) direct enzymatic removal, e.g. mercury, iron, chromate [117,118]. With respect to the former the best characterized system is that for the essential metal ion Cu(II) and its concentration in the cell is delicately balanced by means of CopA, CopB, CopC, CopD, CopY and CopZ ATPases [119].

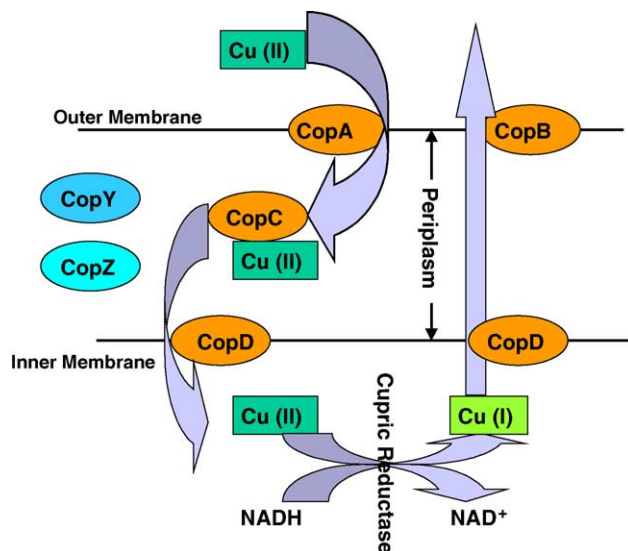


Fig. 11. Copper transport in *Pseudomonas syringae* [119].

Extracellular copper in the form of Cu(II) reaches the periplasmic space (Fig. 11) through the porous outer membrane assisted by CopA. A second ATPase, CopC, transports the copper to the inner membrane and to CopD that allows the copper to enter the cytoplasm. Here the Cu(II) is reduced to Cu(I) via a NADH-cupric reductase system. Within the periplasm are two blue copper-binding proteins—CopY (repressor) and CopZ (chaperone) that sense levels of copper and switch off (or on) the respective operons. This allows for the export of Cu(I) through CopB [120,121]. The assay for cupric reductase is reported [122] (EC.1.16.1.1, Table 2).

Enzymatic processes, for the removal of metal ions from sludge waste effluent streams, use metal-desolubilising ligands on a continuous basis with extracellular or intracellular deposition of metals as a metal-ligand precipitate. Metal reduction usually results in the precipitation of low valence, reduced, forms of metals, and has therefore been proposed as a strategy to treat water contaminated with a range of metals and radionuclides. Mercury is a model example of an enzymatic removal process as it lends itself to inactivating essential thiols that are part of enzymes and proteins [123]. Some bacteria (*Staphylococcus*, *Bacillus*, *Escherichia*, *Pseudomonas*, *Serratia*, *Thiobacillus*) contain a set of genes that form a Hg(II) (*mer*) resistance operon which not only encodes for the production of a periplasmic metal binding enzyme that collects Hg(II) from the surrounding environment but also for a membrane associated transport enzyme that carries the Hg to the cytoplasm for removal. The first, organomercurial lyase (EC.4.99.1.2, Table 2) [124,125] is effective in hydrolyzing the stable mercury-carbon bond by binding Hg(II) in the active site with cysteine sulphhydryl residues. The mercury thiolate product is then utilized as a substrate for mercuric reductase (EC.1.16.1, Table 2) that contains the cofactor flavin in each of its subunits [121] and reduces Hg(II) to Hg(0) (metallic mercury), a process that involves hydride transfer from the electron carrier NADPH to flavin. Metallic mercury is then released to diffuse through the cell membrane and into the surrounding environment.

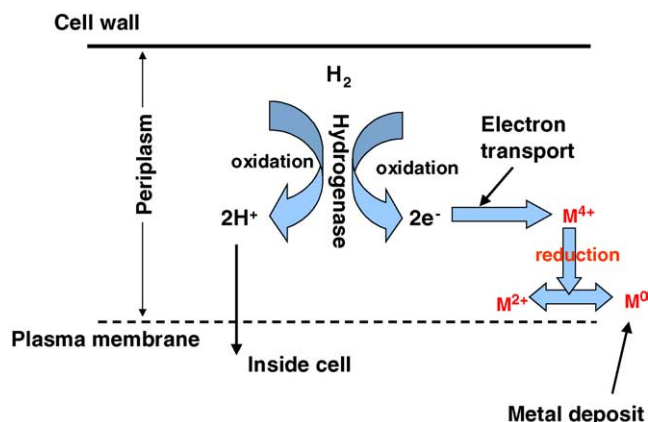


Fig. 12. Scheme of metal reduction by hydrogenase in sulphate reducing bacteria.

Chromium (VI) salts originate from industrial sources such as leather tanning, photographic-film making, wood preservation, car manufacturing, petroleum refining and agricultural activity [126]. Its reduction can occur under aerobic conditions using a soluble chromium reductase enzyme with either NADH or cytochrome c_3 to supply electrons [127,128] (EC.1.97.1, Table 2) or under anaerobic conditions with a hydrogenase or cytochrome c_3/c_7 type enzymes [129,130] (Fig. 12). The majority of hydrogenases are metalloenzymes, and their metal sites belong to two main categories. First, the classical [2Fe–2S], [3Fe–4S] and [4Fe–4S] iron sulphur clusters that shuttle electrons between the H_2 -activating site and the redox partners of hydrogenases [131] and second the H_2 -activating sites that appear as [NiFe], [Fe] [132] or metal free. The [4Fe–4S] cluster that is proximal to the active site is essential to H_2 activation in [NiFe]-hydrogenases [133–137]. Protons generated from the oxidation of molecular hydrogen by hydrogenases are used for metabolic activities of the cell while the electrons released are channeled away to an electron acceptor through an electron transport chain [138] (Fig. 13). Ordinarily, this electron acceptor is the sulphate ion, but if these bacteria are presented with certain metal ions, they will use these as the electron sink,

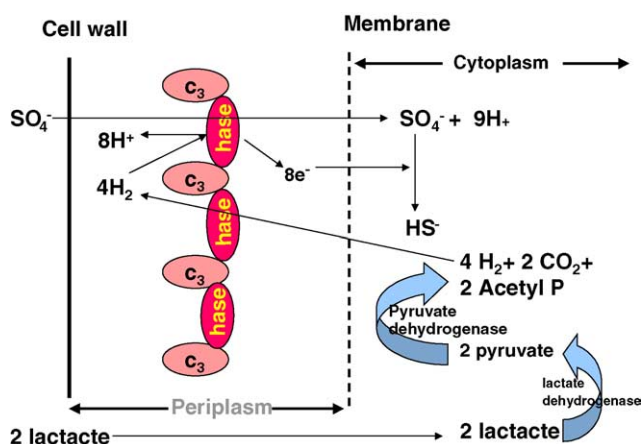


Fig. 13. Hydrogen cycling in *Desulphovibrio vulgaris* (c_3 , cytochrome c_3 ; Hase, hydrogenase) [138].

leading to the reduction of the base metal, which is then precipitated from solution [139,140]. On the other side, lactate enters the cell and is oxidized to pyruvate by lactate dehydrogenases located on the internal aspects of the cytoplasmic membrane or cytoplasm [140]. Pyruvate is then converted to carbon dioxide, acetyl phosphate and electrons that are used to reduce protons to molecular hydrogen. The bioremediation of vanadium [141], uranium [142], cobalt and manganese [143], iron [144] and technetium [145–147] are reputed to follow similar enzymatic pathways.

Many explosives, such as trinitrotoluene (TNT) are enzymatically biodegraded either by an aerobic removal of the aromatic nitro groups [148] or by an anaerobic reduction to nitroso, hydroxylamino or amino groups [149,150]. In both cases, there is concomitant oxidation of NAD(P)H (EC.1.7.2.2; 1.7.99.3, Table 2). Explosives of this kind maybe the sole source of nitrogen in various anaerobic nitrate reducers that use nitrate (NO_3^-), nitrite (NO_2^-), nitric oxide (NO) and nitrous oxide (N_2O) as terminal electron acceptors. NADPH dependent nitrite reductase, which may either be a heme cytochrome cd1 (EC.1.7.2.2) or copper containing enzyme (EC.1.7.99.3) [151,152] has been isolated from *Thiosphaera pantotropha* and *Paracoccus denitrificans* while a [Fe] only hydrogenase has been found in *Clostridium acetobutylicum* [153].

The Gram-negative eubacterium *Thiobacillus ferrooxidans* is important for industry because it is able to solubilize copper, uranium, cobalt and iron metals from ores and contaminated industrial effluents by enzymatic oxidation. Two indirect oxidation mechanisms for this bioleaching exist. The one mechanism is exclusively based on the oxidative attack of iron(III) ions on the acid-insoluble metal sulfides, through a tentative thiosulphate oxidase and thiosulphate intermediate while the second mechanism allows for a dissolution by an attack of iron(III) ions and/or by protons with sulphur dioxygenase (EC.1.13.11.18, Table 2) and a polysulphide as an intermediate [154–156].

5. Concluding comments

The development and preparation of novel enzymes for use in biological remediation or for the industrial solubilisation of activated sludge remains a key challenge and a safe and economic alternative to commonly, and perhaps now redundant, physico-chemical strategies. There are perhaps two approaches: rational and evolutionary. With the former, amino acid sequences, functional properties and structural features of different enzymes are compared, combined, then tested to see if the desired effect is accomplished. In the evolutionary design, a large library of random mutations in proteins is made followed by a selection of enzymes that work well with a particular contaminant. In principal, multiple environmental factors would ‘select’ enzymes to meet these challenges. Molecular evolution [157,158] is a useful tool for evolving enzymes with extended substrate specificities for any recalcitrant pollutant. Furthermore, this technology is more likely to ‘succeed’ than rational approaches as the latter requires multiple sets of structural and biochemical information on every enzyme involved. Sequences encoding specific enzymes can be retrieved direct from environmental samples

thereby circumventing the process of isolating and screening wild-type organisms. Degenerate primers can be used to amplify central segments from these genes by PCR and inserted into the original functional gene. Such an approach allows rapid exploitation of the natural sequence diversity already present in the environment for creation of novel hybrid enzymes [159]. With the advent of molecular engineering the principle of developing a new “designer” enzyme and the creation of micro assemblers or microchips with the role of the computer as a delivery vehicle cannot be too far into the future.

One major feature to consider is to generate a new novel structure for use in activated floc solubilisation. In view of the extreme conditions that the activated sludge digesters may operate, the new enzyme molecules often have to be stable and active under unusual and extreme conditions of temperature, acidity, solvents, chemicals and pH. Enzyme properties, can be exploited to engineer active-site topology, to enlarge binding pockets and to alter the substrate specificity and stability. Consequently, the ability to modify a protein or structure to make it more stable to such conditions, or make it more resistant to self destruction, or make it target directed and functional in the presence of other toxic elements creates enormous challenges for enzymologists. Over the next 20 years, the enzyme–floc model will be exploited at a molecular level from a rational design to specific delivery of enzymes to the active areas disguised in vectors called nanoparticles. These will be the tools and scientific technological platforms for the investigation and transformations of any activated wastewater or biological system.

Under these pretexts biological remediations can only be rationalised by specific finite measurements, for each floc enzyme in the study, of maximal enzymatic rate (V_{max}), substrate specificity (K_m), turnover number (k_{cat}), enzyme efficiency (k_{cat}/K_m).

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