Antifouling enzymes and the biochemistry of marine settlement

Jakob Broberg Kristensen\textsuperscript{a,b}, Rikke Louise Meyer\textsuperscript{a,c}, Brian Søgaard Laursen\textsuperscript{b}, Stepan Shipovskov\textsuperscript{a,d}, Fleming Besenbacher\textsuperscript{a,c}, Charlotte Horsmans Poulsen\textsuperscript{a,b,⁎}

\textsuperscript{a} Interdisciplinary Nanoscience Center (iNANO), University of Aarhus, DK-8000 Aarhus C, Denmark
\textsuperscript{b} Genencor, Danisco A/S, Edwin Rahrs Vej 38, DK-8220 Brabrand, Denmark
\textsuperscript{c} Department of Biological Sciences, University of Aarhus, DK-8000 Aarhus C, Denmark
\textsuperscript{d} Department of Physics and Astronomy, University of Aarhus, DK-8000 Aarhus C, Denmark

ABSTRACT

Antifouling coatings are used extensively on marine vessels and constructions, but unfortunately they are found to pose a threat to the marine environment, notably due to content of metal-based biocides. Enzymes have repeatedly been proposed as an alternative to traditional antifouling compounds. In this review, the enzymes claimed to hold antifouling activity are classified according to catalytic functions. The enzyme functions are juxtaposed with the current knowledge about the chemistry of settlement and adhesion of fouling organisms. Specific focus will be on bacteria, microalgae, invertebrate larvae and macroalgae zoospores.

Two main concepts in enzyme-based antifouling are identified: breakdown of adhesive components and catalytic production of repellent compounds \textit{in-situ}. The validity of the various modes of action is evaluated and the groups of enzymes with the highest potential are highlighted.

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1. Introduction

Attachment and growth of living organisms on man-made surfaces causes hygienic and functional problems to many types of equipment and devices ranging from medical implants and electronic circuitry to larger constructions, such as processing equipment, paper mills and ships. Such build-up of unwanted living organisms and various organic and inorganic compounds is generally referred to as biofouling.

In many cases, biofouling will consist of microscopic organic impurities or a visible slimy layer containing bacteria and other microorganisms. This category of biofouling is called microfouling, or more commonly biofilm, and occurs everywhere in both natural and industrial environments where surfaces are exposed to water (Costerton, 2007). When fully developed, biofouling in marine environments also includes macroscopic organisms, such as algae and barnacles. This type of biofouling is a particular problem for submerged structures, such as pipelines, cables, fishing nets, the pillars of bridges and oil platforms and other port or hydrotechnical constructions (Railkin, 2004). Fuel consumption of ships may be increased by up to 40% due to biofouling (Champ, 2000). In the following, we distinguish between “biofilm”, including only microorganisms, and “biofouling”, referring to fouling with both microscopic and macroscopic organisms.

While the control of biofilm and biofouling is important in many contexts, this review will focus on solutions for preventing marine biofouling. Biofouling in the marine environment is initiated by a conditioning film consisting of organic compounds followed by the initial colonizers – bacteria and microalgae – on which macroscopic algae and invertebrates settle and develop a complex community (Abarzua and Jakubowski, 1995). This sequence of steps in biofouling is of course a simplified view of a complex process (Railkin, 2004). Traditional approaches to marine biofouling control through application of antifouling paints rely on the release of toxins that kill attaching organisms. Recent efforts have been directed towards developing environmentally friendly alternatives, which include modification of surface structure and chemistry to obtain non-stick surfaces, or replacing environmentally persistent toxins with naturally derived, degradable repellent compounds or enzymes (Yebra et al., 2004).

Ship hulls are coated with paint for corrosion protection, but antifouling paints have also been employed for many years to hinder the attachment of marine organisms, which affect fuel efficiency by increasing the ship’s drag in the sea. The antifouling paints kill or repel the organisms before they can attach to the hull. The most efficient active compound in such paint is tributyltin (TBT), which is released continuously to the surrounding sea (Yebra et al., 2004). However, in November 1998, following local bans in several countries, the International Maritime Organisation banned the use of TBT on ships because the substance is toxic to non-target marine organisms and is accumulated in the environment. The ban started in 2003 with an agreement to terminate the sales of tin-containing paints, and all TBT-based paints in use are to be phased out by 2008 (Champ, 2000). Currently, the main alternatives to TBT paints are the copper-based paints. However, copper is under increased scrutiny regarding its environmental effects and is often considered as a bridging solution to a paint that is non-toxic (Fusetani and Clare, 2006). As copper is active against a smaller range of fouling organisms compared to TBT, the effect of copper-based paints is often enhanced by the addition of so-called booster biocides, e.g. copper pyrithione or isothiazolone (Yebra et al., 2004). The boosters are equally under suspicion for being harmful to the environment, and the safety of booster biocides has been reviewed by several authors (Boxall, 2004; Karlsson and Eklund, 2004; Kobayashi and Okamura, 2002; Konstantinou and Albanis, 2004; Ranke and Jastorff, 2002).

Whereas traditional antifouling paints rely on cytotoxic effects, new environmentally safe alternatives aim to interfere with the adhesion and/or growth of the fouling organisms. Generally, the research has followed two avenues:

1) Modification of surface structure and chemistry to obtain non-stick surfaces that minimize adhesion strength of the settling organisms (Holland et al., 2004; Ista et al., 2004; Yebra et al., 2004), and

In the latter approach, enzymes have been proposed as a viable solution (Bonaventura et al., 1999; Kato, 1988; Okamoto et al., 1992; Pettitt et al., 2004; Poulson and Kragh, 2002; Schneider and Allermann, 2003; Wever and Dekker, 1995).

Enzymes are widely used in industries ranging from food over fabric and household care to large-scale biocatalysis (Aehle, 2003). The idea of using enzymes for antifouling coatings reaches back as far as to 1983 (Noël, 1984), and the concept has received increased interest in recent years (Dobretsov et al., 2007; Leroy et al., 2008; Olsen et al., 2007; Pettitt et al., 2004; Schneider and Allermann, 2003; Yebra et al., 2004).

This review offers an analysis of the relevance of various proposed enzymes. Their modes of action will be discussed and the scientific support for the stipulated effects evaluated in view of the current literature, with emphasis on the chemistry involved in each step in the biofouling process. A key point is to determine which events in biofouling may be affected by enzymes, and how the manipulation of these steps influences the biofouling process in general.

2. Biofouling organisms and their adhesive strategies

In this section, a brief introduction to the field of marine fouling biology is given to serve as a presentation of the chemistry involved in each step of biofouling. Specific emphasis relates to the biochemistry of marine adhesion, which is an important factor to subsequent discussions of anti-adhesive enzyme function. As this section can only touch the molecular and general aspects of biofouling, the reader is directed to the books by Railkin (2004) and Smith & Callow (2006) for further comprehensive and detailed insight into fouling biology and biological adhesives.

The first step in marine biofouling is the formation of a conditioning film composed of organic material. This film is primarily composed of protein and carbohydrate. The second step after formation of the conditioning film is the adhesion and development of biofilm by bacteria and microalgae, primarily diatoms in marine environments (Abarzua and Jakubowski, 1995). There are reports of increases of up to 18% in fuel consumption caused by microfouling alone (Candries, 2000). As the biofouling grows and macrofouling formations of barnacles and seaweed etc. join the microorganisms, it becomes increasingly visible and disturbing to the craft’s efficiency (Abarzua and Jakubowski, 1995).

It should be noted that although it is tempting to conclude that biofouling is a successional process, it has been demonstrated that larger colonizers may settle on a surface completely free of biofilm, or a biofilm may form on a substrate absent of conditioning film, so the succession of conditioning film followed by biofilm and then macrofouling should be regarded as a guideline rather than an absolute (Yebra et al., 2004). For example, bryozoans (Maki et al., 1989), and polychaetes (Lau et al., 2003) include species with larvae that will not settle on biofilm or may settle equally well on biofilm and biofilm-free surfaces, while other organisms may prefer to settle on dead biofilms (Hung et al., 2005).

2.1. Bacteria

The bacterial biofilm is formed as a result of planktonic cells encountering a surface. The cells use extracellular “sticky” polymers to adhere reversibly. These polymers being primarily glucose and fructose-based polysaccharide fibrils (Abarzua and Jakubowski, 1995). Sessile bacterial colonies will change their phenotype from planktonic behaviour to an adapted biofilm metabolic state, including...
increased production of extracellular polymeric substances (EPS) (Marshall, 2006).

The phenotypical change is closely linked to a cell density-dependent system called quorum sensing, which has been demonstrated with many distinct bacterial species. As the name indicates, the bacterial cell can “sense” that it is part of a concentration of cells of a certain size (the “quorum”), because low-molecular-weight signal compounds are secreted, accumulated, and recognized by the cells in the quorum. Quorum sensing is considered to enable improved access to nutrients, more resilient colonisation and higher levels of resistance to hostile environments and antibiotics (Reading and Sperandio, 2005; Waters and Bassler, 2005).

Bacterial biofilms are organised communities, which form intricate architectures with microcolonies of homogenous and mixed species, and water channels inside the matrix that can transport nutrients or metabolites through convective flow (Costerton, 1995). The community is analogous to eukaryotic tissues in that cells achieve physiological efficiency and a high level of protection from outside threats. Biofilms are ubiquitous in aquatic environments (Costerton et al., 1995), and this is certainly true for the sea, where no known arti
cial substitutes are polymer-degrading enzymes, and serve to release cells and provide nutrients for the immobilized cells (Allison, 2003). Further documentation of the protein component of biofilm is scarce, as the composition of these proteins is as variable as that of polysaccharides, but recent evidence indicates that a group of proteins containing domains of the sequence GGDEF and EAL (GlyGlyAspGluPhe/GluAlaLeu) is recurring in distinct biofilm species, where the protein group is important in the regulation of exopolysaccharide production (Lasa, 2006). Homologues of the surface protein Bap of Staphylococcus aureus have been identified in various biofilms, and the protein is attributed a structural function in the S. aureus biofilm (Lasa, 2006; Latasa et al., 2006). Furthermore, high abundance of amyloid fibril proteins acting as adhesins has been documented in diverse aquatic habitats (Larsen et al., 2007). The inherent hydrophobicity, structural stability and plasticity of amyloid fibrils (Wetzel et al., 2007) could be important to biofilm structure.

Not only the structures but also the ratios between protein, polysaccharide and lipid in biofilm can vary greatly. Pseudomonas putida biofilm, for instance, is reported to consist of up to 75% protein in the water-soluble extractable EPS (Jahn et al., 1999). Pseudomonas aeruginosa, the best studied biofilm forming bacterial species, has been demonstrated to produce EPS consisting of about 40% (dry weight) neutral polysaccharides, while primarily proteins and lipids as well as some extracellular DNA composed the rest of the EPS (Chang and Gray, 2003).

2.2. Microalgae

The eukaryotic microorganisms in biofouling consist of diatoms, protozoa, fungi, and spores of macroalgae. The diatoms are the dominant species in eukaryotic microfouling, while protozoans are less abundant (Abarzua and Jakubowski, 1995). The spores are the initiators of macroscopic fouling, and will be discussed later.

As diatoms lack flagella, they are unable to actively approach a given surface, but “land” randomly on the substratum. However, upon contact the benthic diatoms initiate a process which allows them to settle on a surface through an active commitment where the initial, reversible contact (primary adhesion) determines if the organism will continue its life-cycle on that surface (secondary adhesion) (Wetherbee et al., 1998; Chiovitti et al., 2006). During primary adhesion, the diatom will use an adhesive complex of EPS to reorient itself, to enable optimal excretion of further EPS for secondary adhesion (Wetherbee et al., 1998). In secondary adhesion, motile diatoms may use the EPS to “glide” over the substratum, adjusting their position after physical
stimuli, while sessile diatoms remain attached to a single site for extended periods (Chiovitti et al., 2006). Whereas the primary adhesion and gliding is based on localized secretion of EPS, sessile adhesion is effectuated by excretion of copious amounts of EPS to form a more stable matrix (Wetherbee et al., 1998; Hoagland et al., 1993). The algae are capable of forming a homogeneous biofilm, which may be of the order of 500 μm in thickness (Callow, 2000), but individual cells may often exhibit “stalks” or “pads” of EPS in their sessile state (Chiovitti et al., 2006). Fig. 2 gives a schematic representation of the constituents of algal EPS, which will be discussed in this section.

Algal EPS is composed of acidic polysaccharides that are frequently carboxylated or sulphated, and of proteoglycans that influence pre-settlement motility of the cells (Lind et al., 1997) and cross-linking stabilisation of the matrix (Callow, 2000). It has been demonstrated that the proteoglycans of Craspedostauros australis consisted of heterogeneous xylan-rich glycans associated with larger protein structures (Chiovitti et al., 2003a). Both polysaccharides and proteinaceous compounds may be involved in initial adhesion, depending on the organism (Callow, 2000).

The composition of the extracellular mucilage covering the diatom Pinnularia viridis gives an impression of the complexity of diatom EPS (Chiovitti et al., 2003b). The polysaccharide EPS is composed of pentoses, hexoses, 6-deoxyhexoses, methylated and aminohexoses. These sugars are linked in multiple ways corresponding to highly heterogeneous structure, with the most prominent linkages being 2-, 4-, 2,4-linked and terminal xylosyl, 2,3-linked rhamnosyl, terminal and 2-linked fucosyl, and a heterogeneous mixture of minor galactosyl and mannosyl residues (Chiovitti et al., 2003b). The EPS composition will also vary widely between species with large differences in residues and linkages (Wustman et al., 1997), generally including protein fractions as well as complex, anionic polysaccharides with heterogeneous combinations of monosaccharides, sulphate esters and/or uronic acids (Chiovitti et al., 2006). The protein fraction of diatom EPS appears not to have been characterized in detail, and other compounds may play a significant role in diatom biofilm. For instance, Amphora rostrata EPS dry matter contained around 50–60% uronic acid, pyruvate and sulphate (Khandeparker and Bhosle, 2001).

Although diatom EPS is a highly complex matrix, research suggests that different species share common features. Research based on time-of-flight secondary ion mass spectrometry (TOF-SIMS) has shown that the EPS of the three species Cylindrotheca closterium, Navicula mutica and Nitzschia cf. brevissima share specific, but uncharacterized, structural components, although the overall structures of the three unique EPS were quite distinct (de Brouwer et al., 2006). Thus, the common traits could be a target for a generally effective method to combat diatom biofouling.

2.3. Macrofouling

The most prominent event in marine biofouling is the colonization of a surface by macroscopic organisms. The spores of macroalgae and the larvae of macrofouling barnacles, bryozoans, molluscs, polychaetes, tunicates and coelenterates etc. behave in much the same way with regard to surface settlement (Abarzua and Jakubowski, 1995), as we shall see in the following. Important elements of macrofouling settlement will be discussed in the following and summarized in Fig. 3.

A first common denominator is the importance of biofilm cues for settlement. The spore and larval precursors of macrofouling will generally settle selectively in response to the dominant species in biofilm on a given surface. Some biofilm species will attract certain larvae, while others will repel the same settlers. This response has been proven for macrofouling species of scleractinian coral (Negri et al., 2001), scyphomedusae (Hofmann et al., 1996), polychaete

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**Fig. 2.** Schematic overview of the structural component chemistry of extracellular polymeric substances (EPS) involved in microalgal biofilms.

**Fig. 3.** Macrofouling species settle selectively in response to a combination of biological and physico-chemical cues (left). The adhesives used for settlement are based on protein and glycoprotein, both of which contain extensive cross-linking (right). The dominant type of cross-linking varies between settling species.
while the majority of the settlement inhibiting bio-
proteases (Dobretsov et al., 2007).

Barnacles, e.g., Balanus improvisus, have a good relation between light intensity, water tem-
perature, and the number of barnacles that settle. They use antennulae to move across a surface, adhering
momentarily by means of a proteinaceous glue on the ends of the
antennulae. Their choice
of substratum is determined by the topology of the surface, light
conditions, water streaming conditions, and to some extent even
chemical cues at the surface (Crisp, 1974).

While the cues for settlement are quite homogeneous, the methods
of settlement vary on crucial points, as will be apparent from the
following examples of notable macrofouling species: Barnacles, repre-
senting invertebrate fouling, and a species of green algae, representing
macroalgal fouling.

Barnacles have been studied in great detail, and their settlement is
well described (Walker, 1995), although new details continue to
emerge. Barnacle cyprids (the final larval stage before settling and
metamorphosis into adult barnacles) use antennulae to investigate
the surface. These have highly developed chemo-
and mechanore-
ceptor systems and are also used for motility (Lagersson and Høeg,
2002). They use antennulae to move across a surface, adhering
momentarily by means of a proteinaceous glue on the ends of
the antennulae while exploring the surface. When a proper surface
is found to have a good relation between light intensity, water flow,
surface texture and topography, primary microfouling and attractive
chemical cues, the cyprids will start to settle (Walker, 1995).

Balanus improvisus cypris larvae secrete their adhesive in granules
containing highly concentrated cement protein. The granules swell
dramatically during secretion, and it is thought that hydration of the
granules facilitates curing reactions that result in the adhesive effect
of the cement (Ödling et al., 2007). The cement flows out and embeds
the antennular attachment organs, and over 1–3 h the cement hardens,
presumably due to protein polymerization/cross-linking. The fixed
cyprids then metamorphose into juvenile barnacles, which later
become adults. As the organisms mature, a highly tenacious hard
foul ing consisting of barnacles in their cured, volcano-like shells
becomes increasingly rough (Walker, 1995). Studies of the barnacle
Megalabalanus rosa have revealed a complex of proteins involved in
barnacle adhesion. Two of the identified proteins, cement protein cp-
100 k and cp-52 k are highly insoluble, held together by disul-
bridges, a common
covalent cross-linking in proteins (Crisp, 1984).

3. Enzymes claimed to prevent biofilm formation and biofouling

The literature on enzymes for antifouling applications can be
divided into two categories.

One group has a broad focus on the general protection of surfaces
against biofouling of any kind. Enzymes mentioned in this category
of sources are claimed to have antifouling activity, and could be expected
to have activity against macroscopic and microscopic organisms alike.

The other group of sources is more narrowly focused on preventing
the formation of biofilm, and these enzymes are thus targeted at
microorganisms.

In Table 1, the distinction between antifouling activity and biofilm
prevention is used to organise the information about the different
types of enzymes proposed to hold potential for preventing biofoul-
ing. Both categories share a focus on the protection of surfaces, and
references that do not relate to surface settlement have thus been
omitted. Moreover, the enzymes claimed to hold a narrow microfoul-
ing preventing activity are often also mentioned in other sources to
hold broad antifouling activity.

In Table 1, the enzymes have been sorted according to their enzyme
classification number (EC-number), which is related to the similar
catalytic activities of various enzymes (Moss, 2006). Note that claims
of antifouling activity or biofilm prevention have been stated for all
classes of enzyme functionality. The following sections will explore
the theoretical and empirical support for these claims.

Enzyme class 1 (EC 1) covers the catalysis of oxidation or reduction.
Hence, these enzymes are collectively referred to as oxidoreductases.
Enzymes in class 2 catalyse the transfer of functional groups from one (organic) molecule to another, and are thus called transferases. The hydrodrolases in class 3 catalyse the hydrolytic breakdown of various polymers. Class 4 covers enzymes that cleave covalent bonds by non-hydrolytic reactions. Changes within one molecule, which do not alter the molecular mass, are catalysed by the isomerases of EC 5. The sixth and last class of enzymes is called ligases, catalysing the formation of covalent bonds under concomitant hydrolysis of ATP or a similar cofactor (Moss, 2006).

4. Strategies for enzyme-based antifouling

The problems related to fouling are caused by one of two types of events. Either by the settlement of individual organisms or by the proliferation of settled organisms. The former relates to macrofouling larvae (e.g. barnacle cyprids to juvenile and adult barnacles) and spores (e.g. Ulva zoospores to plants), while the latter concerns microfouling, e.g. bacteria and diatoms. Strategies to prevent biofouling can thus basically interfere with the first contact between organisms and surfaces or stop settled organisms from developing to problematic levels, although one strategy does not necessarily exclude the other. Enzymes may affect settlement and adhesion in four different ways. Firstly, they may attack the adhesive of settling organisms (see Fig. 4, left and centre), thus preventing the settlement event.

Secondly, enzymes may degrade the polymers in the biofilm matrix formed by proliferating, settled organisms (as in Fig. 4, right).

Thirdly, enzymes may catalyse the release of antifouling compounds from the surface (Fig. 5). These compounds may be non-toxic or toxic, but since the compounds are produced in-situ, they can be much less stable than conventional biocides, which should eliminate the bioaccumulation of harmful chemicals.

Finally, the intercellular communication during colonization of a surface may be obstructed by specific enzymes.

The following discussion on the antifouling potential of the different enzymes has been divided into four sections reflecting the expected mechanism.

4.1. Enzymes degrading adhesives used for settlement

Whereas the event in settlement defined in Section 2.2 as primary adhesion is a reversible initial contact, secondary adhesion of biofouling organisms is the irreversible onset of tenacious colonies. Secondary adhesion is especially problematic in terms of macrofouling, and as described earlier proteins and proteoglycans are the dominant adhesives in this event. Proteases hydrolyse peptide bonds at sites varying from one protease to another (Rawlings et al., 2006). They may thus degrade the peptide-based adhesive compounds, and a single or a few proteases are likely to act efficiently. Spore adhesion strength and settlement of Ulva zoospores was particularly highly inhibited by serine-protease in a comparative lab assay including various types of proteases in solution (Pettitt et al., 2004). Also the settlement of Balanus amphitrite cyprid larvae was significantly reduced in the extensive laboratory trial studying a range of proteases performed by Pettitt et al. (2004), while similar effects were observed against larval settlement of the bryozoan Bugula neritina (Dobretsov et al., 2007). It has been confirmed that the activity against B. amphitrite cyprids is caused by reduction of adhesive effectiveness rather than any toxic or deterrent effect (Aldred et al., 2008), and field experiments have shown an antifouling effect of proteases when these were incorporated into water-based paint (Dobretsov et al., 2007).

For microfouling, the core issue is the formation of a biofilm. However, the events of primary and secondary microorganism adhesion that precede biofilm formation are also significant to microfouling and it is therefore relevant to know the chemistry of this microbial adhesion. Similarly to macrofouling, bacteria and diatoms also use proteaceous glue, but in their case, polysaccharide-based adhesives are at least equally important for secondary adhesion.

Enzymatic degradation of polysaccharide is performed by glycosylase, which specifically hydrolyse ester-bonds in various oligo- and polysaccharides. Exoglycosylases cut off terminal saccharide units, while endoglycosylases break up chains at internal sites. They may act on either α- or β-glycosidic linkages, and enzymes are also specific to the sites to which the glycosidic bond is attached, e.g. (1→4) bonds (Moss, 2006). This sub-class includes variations of amylase, xylanase,
endoglucanase, cellulase, chitinase and chitosanase, lysozyme, glucosidase, galactosidase, mannosidase, pectinase, collagenase, hyaluronidase, laminarinase/pullulanase, glucosaminidase, isoamylase, glucanase, agarase, and fructan hydrolase, which have all been mentioned in the sources listed above and shown in Table 1.

The polysaccharide portion of secondary adhesive will be a difficult target for glycosylases, since polysaccharides have complex chemistry, as exemplified earlier for EPS used for adhesion by diatoms (Chiavitti et al., 2003b). This assessment was supported by trials on adhesion strength of Navicula perminuta diatoms: broad-spectrum glycosylase formulations in solution had some effect in promoting diatom release from a surface under shear, but serine-proteases were superior (Pettitt et al., 2004). Equally, solutions of serine-protease and glycosylase were compared for prevention of Pseudoalteromonas bacterial biofilm-formation showing effect of both, but results were superior using the protease (Leroy et al., 2008).

4.2. Enzymes disrupting the biofilm matrix

EPS in the biofilm matrix is the essential manifestation of microfouling, and the reason why microbial proliferation is so resilient on ship hulls and other surfaces. The complexity and variability of biofilm polymers described above would be expected to call for very broad combinations of hydrolases and lyases in order to achieve a sufficient disintegration of the polymeric networks constituting the biofilm matrix (Manyak et al., 2005). A lyase enzyme with potential relevance to antifouling purposes is alginate lyase (EC 4.2.2.3 or EC 4.2.2.11) (Manyak et al., 2005), which breaks up the polysaccharide alginate by an elimination reaction (Moss, 2006).

As described earlier, lipids are rarely attributed structural significance in biofilms, and propositions of lipases for antifouling purposes have not been very abundant. Lipases hydrolyse the ester bonds connecting fatty acids to glycerol in lipids (Moss, 2006). The two most prominent components, proteins and polysaccharides, are more popular targets of antifouling strategies.

Glycosylases will individually target a limited range of linkages in polysaccharides, but as described above, the variation of linkage types in a mixed biofilm is great. Choosing the appropriate glycosylases would hence be a complicated matter, and what might work in one trial or location would risk being a failure in another. The same goes for lyase acting on polysaccharide, such as alginate. For example, a glycosylase product, which succeeded in inhibiting the adhesion of Pseudoalteromonas bacteria, was not able to detach cells that had already adhered and formed a biofilm (Leroy et al., 2008).

Proteins quantitatively constitute as important a part of the biofilm matrix as do polysaccharides, and they may be as important for the chemical architecture. Similar to the prevention of adhesion, proteases can therefore be very efficient in breaking down the matrix, which has been observed for a strain of Pseudoalteromonas (Leroy et al., 2008). However, the recent evidence of widely conserved presence of amyloid protein structures may confer limitations to the comprehensiveness of proteolytic breakdown of biofilm matrices (Larsen et al., 2007), because the structural stability of amyloid fibrils is resistant to most proteases (Suzuki et al., 2006).

A mixed biofilm is a living community with a wide range of possible responses to external influences. This means that degrading a key component of the biofilm matrix structure may not be sufficient to destroy the biofilm, since alternative components are likely to arise that may constitute new networks or promote the proliferation of different organisms. An example of this relationship can be found in the paper by Xavier et al. (2005), who explored the background for some conflicting observations that had previously been reported: endogenous alginate lyase from P. aeruginosa had been shown to promote cell detachment from a single strain biofilm, which was at a stage of development between initial settlement and the established state (Boyd and Chakrabarty, 1994). However, the addition of the enzyme had no effect on an identical biofilm, which had already established itself (Christensen et al., 2001). Xavier explained this phenomenon by a kinetic model of cohesiveness in a biofilm versus diffusion of detachment promoting agents (DPA) such as hydrolases or lyases versus the rate of production of new EPS by the biofilm organisms (Xavier et al., 2005). The DPA must diffuse into the biofilm to exert its effect, and the thickness and cohesiveness of the biofilm adversely affect this diffusion. Biofilm cells could synthesize and repair the EPS faster than the alginate lyase could degrade EPS, provided that the biofilm was sufficiently dense.

Criteria for the success of DPA application were (1) the ratio between DPA mass-transport through the biofilm and the decay rate of DPA, (2) the balance between the kinetics of the DPA degeneration of EPS and the EPS production by the cells, and (3) mechanisms of DPA influence on the EPS cohesiveness (Xavier et al., 2005).
4.3. Enzymes generating deterrents/biocides

An area of antifouling research that has received a lot of attention in recent years is the extraction of metabolites of different sorts from marine flora and fauna that appear particularly adept at avoiding biofouling. Researchers then attempt to find antifouling compounds among these natural metabolites (Krug, 2006). Naturally produced antifouling compounds have been divided into two categories:

1) Non-polar metabolites will remain on the surface of an organism and may repel larvae exploring its surface (this is exemplified in Fig. 5, left).
2) Polar metabolites liberated into overlying water may be detected by larval receptors and trigger avoidance behaviour (see Fig. 5, right).

Such antifouling compounds should be classified as deterrents rather than toxins, since their modes-of-action may or may not be related to toxic effects (Krug, 2006).

Enzymes suggested to mimic the above by generating deterrents that diffuse out of a coating include glucose oxidase (Huijs and Klijnstra, 2006; Johansen et al., 1997), hexose oxidase (Poulsen and Kragh, 2002) and haloperoxidases (Weyer and Dekker, 1995), which all belong to the oxidoreductases (EC 1) (Moss, 2006). The oxidases are used to produce hydrogen peroxide, while haloperoxidase catalyses the formation of hypohalogenic acids. Similar to other reactive oxygen species (ROS) H2O2 may induce oxidative damage in living cells (Halliwell and Gutteridge, 1999; Imlay, 2003). Hypohalogenic acids, e.g. HOBr or HOCl, are highly reactive and are thus used as oxidants in water treatment and as important disinfecting agents (Das, 2002; Wojtowicz, 2002).

The oxidative damage exerted by hydrogen peroxide on cells has been employed in a number of applications, e.g. hydrogen peroxide has been shown to efficiently remove ectoparasites from Atlantic salmon (Thomassen, 1993). A moderate toxicity to aquatic organisms such as algae, daphnia and fish has also been reported for hydrogen peroxide (ECETO, 1993). The compound has even been successfully used to reduce macrofouling in marine cooling water systems, especially when combined with ferrous ions; a reaction that produces an active oxygen radical (Nishimura et al., 1988). Hydrogen peroxide will disproportionate to oxygen and water in aqueous medium, and the velocity of this process is more than 1000-fold higher at pH 7 than at pH 14 (Shipovskov et al., 2004). The pH of natural seawater is in the range 7.5–8.5 (Anderson, 2004). Bioaccumulation has therefore been judged as unlikely (Jacobi, 2002), which implies the potential for using hydrogen peroxide as an efficient non-ecotoxic biocide, providing a considerable environmental improvement over current antifouling biocide technologies, which has been supported in the work of Poulsen and Kragh (2002), Johansen et al. (1997), Huijs and Klijnstra (2006). The environmental fate of hypohalogenic acid is comparable to that of hydrogen peroxide (Wojtowicz, 1995), which supports the benefits claimed by Weyer and Dekker (1995).

4.4. Enzymes interfering with intercellular communication

Quorum sensing plays a role in the formation of bacterial biofilm, as noted earlier. Studies have shown that presence of the N-acetyl homoserine lactones (AHL) used by Gram-negative bacteria for quorum sensing are necessary for rapid development of a biofilm (Reading and Sperandio, 2005; Waters and Bassler, 2005). AHL acylases have been discovered, which degrade AHL in vitro (Barton et al., 2004; Hamade and Yamamori, 2000; Xu, 2004; Zhang et al., 2005) by hydrolysing the acyl-amide bonds between carboxylic acids and amines/amino acids (Moss, 2006). The elimination of AHL may thus prevent the development of bacterial fouling.

AHLs may also affect algal zoospore settlement. A recent review (Callow and Callow, 2006) extensively evaluates the evidence of biofilm organisms attracting or repelling zoospores, especially from Ulva. In lab trials, Ulva zoospores (Callow and Callow, 2006) and polychaete Hydroides elegans larvae (Huang et al., 2008) have responded to raised concentrations of AHLs by increasing their settlement activity. These observations might lead to the assumption that if the AHL-acylase concept is successful towards marine bacteria, it may also be able to indirectly reduce embodiments of macrofouling, but it should be stressed that since macrofouling can develop without prior biofilm formation, AHL acylases are not likely to succeed as a comprehensive antifouling solution.

4.5. Other enzymes

The enzyme classes transferases, isomerases and ligases have also been proposed to hold antifouling properties (Barton et al., 2004; Hamade and Yamamori, 2000). However, the enzymes’ modes-of-action and effects in this context have not been elucidated further. Transferases catalyse the transfer of functional groups from one organic compound to another; in the case of transaminase (EC 2.6.1) it is the –NH2 group and –H of e.g. a protein or a carbohydrate-amine that is transferred to a compound containing a carbonyl group in exchange for the –O of that group (Moss, 2006). Isomerase activity is defined by the catalysis of changes within one molecule; subclasses include racemases, epimerases, cis-trans-isomerases, intramolecular oxidoreductases, intramolecular transferases, and intramolecular lyases. Synthetase or synthase are synonyms for ligase. Ligase activity is defined by the joining of two molecules with concomitant hydrolysis of the dipospho bond in ATP or a similar triphosphate cofactor. An example could be a peptide-synthesase (Moss, 2006).

4.6. Challenges for enzyme-based antifouling systems

An observation of interest to enzymatic antifouling is that it is widely recognized that warmer waters are inductive to heavier biofouling than cold (Yebra et al., 2004). On the one hand, enzymes may counteract this trend since, generally, they have increased catalytic activity with increasing temperature. On the other hand, enzymes are generally less stable in warm environments (Aehle, 2003), and the effective lifespan of an enzyme-containing antifouling coating can decrease significantly in tropical waters.

The temperatures of the oceans vary from freezing cold to above 30 °C (Anderson, 2004), which will of course entail significant differences in enzyme functionality. Finding the right balance between activity and stability in these variable conditions will be a major challenge to the production of enzyme-based antifouling coatings.

Designing the right coating matrix in which to contain the enzymes will be another crucial step in the product development. The enzymes need contact with a certain amount of water for catalytic activity and they need to have structural mobility, which is not necessarily compatible with long-term (years) efficiency of an antifouling coating. A recent review deals with these interactions between antifouling paint technology and enzyme functionality, while also discussing the legislative issues that may arise in relation to the production and use of enzyme-based antifouling coatings (Olsen et al., 2007). In the review, four requirements are proposed for enzyme-based antifouling systems: 1) Enzymes must retain activity when mixed with coating components, 2) Enzymes must not deteriorate coating performance, 3) Enzymes must have a broad-spectrum antifouling effect, and 4) Enzyme activity must be long-term stable in the dry coating and after submersion of a coated surface in the sea.

The need for structural mobility is most readily answered by solubilisation of the enzymes from the coating, but this may conflict with the fourth parameter, as soluble enzymes are likely to leach rapidly from the coating. Some form of immobilization is suggested to counter leaching, but will need to allow molecular mobility (Olsen et al., 2007).

Another potentially important aspect of enzyme-based coatings is the necessary distribution of the individual enzymes. Bonaventura et al. (1999) postulates a preferred inter-molecule spacing of 100 Å, which Olsen et al. (2007) assesses will correspond to 0.035 wt.% pure enzyme in a dry coating. Such amounts of enzyme are considered reasonable for
commercialization, but the estimate is of course hypothetical and many factors will affect how much enzyme is needed in a specific coating system. These are for example the polishing behaviour of a self-polishing coating (Kil et al., 2002), interactions between released or surface-bound coating pigments and developing biofilms (Yebra et al., 2006), the efflux of enzyme or enzyme-product from the coating, and the general physical and catalytic properties of the enzyme. The feasibility of an enzyme-based antifouling system should therefore be evaluated from a consideration of the integrated functionality, applicability and cost of the entire enzyme-coating complex.

Furthermore, systems that rely on substrates being provided for the enzyme to produce deterrent compounds face the challenge of achieving enzyme/substrate contact. The substrate may be trapped inside the given coatings, which may hinder access, or substrate may originate from the surrounding seawater, in which case the environmental availability and fluctuations in concentration of the substrate can have critical influence on antifouling performance.

The integration of advanced coating design and enzyme technology to provide a commercially viable antifouling solution may therefore prove to be at least as much of a challenge as is the identification of practically effective antifouling enzymes. Biofouling in marine environments is a complicated matter; more than the bacterial/fungal biofilms in food processing equipment, paper production, water handling and many other industrial areas, where biofouling also causes problems. Many of these areas deal with surfaces that are in almost constant contact with water, experience surface shear similar to a ship sailing through the ocean, and have chemical and physical conditions suitable for active enzymes to work. Very few of these industries have multicellular eukaryotic colonizers, so the targets will be cells that settle primarily by passive encounter and hang on by secreted EPS. The most likely approach to preventing this type of biofouling could be a combination of any of the following: EPS-degrading enzymes, on-site generation of compounds toxic to the colonizers and/or enzymatic interference with quorum sensing. The viability of all these approaches has been assessed above.

5. Conclusions

Biofouling on marine structures, especially ship hulls, are characterized by two critical events: The irreversible, secondary adhesion of biofouling species, and the proliferation of the microorganisms in biofilm. The most widely suggested enzyme-based approach to effective prevention of both of these events is the hydrolytic breakdown of adhesive or structural polymers based on protein and polysaccharide components. Proteases are concluded to bear the highest potential in this respect, although some protein structures may be highly resistant to proteolytic breakdown. Another option is to use glycosylases, which can degrade polysaccharide networks. However, each glycosylase targets individual polysaccharide structures, which reduces the likelihood of finding single or combined glycosylases with comprehensive effect. A different approach, not relying on degradation of polymers, is to produce compounds at the surface of the ship that deter settling organisms, e.g. peroxide compounds. Various peroxidases and oxidases can be applied to this approach. A final possible solution is to degrade signalling compounds that promote biofouling. AHL acylases can inhibit biofilm formation to some extent, and while these enzymes essentially target only bacterial fouling, some evidence suggests that the removal of AHLs from a surface has inhibiting repercussions on other colonizers, which may be of use in conjunction with other agents.

Proteases, glycosylases and oxidoreductases hold potential for broad antifouling effectiveness, while acylase has interesting perspectives but less scientific support for marine applications. Moreover, the described technologies are of practical relevance to the protection against unwanted biological colonization of surfaces in other industries. The coming challenges in the field of enzyme-based antifouling will be to obtain further empirical documentation of truly comprehensive effects of antifouling enzymes, most notably in the form of application trials of immersed surfaces in field studies and on marine crafts. Evidently, the ultimate test of the antifouling potential of various enzymes will be whether they can successfully be incorporated in feasible, effective and durable marine coatings.

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