

Green chemicals for enhanced biofilm mitigation

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Biofilms cause many problems in medical and industrial environments. For example, pathogenic biofilms contaminate catheters, which are difficult to eradicate using antimicrobials alone. Biofilms also cause biofouling and biocorrosion that impact many industries such as the water utilities industry and the oil and gas industry. Sessile cells in biofilms are notoriously far more difficult to treat than planktonic cells because biofilms can employ various defence mechanisms, including diffusional limitation, lowered metabolic rate to reduce intake, formation of persister cells, upregulation of resistance genes, efflux pumps, etc. Biofilms usually require 10X or higher antimicrobial concentrations to treat than planktonic cells. Apart from using combinations of antimicrobials or surfactants for better delivery, some special chemicals, known as antimicrobial or biocide enhancers, can also be used to enhance the efficacies of antimicrobials/biocides. Medical researchers found that ethylenediaminetetraacetic acid (EDTA), a chelator, greatly enhanced the biofilm removal from catheters when used in antimicrobial lock solutions. Laboratory data suggests that EDTA and its more biodegradable substitute ethylenediamine-N,N'-disuccinic acid (EDDS) can also be used as biocide enhancers to enhance biofilm prevention and biofilm removal with reduced biocide dosages that offer environmental benefits. A recent exciting development is the use of D-amino acids as biocide enhancers in biofilm mitigation. All bacterial cell walls contain D-alanine termini in their peptidoglycan molecules. Its substitution by other D-amino acids such as D-tyrosine, D-methionine, D-tryptophan and D-leucine triggers the dispersal of bacterial biofilms such as those of *Bacillus subtilis*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*. It was discovered that biofilm dispersal signalling is not effective for recalcitrant biofilms such as the corrosive *Desulfovibrio vulgaris* (a sulfate reducing bacterium) biofilm formed on a carbon steel surface. A biocidal stress in the form of a biocide such as 50 ppm (w/w) of tetrakis hydroxymethyl phosphonium sulphate (THPS) is required to "convince" the biofilm to disperse. This discovery has led to the enhanced biocide mitigation using D-amino acid(s) + biocide combinations in laboratory tests. This chapter discusses the rationales, mechanisms and efficacies of environmentally friendly antimicrobial/biocide enhancers for biofilm mitigation.

Keywords biofilm; biofouling; biocorrosion; antimicrobial; biocide; biocide enhancer; mitigation

1. Introduction

Most microbes in the natural environment live in a biofilm community rather than as individual cells [1]. A biofilm is comprised of many cells of the same species or different species living together in a community. The cells in the biofilm community, known as sessile cells, live and work together synergistically for the good of the community. The sessile cells are approximately 4 to 10 microns away from one another and held mostly in place by exopolymeric substances (EPS) and pili. EPS consist of primarily of polysaccharides, proteins and extracellular DNA. These cellular communities can form passage ways for the movement of nutrients and wastes in and out of the biofilm. They can also form tower and mushroom shapes with certain cells in certain areas of the biofilm to fulfill their specialized tasks [1].

Biofilms cause biofouling that is a major problem in many fields such as water utilities, oil and gas, and the medical field. The use of vascular catheters has provided life saving treatments to many patients with life threatening illnesses such as cancer. In the United States, it is estimated that each year more than 20 million patients have had vascular catheters inserted during hospital visits [2]. Prolonged use of vascular catheters may result in many infectious complications, including local site infection, septic thrombophlebitis, endocarditis, and catheter-related bloodstream infections (CRBSI) [3]. It is estimated that each year more than 200,000 CRBSI occur in the United States [4]. Each year \$60 million to \$460 million is spent on patient care for those with central-line associated bloodstream infections [5]. The primary organisms responsible for CRBSI are *Staphylococcus epidermidis*, *Staphylococcus aureus*, and *Candida* species [4]. CRBSI in central venous catheters (CVCs) are responsible for 500 to 4000 deaths annually in intensive care units in the United States [5]. Biofilms employ several defence mechanisms to counter harsh environmental conditions including antimicrobial/biocide attacks. They include diffusional barriers to prevent biocide penetration, lowered metabolic rates to reduced antimicrobial intake, formation of persister cells to rebuild the biofilm when the environmental conditions improve, upregulation of antimicrobial resistant genes and efflux pumps, etc. [6] It is commonly acknowledged that 10X or higher antimicrobial/biocide concentrations are usually needed to treat sessile cells compared with that needed for planktonic cells [7]. Concentrations as high as 1,000X have also been reported [8].

Biofilms also cause biocorrosion, that is often termed Microbiologically Influenced Corrosion (MIC). They also prevent inhibitors from reaching the metal surface [9]. Biocorrosion causes pitting corrosion rather than uniform corrosion. It accounts for about 20% of all corrosion of metals and building materials according to Flemming [10]. Walsh et al. [11] estimated biocorrosion damages at \$30-50 billion per year in the US alone. The 2006 Alaska pipeline

leak was caused by a ¼" pinhole that was likely due to MIC [12]. The leak caused a spike in world oil prices. Biocorrosion has been classified into three basic categories by Gu and Xu recently [13]. Type I biocorrosion is caused by XRB including Sulfate Reducing Bacteria (SRB), Nitrate Reducing Bacteria (NRB) and methanogens. "X" in XRB stands for the oxidant that is already available in a biocorrosion system, including sulfate, nitrate, CO₂, etc. XRB use anaerobic respiration in their metabolism. They utilize the extracellular electrons released by insoluble elemental iron (Fe⁰) oxidation for reduction of an oxidant such as sulfate in the cytoplasm.



The reduction potential ($E^{\circ'}$) of Fe²⁺/Fe⁰ is equal to -447 mV and $E^{\circ'} = -217$ mV for SO₄²⁻/HS⁻ [14]. $E^{\circ'}$ is measured at 25°C, pH 7, 1 M for solutes (1 bar for gases) except H⁺. It uses the standard hydrogen potential (SHE) as its reference. SHE is based on the 2H⁺/H₂ potential at 25°C, 1 M H⁺ and 1 bar H₂. The cell potential for the redox reaction combining the two reactions above is +230 mV. This positive value corresponds to the Gibbs free energy change $\Delta G^{\circ'} = -178$ kJ/mol sulfate for the redox reaction. This negative $\Delta G^{\circ'}$ value means the redox reaction generates energy and thus the corrosion process is thermodynamically favourable under the conditions defined for $E^{\circ'}$. The actual conditions may differ. However, the Gibbs free energy change will remain negative for this non-borderline case. Despite the thermodynamic driving force, due to a high activation energy for Reaction (2), biofilm catalysis is needed. Xu and Gu [15] showed that starting with the same mature SRB biofilms grown with a full strength liquid medium, subsequent starvation of carbon source made the SRB more aggressive. This was because that due to a lack of organic carbon, sessile SRB cells switched to Fe⁰ as an electron donor. This means Fe⁰ replaced organic carbon as a fuel molecule. Similarly for NRB, Type I biocorrosion occurs due to the following electrochemical reactions,

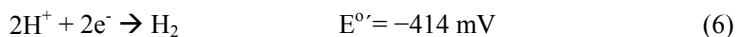


The redox reaction coupling Reaction (1) with nitrate reduction Reaction (3) or (4) is thermodynamically favourable with a positive cell potential of +1207 mV or +807 mV under the conditions defined for $E^{\circ'}$. For methanogens or SRB that reduce CO₂ to methane,



the cell potential for the redox reaction coupling Reaction (1) with Reaction (5) is +203 mV under the conditions defined for $E^{\circ'}$. In Type I biocorrosion, electrogenic sessile cells are needed on the metal surface to transfer extracellular electrons across the cell wall into the cytoplasm. In the mitigation of biofilm involved in Type I biocorrosion, eradication of the bottom layer of electrogenic cells is necessary. Top layer sessile cells may not be directly responsible for the uptake of electrons from iron oxidation. Because of the aqueous water barrier between planktonic cells and the insoluble iron matrix, planktonic cells cannot transport the extracellular electrons released by iron oxidation to their cytoplasm. Sessile cells in a biofilm are responsible for Type I biocorrosion.

In Type II biocorrosion, corrosive metabolites secreted by microbes such as Acid Producing Bacteria (APB) are responsible. Fermentative microbes often produce acids such as volatile fatty acids (formic acid, acetic acid, etc.) that serve as proton reservoirs. Proton reduction in Reaction (6) can replace Reaction (2) to absorb the electrons released by iron oxidation in Reaction (1),



At pH 7, $E^{\circ'} = -414$ mV [14] is too low to cause significant corrosion. A more acidic pH will make the $E^{\circ'}$ value larger (less negative), providing a larger thermodynamic driving force (i.e., a larger cell potential). Organic acids such as free acidic acid (HAc) may also be reduced directly or considered as a reservoir of H⁺,



Recent laboratory experimental electrochemical evidence at Ohio University's Institute for Corrosion and Multiphase Technology Institute supports the treatment of HAc as a H⁺ reservoir (through its dissociation to Ac⁻ and H⁺) rather than the direct HAc reduction mechanism in HAc corrosion. Reaction (6) does not need biocatalysis to go forward. In fact, such corrosion, i.e., Reaction (1) combined with Reaction (6) happens in traditional abiotic chemical corrosion. No cross-cell wall electron transfer is needed because the reduction of proton occurs extracellularly on the metal surface.

Type III biocorrosion is also known as biodegradation. Some biofilms can secrete enzyme to degrade polymers such as polyurethanes and plasticizers and utilize the degradation products as organic carbon and energy sources.

2. Chelaters enhance antimicrobial treatment of medical biofilms

Antimicrobials such as minocycline are used in lock solutions to disinfect biofilms. EDTA is a chelating agent used in many medical and industrial applications. As a chelator, EDTA easily forms complexes with metal ions. This is particularly useful in a variety of applications including detergents, waste water treatment, and the food industry. EDTA has also been identified as an enhancer in antimicrobial treatment of medically important biofilms. Raad et al. showed that low-dose minocycline-EDTA (M_L -EDTA) was more effective at inhibiting biofilm growth on CVCs when compared with a variety of different other treatments [4]. The treatments investigated in their study [4] consisted of streptokinase, heparin, vancomycin, vancomycin-heparin, EDTA, minocycline and M-EDTA. The various treatments were tested against methicillin-resistant *Staphylococcus epidermidis* (MRSE), methicillin-resistant *Staphylococcus aureus* (MRSA), and *Candida albicans* biofilms. A Modified Robbin's Device (MRD) was used to flush 25 catheter segments with 10^6 CFU of the three biofilm producing organisms used in this study for 18 h. The catheter segments were incubated in one of the treatments to determine its effectiveness against MRSE, MRSA, and *C. albicans*. It was found that heparin and streptokinase had no significant effect against any of the three organisms used in this test. Heparin, an anticoagulant, is the most common lock solution used for long-term CVCs. Vancomycin had some effect against all three organisms tested, with the most significant effect against MRSA. The use of vancomycin as a treatment is discouraged because of the increase in the number of gram-positive organisms that have become resistant to this antibiotic. Increased use of vancomycin will only increase the chances of more vancomycin-resistant organisms. Vancomycin-heparin had some effect against both MRSA and MRSE, with a more significant effect against MRSA, but showed no effect against *C. albicans*. Low-dose minocycline proved to be effective against both MRSE and MRSA, but showed no effect against *C. albicans*. EDTA demonstrated some effect against all three organisms tested, but the results were not nearly as successful as M_L -EDTA. M_L -EDTA reduced the number of CFUs to nearly zero for all three organisms tested. High-dose minocycline-EDTA (M_H -EDTA) completely eradicated all three of the organisms in the biofilms on the CVCs. None of the other treatments tested came even close to the results of either M_L -EDTA or M_H -EDTA. One possible mechanism for chelators is that a chelator can remove some Ca^{2+} and Mg^{2+} cations from the outer cell membrane. This makes the cell wall more permeable, and thus more susceptible to antimicrobial penetration [16].

After further research Raad et al. demonstrated that an optimal antimicrobial lock solution could be made by combining minocycline, EDTA and 25% (v/v) ethanol [17] to speed up the treatment. This was determined after testing minocycline, EDTA, and 25% ethanol alone and in combination to determine which created the best lock solution to eradicate MRSA and *Candida parapsilosis* and then prevent their regrowth. Biofilm of each of the two tested strains was allowed to grow on catheter segments from an MRD until it was well developed. One set of catheter segments were then exposed to the one of the tested lock solutions for 15 minutes and then analyzed to determine the effectiveness. Another set were exposed to one of the tested lock solutions for 15 minutes and then incubated for 24 h to determine regrowth. Only minocycline in 25% ethanol solution and M-EDTA in 25% ethanol solution demonstrated complete eradication of both organisms tested in biofilm after only 15 minutes exposure time. M-EDTA in 25% ethanol solution was the only solution tested that not only showed complete eradication of the tested biofilm in 15 minutes, but also demonstrated no regrowth in 24 h. These potential antimicrobial test lock solutions were also tested on silicone disks that were colonized by MRSA and *C. parapsilosis* biofilms. In this test, a set of silicone disks were exposed to the tested lock solution for 1 h and then examined, while another set were exposed to the tested lock solution for 1 h and then incubated for 24 h to allow for regrowth. In this test only M-EDTA in 25% ethanol solution demonstrated complete eradication for both tested organisms in biofilm after 1 h exposure time, and no regrowth after 24 h. A M-EDTA in 25% ethanol antimicrobial lock solution by far was found to be the most successful treatment in these experiments. This shows great promise for use as a lock solution in patients with long-term CVCs.

3. Chelators are biocide enhancers for industrial biofilm mitigation

Industrial biofilm problems are mitigated using various biocides. Pigging is often used during biocide treatment of pipelines. Common industrial biocides include tetrakis hydroxymethyl phosphonium sulfate (THPS), glutaraldehyde, chlorine monoxide, chlorine dioxide, calcium hypochlorite, potassium hypochlorite, sodium hypochlorite, dibromonitripropionamide (dibromonitripropionamide) (DBNPA), methylene bithiocyanate (methylene bis thiocyanate) (MBT), 2-(thiocyanomethylthio) benzothiazole (thiocyanomethylthio benzothiazole) (TCMTB), bronopol, 2-bromo-2-nitro-1,3-propanediol (BNPD), tributyl tetradecyl phosphonium chloride (TTPC), alkyldimethylbenzylammonium chloride (ADBAC), dimethyl benzyl ammonium chloride (DBAC) and acrolein. Some of the biocides such as acrolein are very potent and biodegradable. However, they pose a danger to operators in an offshore environment that is far away from any nearby medical emergency facility.

Large-scale biocide applications such as those in oil and gas fields face strict environmental regulations. THPS and glutaraldehyde are the two most commonly used biocides in oil and gas operations. They are readily biodegradable and are safe if handled properly. THPS is officially labeled as a green biocide since it was awarded the "Designing Greener Chemicals Award" by the United States Environmental Protection Agency in 1997 [18]. It is desirable to reduce the dosage of biocides by enhancing their efficacies. There are different approaches to achieve this objective. For example,

a surfactant can be mixed with a biocide to deliver the biocide where it is really needed (e.g., a pipe surface instead of the bulk fluid). Green chemicals can be used to weaken sessile cells, making them more vulnerable to biocides.

The EDTA chelator enhancer technology patented by Raad et al. (Patent publication numbers: US 6165484 A, US 6509319 B1, US 20110201692 A1) has subsequently been evaluated for use in industrial biofilm mitigation. Because EDTA is slowly biodegradable, it accumulates in aqueous systems. Ethylenediaminedisuccinate (EDDS) is touted as a replacement because it is readily biodegradable [19]. Fig. 1 shows the molecular structures of EDTA and EDDS salts discussed in this work.

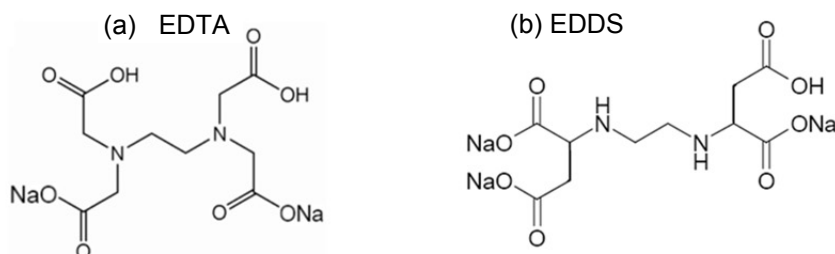


Fig. 1 (a) EDTA disodium salt, and (b) EDDS trisodium salt.

Wen et al. [20] reported that 2,000 ppm (w/w) EDDS considerably enhanced THPS and glutaraldehyde at (an active) concentration of 30 ppm (w/w) against planktonic cells of *Desulfovibrio vulgaris* ATCC 7757 and *Desulfovibrio alaskensis* ATCC 14563 that are two corrosive SRB strains. EDDS was found to be an enhancer for glutaraldehyde in the prevention SRB biofilm establishment and removal of established SRB biofilm on carbon steel surfaces [21]. Methanol also enhanced biocide treatment of biofilms. Since methanol is already used in some oil and gas operations as a winterizing agent, it was tested instead of ethanol. Experimental data from Wen et al. [22] and Xu et al. [23] indicated that when adding 1,000 ppm EDDS and 10% (v/v) methanol, the efficacy of 30 ppm glutaraldehyde was considerably enhanced and far more effective than the binary combination of 30 ppm glutaraldehyde and 1,000 ppm EDDS in the inhibition of planktonic SRB growth, prevention of SRB biofilm establishment and mitigation of souring caused by SRB. Fig. 2 shows that the treatment using 30 ppm glutaraldehyde + 1,000 ppm EDDS reduced MIC pit sizes on the C1018 carbon steel coupon considerably compared with the control without biocide treatment, while the treatment using 30 ppm glutaraldehyde + 1,000 ppm EDDS + 10% methanol almost eliminated pitting by *D. vulgaris* in 1/4 strength ATCC 1249 medium at 37°C [23].

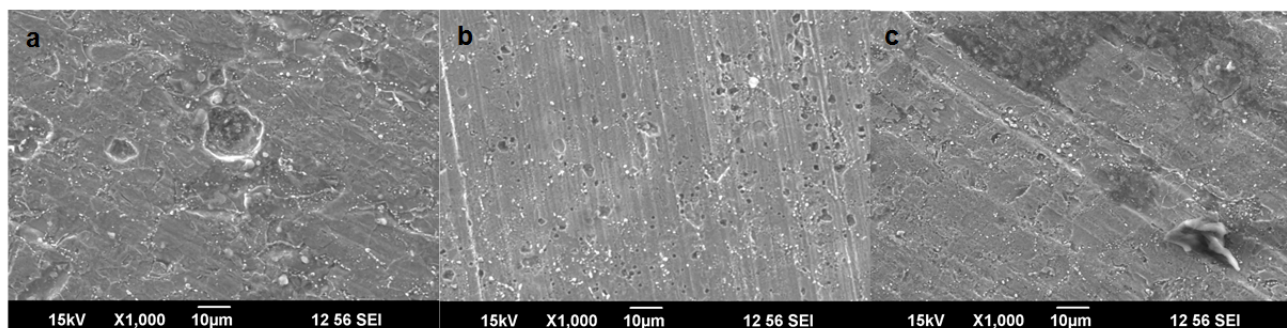


Fig. 2 SEM images of pits on C1018 coupons taken from a 7-day 37°C *D. vulgaris* culture in 1/4 strength ATCC 1249 medium: (a) no biocide treatment, (b) with 30 ppm glutaraldehyde + 1,000 ppm EDDS in the medium, and (c) with 30 ppm glutaraldehyde + 1,000 ppm EDDS + 10% methanol in the medium (reprinted from [23] with permission from NACE International, Houston, TX).

4. Some D-amino acids are biocide enhancers

4.1. Prevalence of D-amino acids in nature

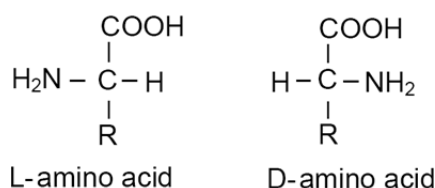


Fig. 3 Structures of L- and D-amino acids

All proteins synthesized right after messenger RNA (m-RNA) translation consist of only L-amino acids. D-amino acids are enantiomers of L-amino acids as shown in Fig. 3. Abiotic organic synthesis in a chemical reactor produces a 50:50 mixture of D- and L-amino acid. Most amino acids are nowadays produced using biosynthesis through fermentation to produce only the D-amino acid form. D-amino acids occur in peptides via two different mechanisms: (1) posttranslational conversion of L- to D-amino acids in the peptides originally synthesized (primarily in eukaryotic ribosomes), and (2) peptide synthesis through nonribosomal peptide synthetases, independent of m-RNA. The second method is used frequently in bacteria [24]. With the advances in analytical methods, especially high performance liquid chromatography, and growing interests in D-amino acids, researchers have discovered that D-amino acids are far more prevalent than previously thought. D-amino acids are distributed widely in nature. In fact, D-amino acids are not only found in microorganisms, but also in animals and even humans [25]. Depending on the age and environmental conditions, biological materials such as silk, bone, shells and teeth have a D/L ratio for each amino acid due to an intrinsic first-order racemization reaction. This property has been used to date archaeological objects [26].

Among the 20 common amino acids, only L-Gly is not racemic. Lam et al. [27] measured the concentrations of all 19 D-amino acids in several bacteria. They found that concentrations of D-valine, D-tyrosine, D-threonine, D-phenylalanine, D-methionine, D-leucine, D-isoleucine and D-alanine were higher than 0.01 mM.

4.2. D-amino acids signal biofilm dispersal

It is well known that all bacterial cell walls contain peptidoglycan molecules. As seen in Fig. 4, peptidoglycan is a polymer of $\beta(1-4)$ -linked N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc). All the lactyl groups in MurNAc are substituted with stem peptides consisting of four alternating D- and L-amino acids [28]. Peptidoglycan molecules in a cell wall maintain the bacterial cell's shape, strength and resistance to the high osmotic pressure of its protoplast [24]. Gram-positive bacilli and Gram-negative bacteria possess meso-diaminopimelic acid (DAP) as the third amino acid (Fig. 4a), while most other Gram-positive bacteria possess L-lysine as the third amino acid (Fig. 4b) [28]. In both cases, D-alanine is the terminal amino acid of the peptide chain. Peptidoglycan synthesis has been a key target in many antibiotics. D-amino acids at high concentrations have been used to alter peptidoglycan synthesis in order to inhibit bacterial growth [24]. Thus, it is not surprising that D-amino acids are found in some antibiotic peptides [29]. In fact, D-amino acids are commercially available to the pharmaceutical industry for drug synthesis.

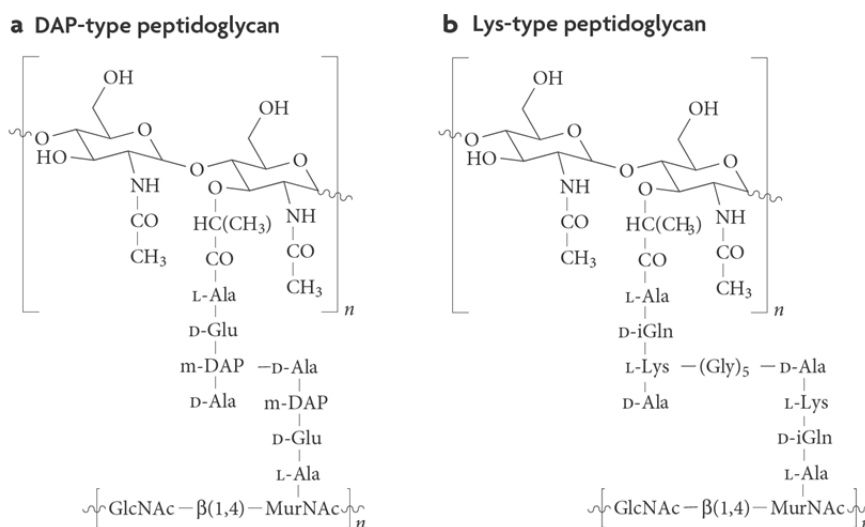


Fig. 4 Two types of peptidoglycan in bacterial cell walls (reprinted from [28] with permission from Nature Publishing Group)

In 2009, Lam et al. [27] found that D-amino acids play a key role in peptidoglycan synthesis and speculated that synthesis of D-amino acids may be a common strategy for bacteria to adapt to the environmental conditions. Cava et al. [24] speculated that in times of nutritional limitation and other cellular stress, bacteria release extracellular D-amino acids that signal to the biofilm community to regulate peptidoglycan amount, composition and strength. Recently, Kolodkin-Gal et al. [30] discovered that some D-amino acids (D-tyrosine, D-methionine, D-tryptophan and D-leucine) dispersed bacterial biofilms at very low concentrations (μM to mM) for *Bacillus subtilis*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*. They also prevented biofilm formation. Kolodkin-Gal et al. hypothesized that these D-amino acids can substitute the D-alanine terminus, thus sending a biofilm dispersal signal. This hypothesis was supported by their experimental data showing that adding a high concentration of D-alanine in a D-tyrosine solution rendered the D-tyrosine treatment ineffective. They proposed a strategy of biofilm mitigation by applying D-amino acids first to convert sessile cells to planktonic cells and subsequently applying an antimicrobial for an easier kill.

Xu and Liu [31] used D-tyrosine for biofilm dispersal on fouled nylon microfiltration membranes used to filter activated sludge. Dosages of 10 ppm (w/w) and 500 ppm D-tyrosine reduced 24-h old biomass on the filter by 25% (w/w) and 60% after treatment, respectively. The treatment inhibited autoinducer-2 (a universal interspecies quorum-sensing chemical) and EPS secretions considerably.

4.3. D-tyrosine enhanced THPS mitigation of SRB biofilms

Pure D-tyrosine can be isolated from a D/L-tyrosine mixture produced from organic synthesis using selective degradation of L-tyrosine by microbes [32]. It can also be produced from hydroxyphenylpyruvate using enzymes [33]. D-tyrosine exists in many food products such as casein, soybean, wheat gluten and fish due to the conversion of L- to D-tyrosine during alkaline or heat treatment of food products [34]. L-tyrosine has an isoelectric point of 5.6. Between pH 3 and 8, L-tyrosine has a small solubility ≤ 3 mM [35]. Its enantiomer, D-tyrosine has similar physical properties. D-tyrosine stands out among various D-amino acids as having a particularly low solubility at pH 7. In biofilm dispersal tests, its required minimum concentration for efficacy is particularly low (≤ 1 ppm by mass) [6, 30], probably by nature's design. In order to make a concentrated stock solution in lab tests, it is necessary to adjust pH to a far more acidic or alkaline pH.

Table 1 D-tyrosine and THPS for the prevention and removal of *D. vulgaris* biofilm (data from [6])

Treatment	Sessile cell count (cells cm ⁻²)*	Sessile cell count after 1-hour treatment (cells cm ⁻²)**	Sessile cell count after 3-hour treatment (cells cm ⁻²)**
No treatment (control)	$\geq 10^7$	$\geq 10^6$	$\geq 10^6$
100 ppm D-tyrosine	$\geq 10^6$	$\geq 10^5$	$\geq 10^5$
50 ppm THPS	$\geq 10^4$	$\geq 10^4$	$\geq 10^3$
50 ppm THPS + 1 ppm D-tyrosine	<10	<10	<10
100 ppm THPS	$\geq 10^2$	<10	<10

*Sessile cell count on carbon steel coupon surface in 37°C ATCC 1249 medium for 7 days in biofilm prevention test

**Sessile cell count on carbon steel coupon surface with established mature SRB biofilm after 1-hour treatment and 3-hour treatment in an anaerobic chamber at room temperature, respectively.

Table 1 (Column 2 data) and Fig. 5 show the results of D-amino acid + THPS for biofilm prevention [6]. D-tyrosine alone only achieved 1-log reduction (90% removal) of SRB sessile cells indicating that D-tyrosine alone did not adequately trigger SRB biofilm dispersion. When treated with 100 ppm THPS, a 5-log reduction (99.999% removal) of sessile SRB cells was achieved. Compared with the 3-log reduction obtained by 50 ppm THPS without D-tyrosine, the binary combination of 50 ppm THPS and 1 ppm D-tyrosine achieved a 6-log reduction, resulting in undetectable sessile cells. For the established biofilm removal test data shown in Columns 3 and 4 of Table 1, similar results were obtained in both 1-hour and 3-hour tests. The binary combination of 50 ppm THPS + 1 ppm D-tyrosine achieved the same biofilm eradication effect as 100 ppm THPS without D-tyrosine (5-log reduction). This means 1 ppm D-tyrosine successfully halved the THPS dosage. The data suggest that D-tyrosine strongly enhanced THPS in SRB biofilm prevention and removal. Fig. 5c indicates that D-tyrosine alone, even at a high concentration of 100 ppm, was insufficient for *D. vulgaris* biofilm dispersal. Apparently, this SRB biofilm was more recalcitrant than those biofilms tested by Kolodkin-Gal et al. [30]. A biocide stress was needed to “convince” the *D. vulgaris* biofilm to disperse. It has been speculated that D-alanine substitution by another D-amino acid sends a signal for biofilm dispersal [30]. Fig. 6 demonstrates that the introduction of 1,000 ppm D-alanine to the binary combination of 50 ppm THPS + 1 ppm D-tyrosine inactivated the efficacy of the binary biocide cocktail. The existence of the high concentration D-alanine suppressed the D-tyrosine substitution of the D-alanine terminus in the peptidoglycan of the bacterial cell wall. Kolodkin-Gal et al. [30] demonstrated this inhibition phenomenon for D-methionine dispersal of *B. subtilis* biofilm.

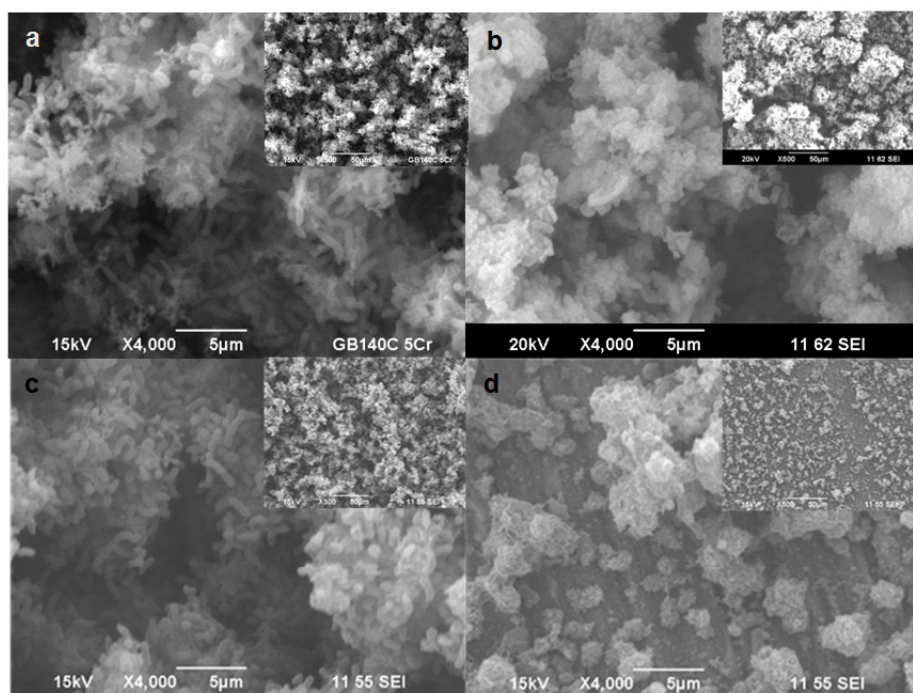


Fig. 5 SEM images for 7-day coupons from 37°C *D. vulgaris* cultures in ATCC 1249 medium in the biofilm prevention test. (A) control with no treatment chemicals added to the culture medium, (B) 100 ppm THPS added, (C) 100 ppm D-tyrosine added, and (D) 50 ppm THPS + 1 ppm D-tyrosine added. Scale bars for the small inserted images are 50 µm (reprinted from [6] with permission from Springer).

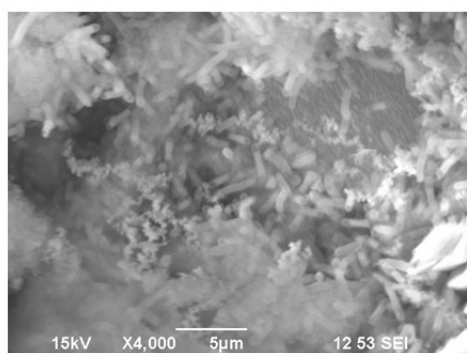


Fig. 6 SEM images for coupons (initially covered with mature SRB biofilms) after undergoing 3-hour shock treatment in a Petri dish at room temperature in an anaerobic chamber with 50 ppm THPS + 1 ppm D-tyrosine + 1,000 ppm D-alanine (reprinted from [6] with permission from Springer).

4.4. D-methionine enhanced THPS in the mitigation of an SRB biofilm and MIC

D-methionine stands out as a D-amino acid with a well-known safety profile. L-methionine is a nutritional supplement in animal feed with a very large demand. L-methionine is commercially produced through organic synthesis that results in a 50:50 racemic mixture of D/L-methionine. After synthesis, D-methionine is enzymatically converted to L-methionine [36]. To reduce cost, D-methionine is directly added to some pet food products because cats, dogs and other animals can convert it to L-methionine in their digestive systems. At a dosage of 1.16 g/day, D-methionine added to human food did not exhibit any side effects [37, 38]. Furthermore, D-methionine has no documented OSHA hazards [36, 39, 40]. Due to its existing large-scale use in the pet food industry, D-methionine appears to have the best safety and environmental profiles for field applications among all the D-amino acids, because some other D-amino acids have not seen much use beyond the pharmaceutical industry and their full safety profiles have yet to be established.

D-methionine has been found to be an effective biocide enhancer of THPS for *D. vulgaris* biofilm treatment and MIC pitting mitigation [41]. In the biofilm prevention test, the combination of 50 ppm THPS and 100 ppm D-methionine successfully prevented *D. vulgaris* biofilm establishment (Fig. 7a vs. Fig. 5a). The sessile cell count shown in Table 2 is consistent with the SEM image in Fig. 7a. The binary biocide cocktail resulted in a 5-log reduction of sessile cells (from 10^6 to less than 10 cells/cm²) compared with the untreated control (Table 2). In the removal of established biofilm test, coupons were incubated in the *D. vulgaris* culture for 7 days to allow biofilm coverage before the coupons were taken

out for biofilm treatment. After treating with the 50 ppm THPS + 100 ppm D-methionine binary biocide cocktail, sessile cells were hardly seen on the coupon surface in different media (Fig. 7bcd).

The combination of 100 ppm L-methionine and 50 ppm THPS did not remove the SRB biofilm with cells obviously left on the coupon surface (Fig. 8a). Although L-methionine was ineffective to enhance THPS, the biocidal effect of binary cocktail biocide of D-methionine + THPS was not adversely impacted by the presence of L-methionine (Fig. 8b). This means a racemic mixture of L- and D-methionine may be used without removing L-methionine to cut costs. Adding 1,000 ppm of D-alanine to the binary biocide cocktail rendered the binary biocide cocktail ineffective for biofilm removal (Fig. 9). This observation again supports the hypothesis that D-alanine substitution by another D-amino acid leads to the signaling of biofilm dispersal.

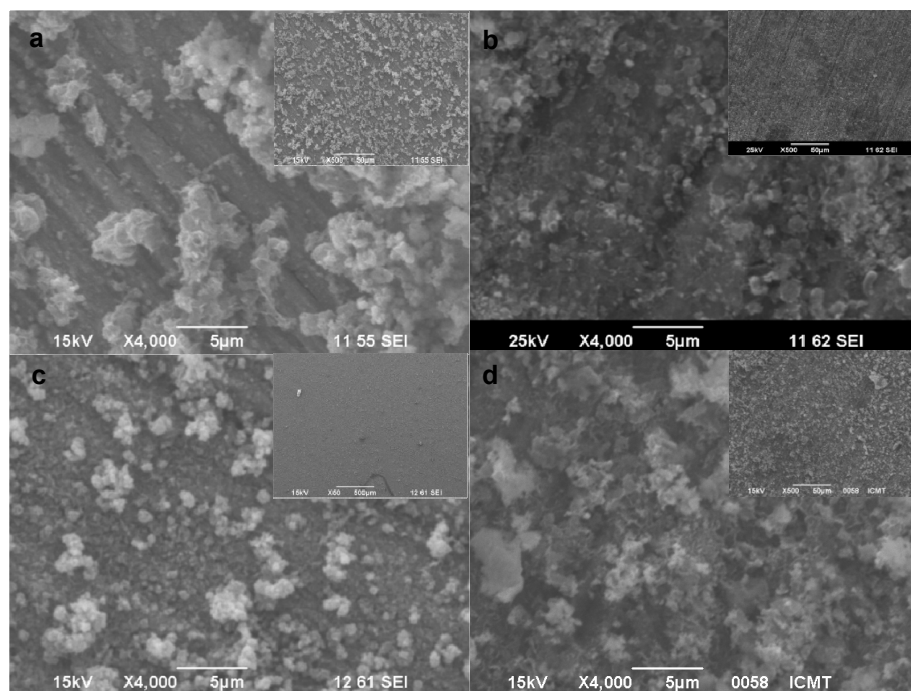


Fig. 7 (a) SEM image of a 7-day coupon taken from a 37°C *D. vulgaris* culture with ATCC 1249 medium added with 50 ppm THPS + 100 ppm D-methionine biocide cocktail for biofilm prevention, (b) coupon (initially covered with established biofilm) in 37°C ATCC 1249 medium (full strength medium) treated with the binary biocide cocktail for 7 days for biofilm removal, (c) coupon (initially covered with established biofilm) in 1/4 strength medium treated at 37°C with the binary biocide cocktail for 7 days for biofilm removal; (d) coupon (initially covered with established biofilm) after being soaked for 3 hours in a Petri dish containing the binary biocide cocktail in an anaerobic chamber at room temperature for biofilm removal. Scale bars for the small inserted images are 50 μm, respectively (reprinted from [41] with permission from Wiley).

Table 2 Sessile cell counts for 7-day coupons taken from 37°C ATCC 1249 medium with and without addition of a binary biocide cocktail for the prevention of SRB biofilm establishment test (data from [41])^{*}

Treatment	Sessile cell count (cells cm ⁻²)
No treatment	≥10 ⁶
50 ppm THPS + 100 ppm D-methionine	≤10

^{*} The experiment was repeated three times.

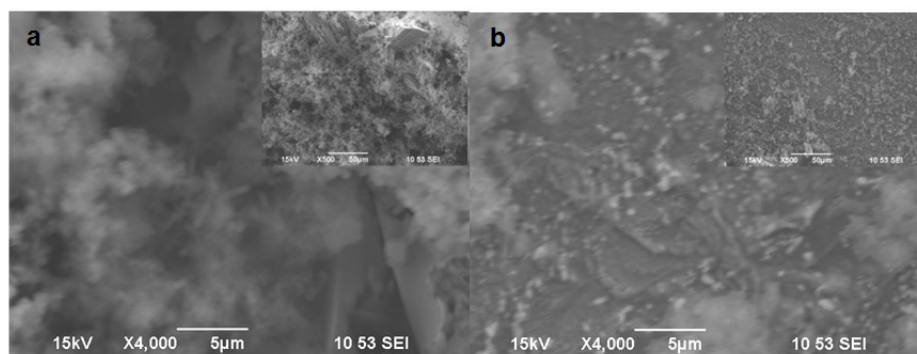


Fig. 8 SEM images for coupons (initially covered with established biofilms) after being soaked for 3 hours in a Petri dish (in an anaerobic chamber at room temperature) containing (a) 50 ppm THPS + 100 ppm L-methionine, and (b) 50 ppm THPS + 100 ppm D-methionine + 100 ppm L-methionine, respectively. Scale bars for the small inserted images are 50 µm (reprinted from [41] with permission from Wiley).

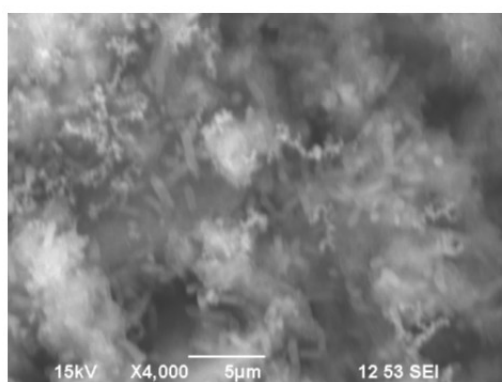


Fig. 9 SEM images for coupons (initially covered with established *D. vulgaris* biofilms) after being soaked for 3 hours in a Petri dish containing 50 ppm THPS + 100 ppm D-methionine + 1000 ppm D-alanine in an anaerobic chamber at room temperature (reprinted from [41] with permission from Wiley).

Apart from biofilm mitigation, the binary combination of 50 ppm THPS and 100 ppm D-methionine performed better in MIC pitting mitigation compared with 50 ppm THPS alone and 500 ppm D-methionine alone (Fig. 10abc). The binary biocide cocktail also had the lowest normalized weight loss of the corrosion coupons compared with 50 ppm THPS treatment and 500 ppm D-methionine treatment (Fig. 10d).

4.5. D-amino acid mixtures

Xu et al. found that a 6.6 ppm (w/w) D-amino acid mixture consisting of equimolar D-tyrosine, D-methionine, D-tryptophan and D-leucine considerably enhanced the 30 ppm THPS + 500 ppm EDDS binary combination in both biofilm prevention and biofilm removal tests [42]. Table 3 shows that the triple combination of 30 ppm THPS + 500 ppm EDDS + 6.6 ppm D-amino acid mixture achieved 5-log and 4-log reductions in biofilm prevention and biofilm removal tests, respectively. The D-amino acid mixture alone did not show significant effects in the two tasks even at a high concentration of 660 ppm, indicating the need of a biocide stress for *D. vulgaris* biofilm dispersal by the D-amino acids.

4.6. Selection of D-amino acids

As mentioned before, the likely mechanism for D-amino acids is the substitution of D-alanine in the peptidoglycan, which sends a biofilm dispersal signal. For a particular bacterial species, it is possible that a particular D-amino acid may work better than other D-amino acids. In a mixed-culture biofilm consortium, it is likely that a mixture of D-amino acids is preferred. Kolodkin-Gal et al. [30] pointed out that D-amino acid mixtures work better at biofilm dispersal compared with individual D-amino acids even at lower doses. As a rule of thumb, D-amino acids having lower solubilities at physiological pH probably require a lower dose. The results above show that for *D. vulgaris*, 1 ppm D-tyrosine is required while for D-methionine, 100 ppm is required for biocide enhancement. At pH 7 and 20°C, D-tyrosine solubility is 0.0025 mol/l (0.45 g/l) at 25 °C compared to 0.35 mol/l (53 g/l) for D-methionine [23]. By nature's design, if a higher concentration is needed, the D-amino acid would likely have a higher solubility.

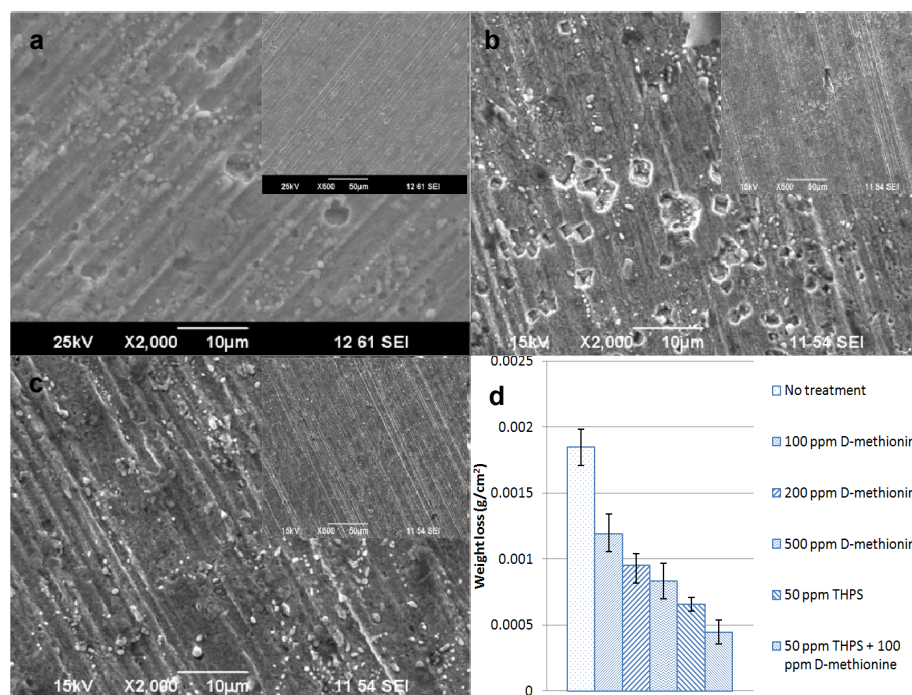


Fig. 10 SEM images of coupon surfaces after *D. vulgaris* biofilm removal for coupons obtained after 7 days of incubation at 37°C from ATCC 1249 medium with the addition of (a) 50 ppm THPS, (b) 500 ppm D-methionine, (c) with 50 ppm THPS + 100 ppm D-methionine, respectively, accompanied by normalized weight loss data shown in (d). Scale bars for the small inserted images are 50 µm. (reorganized and reprinted from [41] with permission from Wiley).

Table 3 D-amino acid mixture enhancement of EDDS + THPS combination for *D. vulgaris* biofilm mitigation (data from [42])

Treatment	Sessile cell count* (cells cm ⁻²)	Sessile cell count** (cells cm ⁻²)
No treatment (control)	≥10 ⁸	≥10 ⁷
30 ppm THPS	≥10 ⁶	≥10 ⁶
30 ppm THPS + 6.6 ppm D-amino acid mixture	≥10 ⁶	≥10 ⁶
30 ppm THPS + 500 ppm EDDS	≥10 ⁵	≥10 ⁵
30 ppm THPS + 500 ppm EDDS + 6.6 ppm D-amino acid mixture	≥10 ³	≥10 ³

* Sessile cell count in the biofilm prevention test for coupons incubated for 7 days in 37°C *D. vulgaris* culture in ATCC 1249 medium with and without treatment chemicals

** Sessile cell count of in biofilm removal test using coupons (initially covered with mature *D. vulgaris* biofilm) in 37°C ATCC 1249 medium with and without any treatment chemicals after an incubation time of 7 days.

4.7. Biofilms containing non-bacterial microbes

In its native wild environment, a biofilm may contain eukaryotic cells (e.g., fungi) and archaea (e.g., methanogens) in a synergistic community. Each microbe contributes to the community differently, either in terms of nutrients, defence or other ways. The cell walls of eukaryotes and archaea do not have the peptidoglycan molecules that are possessed by all species of bacterial cells. Fungal cell walls contain glucosamine polymer chitin, while archaean cell walls contain pseudopeptidoglycan. None of the fungal or archaean cell walls contain D-amino acids. This means D-amino acids cannot signal their dispersal. However, in a synergistic biofilm community, bacterial cells are usually present if not dominating. The dispersal of bacterial cells in the biofilm community will damage the structure of the biofilm and weaken the synergy among various microbial species. This would make the biofilm vulnerable to a biocide attack. Thus, it is likely that in such a situation, D-amino acids can still be effective biocide enhancers. However, experimental results are needed for confirmation.

5. Summary

Chelators and D-amino acids can be used as antimicrobial/biocide enhancers. EDDS is more attractive than EDTA in industrial applications. This is because unlike EDTA, EDDS is readily biodegradable and will not accumulate in the environment. D-amino acids such as D-tyrosine, D-tryptophan, D-methionine and D-leucine are biocide enhancers that are effective at low concentrations. They are hypothesized as biofilm dispersal signaling molecules. By replacing the D-alanine terminus on the stem peptide of the peptidoglycan molecules in bacterial cell walls, they send a biofilm dispersal signal. Thus, D-amino acids can reduce biocide dosages considerably because planktonic cells are much easier to mitigate than sessile cells. For recalcitrant biofilms such as the *D. vulgaris* biofilm, a biocide stress is required together with D-amino acids to “convince” the sessile cells to disperse. This means in the mitigation of recalcitrant industrial biofilms, D-amino acids and biocide(s) should be applied simultaneously instead of sequentially. It is likely that a mixture of several D-amino acids will work more effectively in substituting the D-alanine termini in bacterial cell wall’s peptidoglycan and thus sends a biofilm dispersal signal. More tests are needed to evaluate the efficacies of different D-amino acid(s) + biocide combinations, especially against field biofilm consortia. D-amino acids are naturally occurring. They will likely find field applications in biofouling and biocorrosion mitigation. They may prove to be particularly attractive biocide enhancers in environmentally sensitive applications such as hydraulic fracturing in shale gas production.

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