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Enzyme-Containing Paints Inhibit the Growth of Marine Microorganisms

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Abstract: A new strategy to prevent the biofouling of water-submerged surfaces is presented here. In particular, the authors show that carbonic anydrase from *Methanosarcina thermophila* can be entrapped into polyacrylic paints, preserving enzyme activity. In addition, the authors also show that enzyme-containing paints inhibit the growth of marine microorganims, preventing biofouling.

Key words: Carbonic anhydrase, *Methanosarcina thermophila*, fiberglass, paints, biofouling.

1. Introduction

In conventional practice, paints containing antimicrobial chemicals, e.g., copper [1], zinc pyrithion [2], chlorothalonil [2] or Sea-Nine [2] are used to delay the fouling of water-submerged surfaces. However, considering the environmental impact of traditional antimicrobials, alternative antifouling strategies were recently considered. In particular, the addition to paints of antimicrobial nanoparticles [3] or enzymes [4] was investigated. Interestingly, enzyme-containing paints were patented as antifouling agents (Allermann, K. and Schneider, I.: Antifouling paint composition comprising rosin and enzyme, WO/2001/072911). More recently, encapsulated enzymes were used to prevent biofouling [5]. Nevertheless, a major problem concerning the addition of enzymes to antifouling paints is the presence of organic solvents, whose use generally inactivates the enzymes [6]. Moreover, in order to exert their action, the antifouling enzymes have to bind their molecular targets. This implies that the effectiveness of enzymes is limited to a single nanolayer of paint. Here we present a radically new, acting at a distance, antifouling strategy, i.e., the physical inhibition of microbial colonization of water-submerged surfaces. The authors indeed show here that the CO_2 released by paint-entrapped carbonic anhydrase is an effective mean to prevent the fouling of water-submerged surfaces. This technology has been patented (Hochkoeppler A., Panizza L., Roda, E., Stefan, A.: Method for preventing and controlling biofouling on marine objects, WO/2010/145905 A1 (2010), also published as EP2432323 (Al)—2012-03-28). The patent is based on the in situ generation of a low-risk antifouling agent. According to European Biocides Legislation, CO_2 has already been identified as a low risk molecule active as a biocide. The use of carbonic anhydrase seems ideal to water environments, due to the natural presence of carbonated salts, leading to the production of CO_2,

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preventing biofouling. Further, CO_2 can feature biocide effects on the fouling organisms present on the water-submerged surface of manufactures, but is devoid of any significant eco-toxicological effect.

2. Materials and Methods

As a model enzyme, the authors selected the carbonic anhydrase from *Methanosarcina thermophila* [7, 8]. Accordingly, the authors constructed and synthesized an artificial gene, the sequence of which takes into account the codon usage of *Escherichia coli*. The primary structure of the carbonic anhydrase from *Methanosarcina thermophila* (Cam) was back-translated, and the nucleotide sequence was optimized, according to the *Escherichia coli* codon usage, using the software Leto® (Entelechon GmbH, Bad Abbach, Germany). The optimized gene was synthesized (Entelechon GmbH, Bad Abbach, Germany), and then cloned between the *Nde*I and *Bam*HI sites of the pET9a expression vector, generating the pET9a-*camop* plasmid. This was used to transform the *E. coli* strains BL21 (DE3) and BL21AI. In these strains, the overexpression of Cam is induced by supplementing the growth medium with IPTG (isopropyl-thio-β-D-galactopyranoside) or arabinose, respectively [9-11]. Transformants were selected using LB-agar Petri dishes supplemented with kanamycin (40 μg/mL). Single colonies of transformants were pre-cultured at 37 °C in LB medium (2 mL) supplemented with kanamycin, the pre-cultures were diluted (1:1,000) and grown to mid-log phase, and the overexpression of Cam was finally induced for 3 h using 1 mM IPTG or arabinose. Induced cells were harvested (5,000 g, 20 min) and stored at -20 °C until used. To extract Cam, cell pellets were resuspended in 50 mM Tris pH 8, 50 mM NaCl, 1 mM EDTA and 1 mM PMSF (phenylmethylsulfonyl fluoride). The cells suspension was subjected to 3 cycles of sonication (18 W, 20 kHz, 15 s pulse, 15 s rest, 4 min total) at 4 °C. Protein extracts were centrifuged at 10,000 g for 20 min, and aliquots of pellet and supernatant were subjected to SDS-PAGE. The great majority of Cam was detected in the soluble protein fraction (supernatant). Soluble protein extracts were frozen and stored at -20 °C until used.

To assay the release of carbon dioxide catalyzed by Cam, a CO_2-sensitive electrode (Mettler-Toledo, model 9031345 equipped with a CO_2-selective membrane, type 51340013) was used. The electrolyte solution contained 150 mM glycine, 100 mM NaCl, 20 mM $NaHCO_3$, pH 10.33. The assay buffer was 200 mM Tris, 1 μM $ZnCl_2$, pH 7.6. Reactions were started by the addition of 5 mM $NaHCO_3$. The electrode was calibrated using standard solutions of $NaHCO_3$ (ranging from 0.05 to 5 mM) dispensed into 150 mM citrate buffer, pH 5. The electrode was interfaced to a potentiometer (Eutech Instruments, model Cyberscan pH 2100) controlled by a laptop using the software Cyber Comm Pro. Using the same equipment, the release of CO_2 by fiberglass slides was assayed by submerging half of them into a vessel (100 mL capacity) containing the assay buffer, and dipping the CO_2-sensitive electrode near the painted surface of the fiberglass. Reactions were started by the addition of 5 mM $NaHCO_3$.

To test the competence of Cam in inhibiting the growth of marine microorganisms, *Phaeodactylum tricornutum* was chosen as a model target, according to the UNI EN ISO 10253 (2006) procedure. Briefly, aliquots of a culture of *P. tricornutum* were diluted (yielding 10^4 cells per mL) into 500 mL glass Beakers containing or not 1 fiberglass slide (25 cm^2) and 100 mL of sea water, supplemented with $FeCl_3 \cdot 6H_2O$, $MnCl_2 \cdot 4H_2O$, $ZnSO_4 \cdot 7H_2O$, Na_2EDTA, K_2HPO_4, $NaNO_3$, $Na_2SiO_3 \cdot 5H_2O$, H_3BO_3 (at 0.72, 2.16, 0.675, 15, 3, 50, 14.9 and 17.1 mg/L, respectively), $CuSO_4$ $5H_2O$, $CoCl_2 \cdot 6H_2O$, thiamine, biotin, vitamin B_{12}, (at 2.36, 6.06, 25, 0.005 and 0.05 μg/L, respectively). Samples were kept under continuous light (10,000 lux), at 20 ± 2 °C, using an Ecotherm 80 chamber (Ecotox LDS, Cornaredo, MI, Italy). The number of *P. tricornutum* individuals was evaluated by direct

counting, using a Leica ATC 2000 microscope, equipped with a Bürker chamber. Experiments were carried out in triplicate.

To test the ecotoxicity of Cam, *Artemia franciscana* nauplii (II-III stage) were transferred to 12-multiwell microplates (Iwaki, Japan), previously painted with enzyme-free or enzyme-containing styrene acrylic resin. Samples were then inspected 24 h and 48 h after the transfer of nauplii to the microplates, and the number of dead or swimming-incapable *A. franciscana* individuals was determined. Experiments were carried out in triplicate.

3. Results and Discussion

First, the authors investigated the overexpression levels of *Methanosarcina thermophila* carbonic anhydrase (Cam) in *Escherichia coli*. Interestingly, the authors observed identical production of Cam in *E. coli* BL21 (DE3) and BL21AI (data not shown). Therefore, when considering potential industrial applications of Cam, we decided to produce this enzyme using the overexpression system coupled to the cheaper inducer, i.e., IPTG. Accordingly, we tested in *E. coli* BL21 (DE3)/pET9a-*camop* the yield of Cam as a function of induction time, using 1 mM IPTG. In particular, identical production of carbonic anhydrase was observed 3 h or 5 h after induction (data not shown). Therefore, we decided to induce for 3 h the expression of *camop* in further experiments. Remarkably, under these conditions we were able to overexpress Cam at levels equaling ca. 50% of the total soluble proteins (data not shown). Moreover, about 95% of Cam was found in the soluble proteins fraction, as indicated by the negligible portion of enzyme detected in pellets isolated after centrifugation of protein extracts at 10,000 g (data not shown).

Overall, the performance of Cam overexpression presented here strongly suggests that this enzyme deserves attention as a tool in industrial applications. To further test this potential, the authors decided to evaluate the stability of Cam under a variety of conditions. First, the authors analyzed the stability of Cam at 54 °C. In particular, the authors monitored the protein concentration, according to Bradford assay [12], and the carbonic anhydrase activity (release of CO_2 from $NaHCO_3$) of an enzyme sample (at 1 mg/mL concentration) maintained for 14 days at 54 °C, in phosphate buffer (pH 6.8). Surprisingly, 14 days after the onset of the thermal treatment, Cam did fully retain its initial activity (Fig. 1a). Moreover, the thermal treatment triggered the aggregation and sedimentation of *E. coli* proteins, as indicated by the decrease in protein concentration of the sample (Fig. 1a). Therefore, the specific activity of Cam did consistently increase over the time interval considered (Fig. 1b). The authors then tested the stability of Cam at room temperature in seawater, in the absence of buffer. As a reference, the authors determined the stability of bovine carbonic anhydrase (Bca), under the same experimental conditions. The authors observed a rapid inactivation of Bca, the activity of which was extremely modest, if at all, 48 h after its transfer in seawater (Fig. 1c). On the contrary, Cam did perform well, retaining about 20% of its initial activity after 30 days of residence in seawater (Fig. 1c).

The insensitivity of Cam towards seawater suggests in the authors' opinion, the potential use of this enzyme as an antifouling agent for marine manufactures. Accordingly, the authors dissolved Cam in styrene acrylic resin (Crilat D120S, Vinavil, Milano, Italy), which was used to paint raw fiberglass surfaces previously treated with an epoxy-primer (Marine Primer, Attiva Marine by Bartolomeo Boero, Genova, Italy). Mixtures containing 370 mg of paint and 10 mg of lyophilized Cam were prepared, vortexed, and used to cover 10 raw fiberglass surfaces (squares, 25 cm^2 each). The painted fiberglass slides were left to desiccate for at least 24 h before performing enzyme assays. Control fiberglass squares devoid of Cam were also prepared. The velocity of CO_2 release from control slides was determined and subtracted from the activity observed with

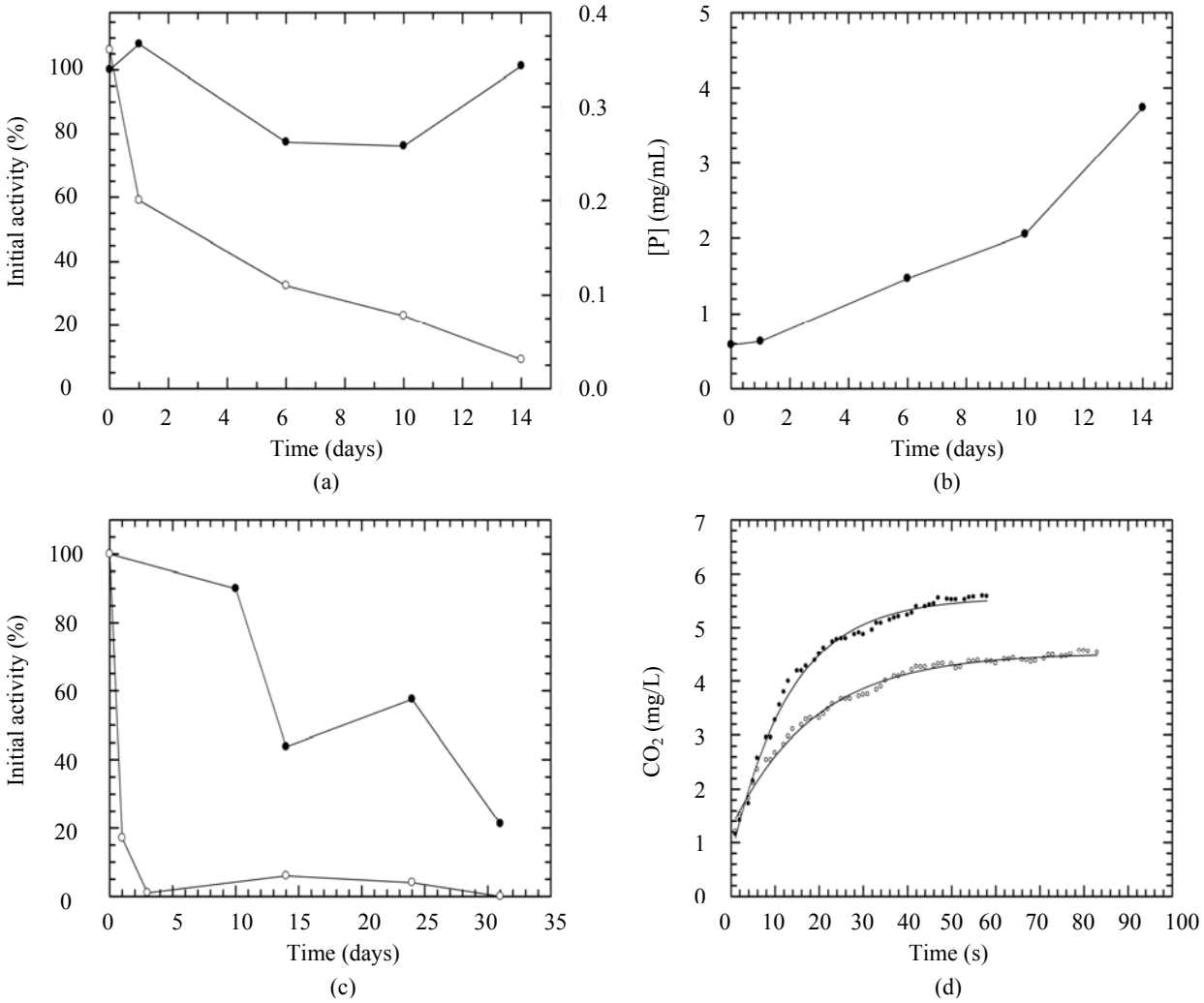

Fig. 1 Stability ((a)-(c)) and carbonic anhydrase activity of Cam entrapped into styrene acrylic resin (d).

Panels (a) and (b): stability at 54 °C of *Methanosarcina thermophila* carbonic anhydrase.

The lyophilized enzyme was dissolved, at 1 mg/mL, in phosphate buffer, and the enzyme solution was kept at 54 °C for 14 days. At predetermined time intervals, aliquots of the sample were withdrawn and used to determine enzyme activity (release of CO_2 from $NaHCO_3$, panel (a), filled circles), and protein concentration according to the Bradford assay (panel (a), empty circles). The specific enzyme activity, as a function of time, is reported in panel (b).

Panel (c): stability of *Methanosarcina thermophila* (filled circles) and bovine (empty circles) carbonic anhydrase in seawater. The enzymes were dissolved in natural seawater and kept at room temperature for 31 days. At predetermined time intervals the activity of both enzymes was assayed using a CO_2-sensitive electrode to determine the release of carbon dioxide from $NaHCO_3$.

Panel (d): CO_2-releasing activity of fiberglass slides (25 cm^2) painted with enzyme-free (empty symbols) or enzyme-containing (filled symbols) polystyrene resin. To obtain enzyme-containing slides, lyophilized carbonic anhydrase from *Methanosarcina thermophila* was dissolved into polystyrene resin (Crilat D120S, Vinavil, Milano, Italy), and the paint accordingly prepared was uniformly distributed over fiberglass slides previously treated with an appropriate primer (Marine Primer, Attiva Marine by Bartolomeo Boero, Genova, Italy). Enzyme-free slides were prepared using the same procedure and a polystyrene resin devoid of carbonic anhydrase. To assay enzyme activity, the slides were half-immersed into a vessel containing 200 mM Tris buffer (pH 7.6). A CO_2-sensitive electrode was then dipped into the vessel, and reactions were started by the addition of 5 mM $NaHCO_3$.

enzyme-containing surfaces (Fig. 1d). Remarkably, the authors detected net CO_2 release from the enzyme-containing slides at a rate equal to 68 ± 34 μg/L·s (Fig. 1d), corresponding to 1.55 ± 0.77 μM/s.

The cost-effective production, the biochemical robustness of Cam, and the capability of this enzyme to withstand entrapment into polyacrylic resins, prompted us to test the potential of this enzyme as an antifouling component of paints. We indeed reasoned that the release of CO_2 by the paint-entrapped enzyme

would physically and chemically inhibit the colonization of water-submerged surfaces by microorganisms. In particular, the authors expected that a CO_2-releasing surface would physically (mechanically) hamper the adsorption of microorganisms, whose the growth would be chemically inhibited by the concomitant increase in CO_2 concentration. To verify these hypotheses, the authors used painted fiberglass slides to determine the growth inhibition of the unicellular marine microorganism *Phaeodactylum tricornutum*, which represents a model microorganism for tests of ecotoxicity. The authors indeed performed a first experiment according to the UNI EN ISO 10253 (2006) procedure (ISO 10253:2006. Water quality. Marine algal growth inhibition test with *Skeletonema costatum* and *Phaeodactylum tricornutum*). Independent slides (painted with enzyme-free or enzyme-containing resin) dipped into seawater devoid of the marine microorganism were also used to determine the concentration of CO_2. As Fig. 2a shows, the growth of *P. tricornutum* was strongly inhibited (65%) for 24 h by the fiberglass slides painted with an enzyme-containing resin, and this inhibition lasted, although at a lower level (35%), for further 24-48 h (Fig. 2a). Nevertheless, 96 h after the onset of the experiment, the growth of *P. tricornutum* was not significantly affected by the slides painted with the enzyme-containing resin (Fig. 2a). During the same experiment a significant difference was observed between the concentration of CO_2 determined in the presence of enzyme-free or enzyme-containing slides. The concentration of CO_2 was indeed found to steadily increase for 72 h in the presence of enzyme-containing slides, and to reach a maximum between 72 h and 96 h after the onset of the experiment (Fig. 2b).

This observation suggests that the enrichment of seawater with CO_2 released by the Cam-containing slides chemically inhibits the growth of the marine microorganism. This chemically-induced inhibition does

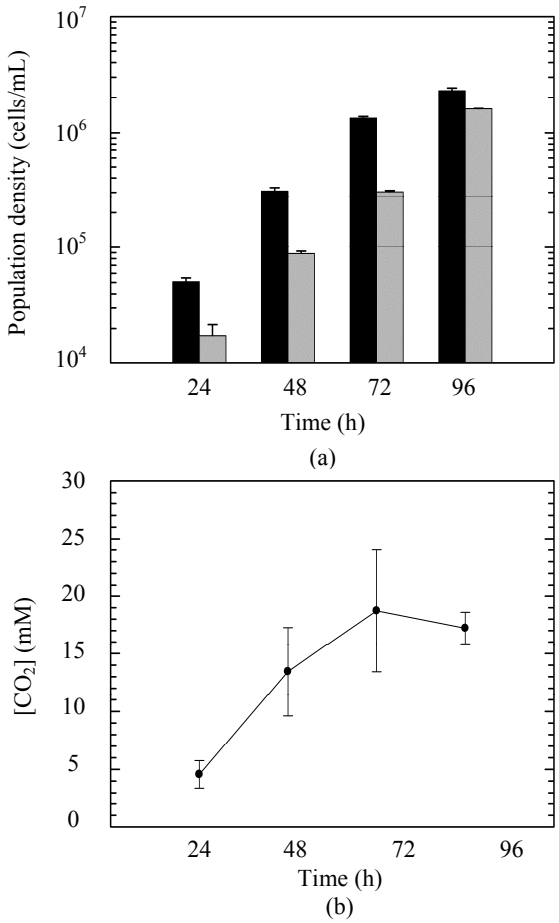

Fig. 2 Inhibition of growth of *Phaeodactylum tricornutum* (Panel (a)) and CO_2 release (Panel (b)) by Cam-containing fiberglass slides.

Panel (a): growth of *Phaeodactylum tricornutum* in the absence (black bars) or in the presence (grey bars) of enzyme-containing fiberglass slides. A single pre-culture of *P. tricornutum* was diluted to 10^4 total individuals into vessels filled with artificial seawater and provided or not with enyme-containing fiberglass slides. Painting of slides was performed as in Fig. 1d. Each 24 h aliquots of the samples were withdrawn, and *P. tricornutum* individuals were counted. Experiments were performed in triplicate. Error bars represent standard deviation ($n = 3$).

Panel (b): CO_2 concentration determined, as a function of time, in samples of artificial seawater hosting enzyme-free or enzyme-containing fiberglass slides. Painting conditions as in Fig. 1d. The concentration of carbon dioxide was determined, by means of a CO_2-sensitive electrode, transferring aliquots of the artificial seawater to a vessel containing 150 mM sodium citrate, pH 5. The difference between the baseline (before addition of sea water) and the final (120 s after addition of sea water) signal was recorded. The CO_2 concentration determined in the presence of enzyme-free fiberglass slides was subtracted from the concentration observed in the presence of enzyme-containing slides. The electrode was previously calibrated with appropriate standars of $NaHCO_3$.

not rule out that the biofouling of water-submerged surfaces could be physically inhibited, i.e., by the continuous release of CO_2 from the painted surfaces.

The toxicity of Cam-containing paints towards *Artemia franciscana* Kellogg was also evaluated, according to the APAT-IRSA-CNR 8060 (2003) procedure (APAT, IRSA-CNR: Metodi analitici per le acque, Poligrafico dello Stato, 2003). In this case, the experiments were performed under dark conditions, at 25 °C. The authors observed a slight, if at all, toxicity of the enzyme-containing paint towards *Artemia franciscana*. In fact, 48 h after the transfer of the nauplii to the microplates, the density of *A. franciscana* populations hosted into enzyme-containing wells was 5% lower when compared with the density of populations hosted in enzyme-free wells (data not shown). This observation suggests that the enzyme-containing paints do not feature toxicity towards marine organisms, although they are competent in preventing the growth of marine microorganisms. This property is, in the authors' opinion, important to produce manufactures provided with a specific anti-biofouling agent.

4. Concluding Remarks

The authors have shown here that enzymes can be effectively entrapped into paints without impairing their catalytic activity, and that this activity can act at a distance to prevent the growth of marine microorganisms whose action is relevant in the fouling of water-submerged surfaces. Further work, carried out on time scales longer than those considered here, could provide insight into the market potential of paints containing carbonic anhydrase. To clarify this point, the authors are currently planning to perform experiments using large surfaces exposed to natural marine environments.

Acknowledgments

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