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Research article

Calcite/aragonite-biocoated artificial coral reefs for marine parks

Volodymyr Ivanov[1,3,*] and Viktor Stabnikov[2,3]

[1] Advanced Research Laboratory, National University of Food Technologies, 68 Volodymyrskaya Str., Kiev 01601, Ukraine
[2] Department of Biotechnology and Microbiology, National University of Food Technologies, 68 Volodymyrskaya Str., Kiev 01601, Ukraine
[3] Previous address: School of Civil and Environmental Engineering, Nanyang Technological University, 50 Nanyang Avenue, Singapore, 639798

* **Correspondence:** Email: cvivanov111@gmail.com; Tel: + 38-066-403-0226

Abstract: Natural formation of the coral reefs is complicated by slow biomediated precipitation of calcium carbonate from seawater. Therefore, manufactured artificial coral reefs can be used for the formation of "underwater gardens" in marine parks for the recreational fishing and diving that will protect natural coral reefs from negative anthropogenic effects. Additionally, the coating of the concrete, plastic or wooden surfaces of artificial coral reef with calcium carbonate layer could promote attachment and growth of coral larvae and photosynthetic epibiota on these surfaces. Three methods of biotechnological coating of the artificial coral reefs have been tested: (1) microbially induced calcium carbonate precipitation from concentrated calcium chloride solution using live bacterial culture of *Bacillus* sp. VS1 or dead but urease-active cells of *Yaniella* sp. VS8; (2) precipitation from calcium bicarbonate solution; (3) precipitation using aerobic oxidation of calcium acetate by bacteria *Bacillus ginsengi* strain VSA1. The thickness of biotechnologically produced calcium carbonate coating layer was from 0.3 to 3 mm. Biocoating using calcium salt and urea produced calcite in fresh water and aragonite in seawater. The calcium carbonate-coated surfaces were colonized in aquarium with seawater and hard corals as inoculum or in aquarium with fresh water using cyanobacteria *Chlorella sorokiana* as inoculum. The biofilm on the light-exposed side of calcium carbonate-coated surfaces was formed after six weeks of incubation and developed up to the average thickness of 250 μm in seawater and about 150 μm in fresh water after six weeks of incubation. The biotechnological manufacturing of calcium carbonate-coated concrete, plastic, or wooden surfaces of the structures imitating natural coral reef is technologically feasible. It could be commercially attractive solution for the introduction of aesthetically pleasant artificial coral reefs in marine parks and resorts.

Keywords: biotechnological coating of surface; artificial coral reefs; marine parks; calcium carbonate precipitation; underwater gardens; tourist attraction

1. Introduction

Coral reefs are declining and degrading around the world due to anthropogenic pollution and technogenic stresses, as well as warming and acidification of seawater due to global climate change. To cope with increasing marine habitat destruction due to anthropogenic and natural impacts, new scientific ideas and novel methods to enhance marine ecosystems are needed urgently. One way for the restoration and protection of natural coral reefs could be the construction of artificial coral reefs for the marine parks, which will protect natural coral reefs from negative anthropogenic effects. Manufacturing and use of the artificial coral reefs in the tropical and subtropical coastal areas could be commercially attractive business for the recreational fishing and diving in "underwater coral gardens" as well as the calcium carbonate-coated frames of shellfish aquaculture farms.

Conventionally, any human-made underwater construction supporting marine life can be considered as an artificial reef. The European artificial reef research network (EARRN) defined an artificial reef as "any structure that has been deliberately submerged on the substrate (sea bed) to imitate some of the characteristics of natural reefs" [1]. The use of artificial reefs suitable for colonization by the coral larvae is an effective way to restore damaged coral reefs to maintain marine biodiversity and productivity [2,3]. The surface of the artificial reef needs to be coated with calcium carbonate to facilitate colonization of marine microorganisms because calcium carbonate forms the skeletal base for marine larval settlement [4]. Meanwhile, the natural precipitation of calcium carbonate by reef-building organisms is extremely slow process. Its annual rate is usually a few millimeter layers. One idea to accelerate this process is to deposit $CaCO_3$ from seawater by electrochemical method [5,6]. However, this method is not effective for large scale formation of artificial coral reefs since the concentration of dissolved calcium in seawater is low.

The idea of present research was the biotechnological coating of the surface of concrete, plastic or wooden frames with the layer of calcium carbonate and testing them as the potential artificial reefs for the colonization with coral larvae or microscopic algae. Three methods of the biotechnological coating have been tested:

1) Microbially induced calcium carbonate precipitation (MICCP) from concentrated solutions of calcium chloride and urea:

$$CaCl_2 + CO(NH_2)_2 + 2H_2O + \text{urease} \rightarrow CaCO_3 + 2NH_4Cl + \text{urease} \quad (1)$$

where urease of urease-producing bacteria is needed to hydrolyze urea producing alkaline pH and forming the crystallization centers of calcium carbonate minerals such as calcite, vaterite, or aragonite;

2) Calcium carbonate precipitation from calcium bicarbonate solution of low concentration. This chemical reaction requires either small shift of pH due to addition of urea and urease or urease-producing bacteria or drying of treated surface to shift equilibrium of the reaction to the right side:

$$Ca(HCO_3)_2 \leftrightarrow CaCO_3\downarrow + HCO_3^- + H^+ \leftrightarrow CaCO_3\downarrow + CO_2\uparrow + H_2O \quad (2)$$

3) Calcium carbonate precipitation due to aerobic microbial oxidation of calcium salts of organic acids, for example calcium acetate:

$$Ca(CH_3COO)_2 + 4O_2 \rightarrow CaCO_3\downarrow + 3CO_2\uparrow + 3H_2O \quad (3)$$

The mechanisms of calcium carbonate precipitation using MICCP were described in reviews [7-15]. The mechanisms of precipitation from calcium salts of organic acids were also described [10-17] but precipitation from calcium bicarbonate solution was just discussed [9-15]. Photosynthetic precipitation of calcium carbonate, which is a common process in microbial lithification and formation of marine stromatolites [16,18], was not studied because it is too slow process to be used in real production of artificial reefs.

The aim of this study was to examine the biotechnological coating of the concrete, plastic, and wooden surfaces with a layer of calcium carbonate to manufacture artificial coral reefs for the recreational "underwater gardens" in marine parks in Singapore or other tourist destinations.

2. Materials and Methods

2.1. Bacteria used for Biocoating

Precipitation of calcium carbonate MICCP from calcium chloride solution and stoichiometrical quantity of urea, and from calcium bicarbonate solution and small quantity of urea to shift equilibrium to the decay of bicarbonate were performed with either live bacteria of alkaliphilic strain of *Bacillus* sp. VS1 [19,20] or with dead but urease active cells of Y*aniella* sp. VS8 from the family *Micrococcaceae* [21]. Their batch cultivation was under shaking at 200 rpm at temperature 25 ℃ in the medium of the following composition: tryptic soya broth DIFCO: 20 g, NaCl: 20 g, $NiCl_2{\cdot}6H_2O$: 24 mg, phenol red: 10 mg, distilled water: 1 L, pH 8.2. This medium was sterilized at 121 ℃ for 15 min. Stock solution of urea, 100 g/L, was sterilized by filtration through Millipore filter with diameter 0.2 μm to avoid urea loss due to thermal treatment. 200 mL of urea stock solution was added to 800 mL of described above medium and this urea-containing medium was used for cultivation. Urease activity was defined as the amount of ammonium produced from 1 M solution of urea per minute determined using electric conductometer showing linear correlation between the molar concentrations of NH_4^+ and the changes of electric conductivity of solutions in mS/cm.

Precipitation of calcium carbonate due to aerobic microbial oxidation of calcium acetate was done using *Bacillus ginsengi* strain VSA1 isolated from tropical soil [13]. Their batch cultivation was under shaking at 200 rpm at temperature 25 ℃ in the medium containing calcium acetate, 159 g/L (1 M), and yeast extract, 1 g/L.

2.2. Biocoating Procedures

To perform MICCP, 500 mL of bacterial suspension was centrifuged at 4 ℃ and 10 000 rpm for 15 min using micro cooling centrifuge 5922 (Kubota, Japan). Precipitate was re-suspended in 100 mL of 0.2% xanthan solution and stored at 4 ℃ before coating experiments. This bacterial suspension was used for the spraying of the surfaces with a dosage of about 0.1 mL/cm^2 (1 L/m^2). Then the surfaces were placed for 20 h in solution containing 111 g/L (1 M) of calcium chloride and 90 g/L

(1.5 M) of urea. Such treatments were repeated usually four times, until formation of mechanically stable layer of calcium carbonate crystals on the surface.

The calcium bicarbonate solution for the coating was produced by shaking or rotation of 0.5 M suspension of amorphous powder of calcium carbonate in atmosphere of CO_2 under pressure of 30 bars (maximum concentration of dissolved calcium was 22 mM) or atmospheric pressure (maximum concentration of dissolved calcium was 10 mM). The rate of calcium dissolution was from 10 to 15 mM/h. Urea was added to the decantated solution of calcium bicarbonate to final concentration 1 mM. Centrifuged suspension of urease-producing bacteria described above was used for the spraying of the surfaces with a dosage of about 0.1 mL/cm^2 (1 L/m^2). Then the surfaces were placed for approximately 10 h in solution of calcium bicarbonate: 10 mM, and urea: 1 mM, until remaining calcium concentration will be lower 1 mM.

The coating using calcium acetate bacterial oxidation was done after spraying of surfaces with centrifuged suspension of acetate-oxidizing bacteria as described above. Bacteria-treated surfaces were placed for approximately 48 h in solution of calcium acetate: 159 g/L (1 M), and yeast extract: 1 g/L, under stirring of solution about 100 rpm until remaining calcium concentration will be lower 10 mM. The dosage of calcium acetate solution must be about 10 mL/cm^2 (100 L/m^2) of surface to produce a layer of calcium carbonate with a thickness of 3 mm. Therefore, the coating treatments were repeated several times until formation of mechanically stable layer of calcium carbonate crystals on the surface.

2.3. Study of the Coated Surfaces

The surfaces of concrete, ceramic brick, plastic artificial corals, and terrestrial plants such as cypress-like *Chamaecyparis lawsoniana* and cactus *Arthrocereus spinosissimus* were coated with calcium carbonate layer as described above. Coated surfaces were placed in the aquarium with either sea water (sea salt from Red Sea was added into tap water to concentration 35 g/L) or fresh water. Live rocks and two types of solid corals and one type of soft corals were added to the aerated sea water aquarium because live coral acting as a source of larvae. Suspension of cyanobacteria *Clorella sorokiana* was added to the aerated aquarium with fresh water.

The surfaces were observed by a scanning electron microscope (SEM) Zeiss EV050, UK, and a Fluoview 300 confocal scanning fluorescence microscope (CSFM) (Olympus, Tokyo, Japan). For SEM, the specimens were sputter coated with Au-Pd using Emitech SC7620, Quorum Technologies, UK.

3. Results and Discussion

3.1. $CaCO_3$ Precipitation Rate and Duration of the Biocoating Processes

The rate of calcium carbonate precipitation from calcium chloride and urea solution was proportional to the urease activity of bacterial suspension. Initial urease activity of urease-producing bacteria decreased quickly in 1 M solution of urea (Figure 1). So, about 90% of urea was hydrolyzed and, respectively, 90% of $CaCO_3$ was precipitated for about 4 h. Therefore, duration of one cycle of the treatment (coating) using MICCP should be shorter 4 h. In this case, bacterial biomass with average urease activity 4 mM/min will precipitate about 0.97 M of calcium for 4 h.

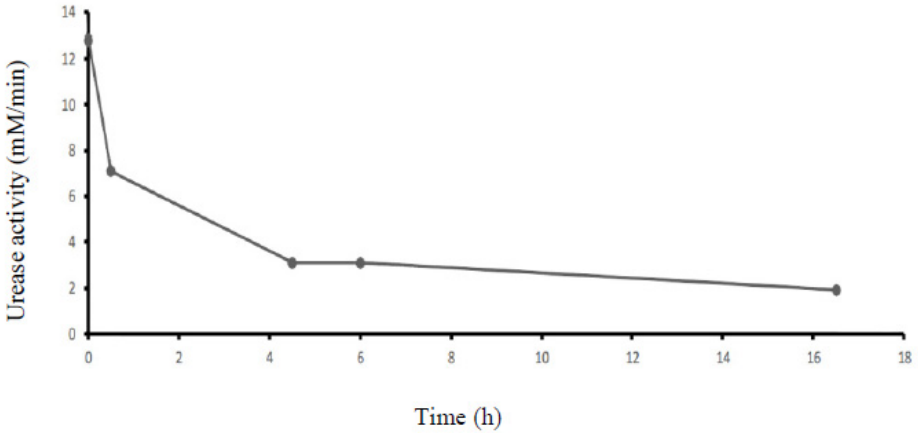

Figure 1. Decrease of urease activity of *Yaniella* sp. VS8 in 1 M solution of urea.

The average rate of $CaCO_3$ precipitation from calcium chloride and urea solution in our experiments was about 60 mM/h or 6 g of $CaCO_3$/h from 1 L of solution. To produce a layer of calcium carbonate with a thickness of 0.35 cm and a density of 2.9 g/cm^3 the coating density should be 1 g of $CaCO_3$/cm^2, so the total calculated dosage of calcium chloride (1 M) and urea solution is about 0.01 L/cm^2 (0.1 m^3/m^2) of surface. The calculated depth of the solution layer above surface in this case is 10 cm which looks as acceptable depth for diffusion and convection transfers of calcium ions from the solution to the surface.

80% of bioactivated precipitation of $CaCO_3$ on surface from the solution of calcium bicarbonate (22 mM) and urea (1 mM) was done for about 10 h. The average rate of $CaCO_3$ precipitation from calcium bicarbonate solution was about 1.6 mM/h in the system with mechanical stirring of solution. To produce a layer of calcium carbonate with a thickness of 0.35 cm and a density of 2.9 g/cm^3 the coating should be 1 g of $CaCO_3$/cm^2, so the total calculated dosage of calcium bicarbonate solution (10 mM) is about 1 L/cm^2 (10 m^3/m^2) of surface. Therefore, the total depth of the solution layer above surface in this case is 10 m, which looks as not acceptable depth for diffusion and convection transfers of calcium ions from the solution to the surface. To decrease this depth to conventionally acceptable 10 cm, the number of the treatments with the solution of calcium bicarbonate must be at least 100 with the duration of all treatments for at least 620 h (26 days).

The average rate of bioactivated precipitation of $CaCO_3$ on the surface from the solution of calcium acetate was about 7 mM/h. So, duration of the coating cycle from 1 M solution of calcium acetate is about 143 h (6 days). To produce a layer of calcium carbonate with a thickness of 0.35 cm and a density of 2.9 g/cm^3 the coating should be 1 g of $CaCO_3$/cm^2, so the total calculated dosage of calcium chloride (1 M) and urea solution is about 0.01 L/cm^2 (0.1 m^3/m^2) of surface. The calculated depth of the solution layer above surface in this case is 10 cm which looks acceptable depth considering diffusion and convection transfers of calcium ions from the solution to the surface.

Comparison of the major parameters of different biocoating procedures is given in the Table 1.

Fast but environmentally not safe method is biotechnology using calcium salt and urea. Slow but environmentally safe method is coating using calcium bicarbonate. Probably, optimal method could be a combination of precipitation of calcium carbonate from calcium acetate solution due to both hydrolysis of urea by urease-producing bacteria and aerobic biooxidation of acetate by acetate-oxidizing bacteria, which could be relatively fast and at the same environmentally safer than method

with urea hydrolysis only. Other biotechnologies of the surface biocoating with calcium carbonate could be developed. For example, most interesting approach could be the delayed decay of calcium bicarbonate using EDTA or other chelates of calcium ions.

Table 1. Comparison of the major parameters of different biocoating procedures.

Type of the biocoating	Total duration of biocoating, h	Number of the treatment cycles	Environmental safety
Precipitation from calcium choride and urea solution (molar ratio 1:1.5) due to urea hydrolysis	4 or more depending on urease activity of attached bacterial cells	1	Unsafe due production of ammonia/ammonium, and increase of pH to 9 but could be relatively safe if release of ammonia and polluted water to environment will be prevented
Precipitation from calcium bicarbonate and urea solution (molar ratio 1:0.05) due to instability of calcium bicarbonate	620 (26 days)	100	Safe
Precipitation from calcium acetate solution due to aerobic oxidation of acetate	143 (6 days)	1	Relatively safe if to exclude release of acetic acid and bacterial cells to atmosphere

3.2 Surfaces after Biocoating and Colonization

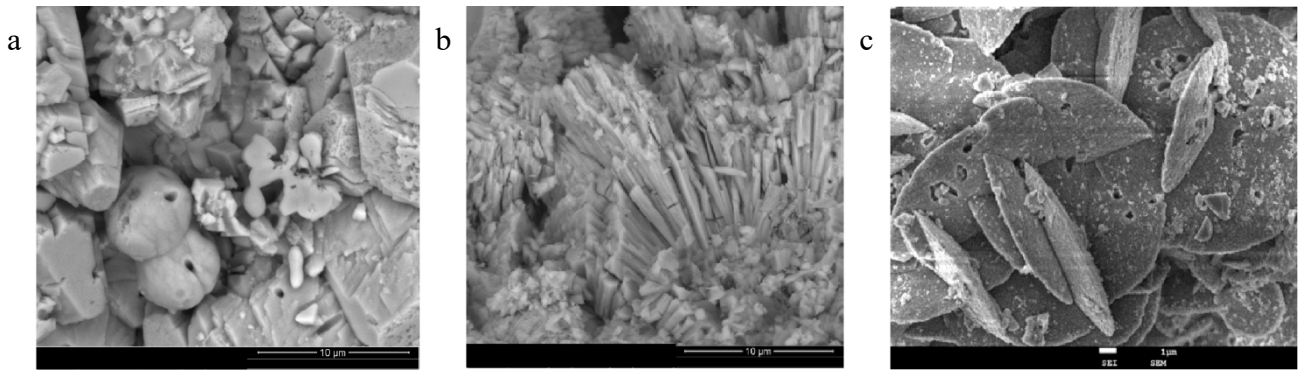

Figure 2. Crystals of calcium carbonate using urea hydrolysis (a and b) or decay of calcium bicarbonate and aerobic oxidation of calcium acetate (c).

Precipitation of calcium carbonate using urea hydrolysis in fresh water is going in the shape of prismatic crystals of calcite but there may be also spheres of vaterite (Figure 2a). The needles-shaped crystals of aragonite were produced in seawater or in case that Mg^{2+} ions were present (Figure 2b). Decay of calcium bicarbonate and aerobic oxidation of calcium acetate produced rose-shaped crystals of $CaCO_3$ (Figure 2c).

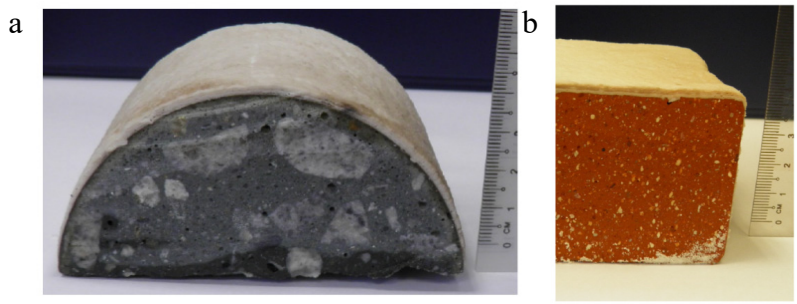

Figure 3. Biocoated concrete (a) and ceramic brick surfaces (b).

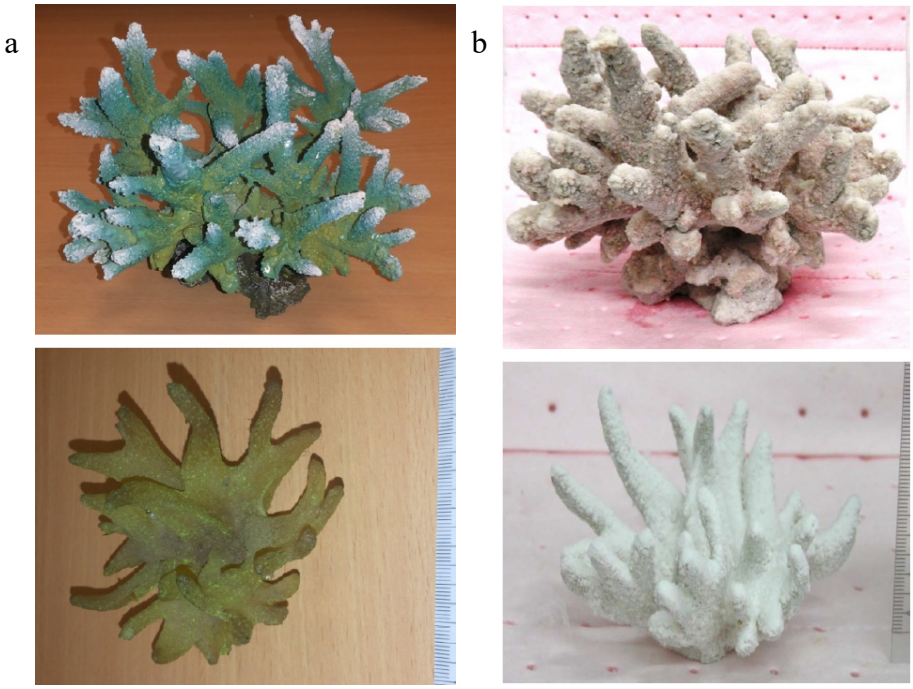

Figure 4. Plastic artificial corals before (a) and after (b) coating.

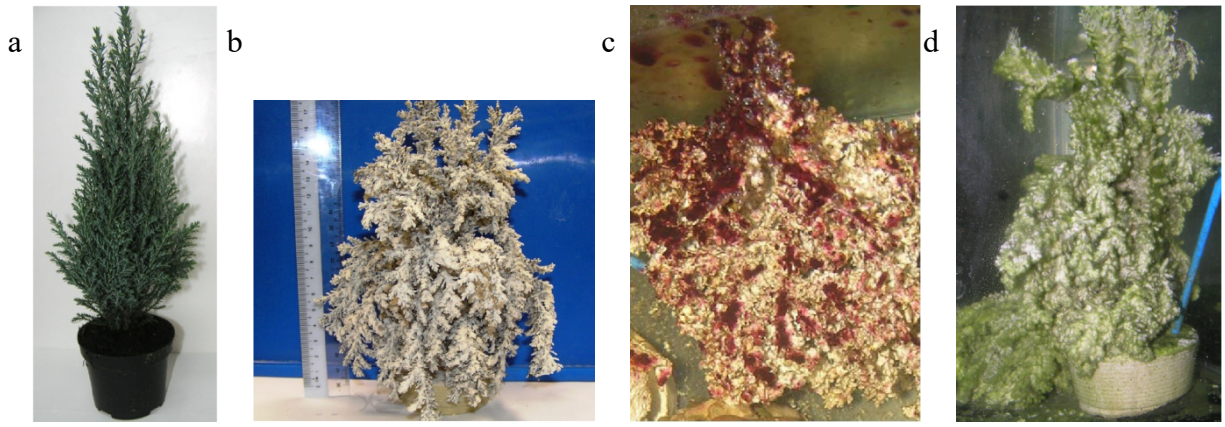

Figure 5. *Chamacyparis ellwoodi* plant before (a) and after biocoating with calcium carbonate (b), and after 6 weeks of surface colonization with photosynthetic microorganisms in seawater (c) and freshwater (d).

Figure 6. Cactus *Arthrocereus spinosissimus* plant before (a) and after biocoating with calcium carbonate (b), and after 6 weeks of surface colonization with photosynthetic microorganisms (c).

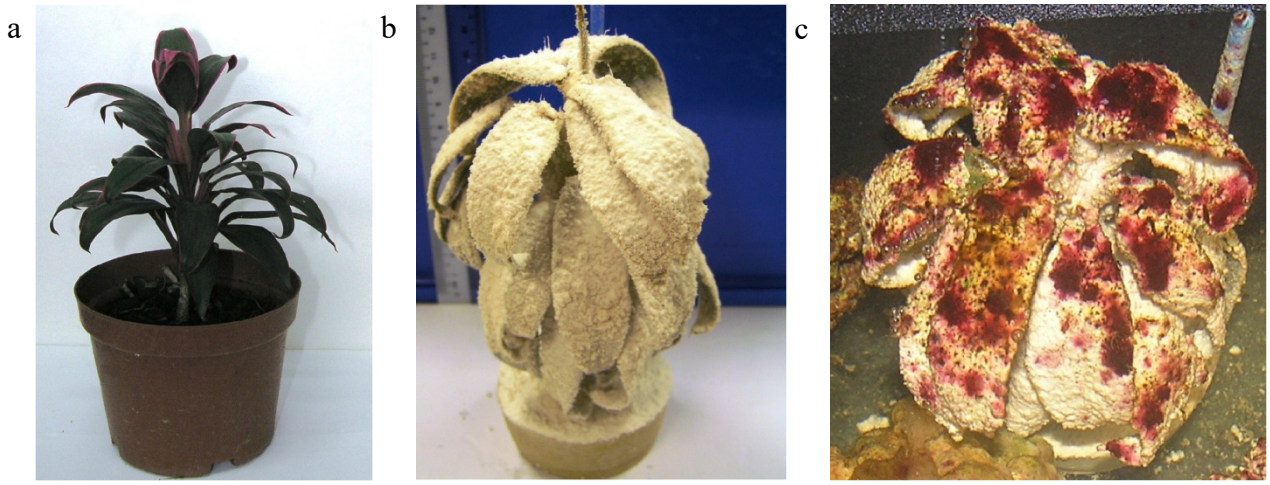

Figure 7. *Cordyline terminalis* plant before (a) and after biocoating with calcium carbonate (b), and after 6 weeks of surface colonization with photosynthetic microorganisms (c).

Biocoatings have been done for the concrete and ceramic surfaces (Figure 3), for the decorative plastic artificial corals (Figure 4), and for the plants *Chamacyparis ellwoodi*, cactus *Arthrocereus spinosissimus*, and *Cordyline terminalis* (Figure 5–7).

The coated surfaces being placed in seawater or fresh water with inoculum of corals or algae were colonized with photosynthetic microorganisms after six weeks of incubation (Figure 5–7). The biofilm on the light-exposed side of the surfaces developed up to the thickness of 250 ± 100 μm. These data show that the formation of a layer of $CaCO_3$ on surfaces of concrete, ceramics, plastic, or wood and its colonization with photosynthetic microorganisms is feasible.

It is expecting that manufacturing of the artificial coral reefs coated with calcium carbonate layer will have positive environmental impact due to increase of aquatic biodiversity in coastal areas, enhanced consumption of CO_2 by photosynthetic epibiota, enhancing natural restoration of corals, and diverting tourists and divers away from fragile natural coral reefs.

Conclusion

Biotechnological coating of surfaces is feasible for the manufacturing of artificial coral reefs. Optimal biotechnology could be precipitation of calcium carbonate due to aerobic microbial oxidation of salts of calcium with organic acids. From the scientific point of view, the development of biocoating biotechnologies will promote studies in new interdisciplinary area involving biogeochemistry, environmental engineering, and marine sciences. The biogeotechnology of calcium carbonate biocoating have the potential to generate new businesses such as "underwater marine gardens" with positive social and economic impacts. For example, the biocoated artificial coral reefs can be linked to the tourist destinations such as Sisters' Island Marine Park development in Singapore or other marine parks, which are planned worldwide.

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