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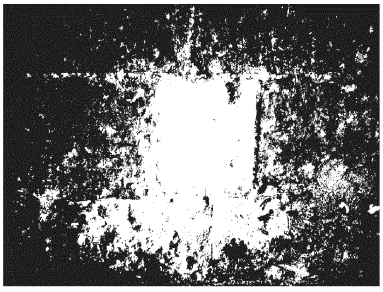
(54) **BIO-CLEANSING KIT AND METHOD FOR REMOVING BIOFILMS FROM SUBSTRATES**

(57) The present invention relates to an enzymatic bio-cleansing kit suitable to remove biofilms as molds, algae, bacteria, lichens and similar organisms from substrates, preferably from stone-like substrates such as marble surfaces. Furthermore, the present invention discloses a method based on said enzymatic bio-cleansing kit for removing pre-existing biofilms from surfaces without affecting the integrity of the substrate and optimizing times and costs in surface maintenance. Said kit and method find application in many areas. Advantageously, the kit according to the present invention is easy to make and use in various embodiment, one of them allows enzyme recovery. In addition, it is safe for the users, the substrate and the environment.



(a)

(b)

Figure 5

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Description

TECHNICAL FIELD

[0001] The present invention relates to a kit for the enzymatic bio-cleansing and a method based on said kit to remove biofilms from surfaces made of stone, wood, ceramic or other materials and preferably from surfaces of delicate and unique works of art e.g. an ancient mosaics or stone works. In particular, the present invention provides a new and inventive solution to the problem of removing biofilms comprising microorganisms such as molds, lichens, cyanobacteria, microalgae, etc. without affecting the integrity of a delicate and valuable surface.

BACKGROUND ART

[0002] Microorganisms such as molds, cyanobacteria, microalgae, lichens, etc., often in combination with organic and inorganic substances, gradually adhere to floors, walls or stone-works in particular when they are exposed to atmospheric agents. These organic deposits, technically defined as 'biofilms', deteriorate the substrate they adhere thereto by altering, often irreparably, firstly the surface aesthetics and then the surface structure of the substrate. The kinetics of the surface deterioration induced by biofilms depends on the nature of the substrate and therefore on the substrate material and porosity.

[0003] The problem of biofilm removal is of great concern in case of sites which cannot be covered by their extension (e.g. archaeological areas) and therefore are constantly exposed to the combined action of atmospheric agents and of microorganisms that gradually colonize their surface.

[0004] Currently, there are commercially available various products, for both professional and domestic use, which are useful to remove biofilms from a surface. Typically, these products are based on biocides such as sodium hypochlorite, tin compositions or other compounds such as Biotin-T and Biotin-R (marketed by CTS, Bresciani or Antares). These known compounds are chemically rather aggressive and generally remove effectively biofilms (often after several applications or with high quantity of biocide), but they may alter the surface colors and even damage the surface structure. For example, it is absolutely not recommended the use of chlorine-based biocidal products when removing biofilms from limestone substrate (e.g. marble or travertine) as they attack the surface of the stone-work.

[0005] Furthermore, these known products present a significant chemical hazard for the user which is increased in aerosol formulations, since they can generate respiratory or skin sensitization as a result of a prolonged use. Finally, these products do not exhibit a selective action against the biofilm constituents. For these reasons, the use of traditional products biocides-based is not recommended in the restoration of delicate, valuable or unique surfaces especially of artifacts belonging to the cultural heritage.

[0006] To overcome the drawbacks of cleaning products based on biocides, in recent years formulations containing enzymes have been commercially introduced. As a main advantage formulations containing enzymes present a selective cleaning action. In fact, biofilm removal from the surface occurs as the result of the enzyme attack on a selective bond recognized by the enzyme chemical structure. In other words, enzymes act on the chemical mechanism of biofilm removal.

[0007] In the state-of-the-art there are known various enzyme systems, for example, gels consisting of enzymes and specific surfactants and systems using microorganisms responsible for enzymatic reactions (see Ranalli, G., et al. Journal of Applied Microbiology 2005 98 pg. 73-83). Particularly, in the field of fine arts and restoration, it is well-known the use of enzymatic systems to remove biofilms from art-works of historical importance such as frescoes, mosaics, prints, but also from delicate floors or walls in traditional or contemporary houses. A list of enzyme-based cleaning compositions can be found, for example, in the book "L'uso degli enzimi nella pulitura di opere policrome" by P. Cremonesi (Il Prato ed. 2002), or in the text-book "La Chimica nel Restauro" of M. Matteini and A. Moles (Nardini ed. 2007). The use of such compositions is particularly suitable, and sometimes it is the only choice when the use of traditional cleaning systems would result in damage or destruction of the work to be restored.

[0008] Despite their attractiveness, enzyme-based coatings present several drawbacks, particularly high cost, composition stabilization, rapid enzyme inactivation and safety concerns. In an effort to overcome the limitation of the prior art enzyme-based cleaning compositions, a high number of patent applications related to cleaning compositions and methods have been filed during the last years.

[0009] For example, in the patent specification US2012276617A1 JIA et al. proposed waterstabilized active coatings containing polymer modified enzyme for stable self-cleaning of organic stains. In particular, this composition includes a base material i.e. a wax-based surface conditioner, polish or protectant and a PEG-modified (polyethylene glycol) enzyme associated with said base. The main issue addressed in said patent is rapidly enzyme inactivation upon exposure to water of the coating materials of the prior art. The inventors overcome this problem by providing coatings stabilization against inactivation by weathering. Noticeably, they discovered that the addition of one or more polymeric moieties on an enzyme prior to incorporation with a base provides for improved waterstability of the resulting coating material.

[0010] The intended use of the active temporary coatings disclosed in US2012276617A1 is clearly preventing organic stains or organic material (e.g. from food, insects, or the environment) stains to adhere to the temporary active coating material. In fact, the PEG-modified en-

zyme is capable of degrading a component of an organic stain in contact with said active coating material (and not in contact with the substrate). Therefore, such coatings are definitely not useful to remove organic stains or biofilm already existing on the substrate, particularly from stone-like works. This limitation in use and the coating composition comprising an organic base material associated to the enzyme, make the teachings by JIA et al. not useful in the field of historic heritage restoration and maintenance, where biofilm removal from ancient works must not alter the original substrate or leave residues such as a wax.

[0011] Another noticeably patent application is WO2014006424A1, in the name of Xeros Ltd, which discloses a novel cleaning material that finds particular application in cleaning processes, preferably of fabrics. The novel cleaning material operates in the presence of limited quantities of water and use a cleaning formulation which comprises solid polymeric cleaning particles in combination with suitable detergent component. Most particularly, the system utilizes a cleaning formulation wherein the detergent component is an enzyme immobilized on the solid polymeric cleaning particles.

[0012] Enzyme immobilization improves cleaning performance, as well as the ability to re-use these detergent components over multiple washes, thereby providing environmental and economic benefits. Despite these remarkable advantages, in WO2014006424A1 the use of the novel cleaning material to stone-like substrates is not provided, and it appears clearly out of the scope of this patent. In addition, the pores of the stone-like substrate can trap the solid polymeric cleaning particles, which degrade and yellow under the effect of solar radiation. For this reason, they are unsuitable in the field of historic heritage where restauration must not alter the original substrate nor leave residues thereon.

[0013] Being proteins, free enzymes are likely to stimulate an immunological response in man and animals, including an allergic response, under certain conditions. Therefore, for its allergenic potential and immunogenicity the use of enzymes raises safety concerns which are addressed, for instance, in the patent WO2014067933A1 in the name of C-Lecta GmbH.

[0014] This patent claims a single-use-composition based on an insoluble enzyme preparation comprising an enzyme immobilized on a solid carrier (preferably fumed silica), wherein the insoluble enzyme preparation is dispersed in the single-use-composition. Compared to free enzyme compositions, safety risk is diminished by the insoluble enzyme preparations of this invention in certain applications that involve direct contact of enzymes with humans either by handling, utilizing, or uptake of such enzymes or enzyme containing compositions.

[0015] The single-use insoluble enzyme preparations of WO2014067933A1 are particularly useful in care compositions and food applications, while washing or cleaning applications are only described generally in a useless way for the skilled in the art. Actually, no useful cleaning compositions is disclosed, although the inventors declare that they can be used for providing enzyme activity to single-use hard surface (e.g. surface of equipment used in the food industry) preparations. Furthermore, removal of biofilms is cited only with reference to biofilms formed in lens cases, which are very different from biofilms formed on stone-like substrate. In this case, the inventors do not disclose any useful composition. Again, in a preferred embodiment of the invention, embedding enzymes in coatings, paint and varnish yields a protection against the settlement of algae and mold. Therefore, such compositions prevent biofilm formation and they are not useful to remove biofilms already adhered to the substrate.

[0016] Finally, the only cleaning composition of WO2014067933A1 is presented in the Example 4 and provides lipase activity to a commercial household cleaner. It is relevant to point out that first, the formulation of Example 4 is an aerosol (and not a liquid or gel), and then that lipase is immobilized on Aerosil® 90 a hydrophilic fumed silica manufactured by Evonik Industries AG, which is characterized by a non-porous structure (see for instance data-sheet of Aerosil® 90). In addition, the results of the performance testing of Example 4 are obscure and are not useful to the skilled in the art wishing to develop a liquid or gel formulation useful to remove biofilms from stone-like substrates.

[0017] In summary, the scope of WO2014067933A1 relates to the use of insoluble enzyme preparations, having reduced safety concerns compared to the free enzyme. Clearly, enzyme activity enhancement and cost optimization are out of the scope of this invention. In fact, it refers to an insoluble enzyme 'single-use-composition', i.e. a composition, which after its primary use is either discarded, used up or rendered in a form unsuitable for subsequent reuse. The teachings provided by C-Lecta GmbH in the international application WO2014067933A1 are not useful to remove biofilms from stone-like substrates, by means of an enzyme composition wherein the enzyme can be reused. Therefore, the compositions disclosed are definitely not a viable alternative to known biocides or enzyme formulations currently used in the restauration and maintenance of historic heritage or in fields having similar problems with regard to biofilm removal from stone-like substrates.

TECHNICAL PROBLEM

[0018] The high cost of enzymes represents one main drawback of enzymatic cleaning systems already available in the market and of prior art biofilm removal methods thereof. In fact, enzymes are expensive products per se and furthermore they cannot be recovered for reuse after surface treatment. In addition, the preparation of the enzyme system and kit incorporating such system requires a long time because enzyme conservation is difficult and therefore the enzyme system must be freshly prepared just before performing the cleaning operation.

[0019] High cost, long preparation time and conservation of enzymatic cleaning systems involves a number of practical drawbacks. First, the effective cleaning effectiveness per unit area of known enzyme systems is reduced. Second, the application on large walls or floors (e.g. found in many archaeological sites) is limited. Finally, the use of prior-art enzyme systems is not flexible and is not compatible with the best practices currently adopted by the agencies in charge of the maintenance and conservation of historical heritage (in Italy, the "Superintendency of Cultural Heritage").

[0020] It will be apparent to those skilled in the art how these drawbacks of the known enzymatic cleaning systems are particularly critical when public spending is restricted, especially in countries rich of cultural heritage in need of constant maintenance such as Italy. Such defects could be mitigated by increasing the cleaning effectiveness of the enzyme systems, or by using manufacturing methods of such cleaning kits that can take advantages from economies of scale and thus cut costs. However, according to the best knowledge of the present inventors, technical teachings useful for such purposes do not result in the scientific and patent literature.

[0021] In conclusion, it is not already available a bio-cleansing formulation including a stable enzyme system that is useful to remove biofilms from stone-like substrates, and that is simple to synthesize and conserve, and compared to prior art solutions, fulfil the requirements of demanding fields such as historic heritage or fine-arts. Such requirements cover improved cleaning efficiency and flexibility in use, low cost per unit surface treated, preservation of substrate integrity (i.e. no substrate damage nor alteration, no residues on substrate after treatment) and finally negligible risks for users and for the environment.

TECHNICAL SOLUTION

[0022] By means of this patent specification, the present inventors intend to overcome the limitations existing in the state of the art related to enzyme cleaning systems useful for removing biofilm from surfaces, particularly of natural stone, stone-like materials, ceramic and wood.

[0023] Accordingly, it is a first and main object of the present invention to provide a bio-cleansing kit including an enzymatic system as set forth in the appended independent claim. Said bio-cleansing kit is useful to remove pre-existing biofilms from various kinds of substrates, preferably stone-like substrates, more effectively and less expansively than known solutions. Particularly, an important task of the present invention is to provide a stable bio-cleansing kit having an improved enzyme cleansing activity and which is storable and reusable several times. Still, another important task of the present invention is to provide a bio-cleansing kit, which can be customized according to the needs of different fields, particularly of cultural heritage.

[0024] A second important object of the present invention is a method for making said bio-cleansing kit, as set forth in the appended independent claim, and particularly a scalable manufacturing method that is easily achievable with known technologies at competitive costs compared to the enzyme-based cleaning solutions already in the market.

[0025] Within the above first and second objects, a third object of the present invention is to provide a method for removing biofilms from substrates, preferably stone-like substrates, which allows cost-cutting and a simplification of restoration plans, particularly in the field of cultural heritage.

[0026] Finally, a further object of the present invention is to provide a bio-cleansing kit and to develop a method for removing biofilm from substrates, based on said kit, which do not damage or alter the substrate, and do not present chemical hazard for users as well for the environment.

[0027] In view of the above disadvantages or drawbacks of the prior art, the present inventors have made many studies related to enzyme systems consisting of enzymes immobilized on inorganic particles. These studies were mainly directed to address optimization of the enzyme system, enhancement of enzyme cleansing activity, and optimization of a formulation comprising such enzyme systems compatible with the working conditions of restorers or other professionals involved in restoration interventions.

[0028] After long terms of practice, the inventors have prepared an enzymatic bio-cleansing kit suitable to remove biofilms from substrates, preferably stone-like substrates, comprising: an enzyme system consisting of immobilized enzymes on inorganic particles, preferably silica nanoparticles, a formulation comprising said enzyme system and a support for said formulation. Preferably, said enzyme is trypsin, but it can be selected from those known in the art according to the characteristics of the biofilm to be removed from the substrate. In the preferred mode, said formulation impregnates the support consisting of a hydrophilic fabric but in another embodiment, the formulation is coated on a flexible or rigid support, such as a plastic film, so that the formulation forms a substantially distinct phase from such support. Still in another embodiment, the kit does not have a support (i.e. a 'support-less' kit configuration) and said formulation is dispersed in a medium being a component of said formulation. Said medium is in form of a liquid, or a gel, or a solid medium suitable to deliver and to put in contact the enzyme system with the substrate to be cleaned.

[0029] Furthermore, in other embodiments, additional components are included in the bio-cleansing kit such as means to control the cleaning process parameters (i.e. temperature, moisture content and pH), in order to optimize the enzyme action and thus substrate cleaning, according to the characteristics of the enzyme system and the external climatic conditions. Additional components include also means to facilitate adhesion to the sub-

strates, particularly on vertical substrates, and protective films to prevent contamination of the formulation by external agents, as well as to maintain the proper moisture content and temperature in the impregnated support to enable an effective cleaning action.

[0030] The present invention also claims a method for removing biofilms comprising microorganisms such as molds, cyanobacteria, microalgae, lichens, etc. without affecting the integrity of a delicate and valuable surface, in particular works of art. In the best mode said method starts with an analysis of the substrate and the environmental conditions which help to select the proper enzymatic system and bio-cleansing kit configuration. Said enzyme formulation and the bio-cleansing kit are then freshly prepared. The method proceeds with kit application on the biofilm-contaminated substrate. After the enzymatic cleansing process has concluded, the kit is removed from the substrate and stored for future use.

[0031] Alternatively, in another embodiment, the enzyme formulation is not freshly prepared, and a prestored formulation or kit is used. In this embodiment, the method includes activation of the enzymatic system. In one embodiment, the enzyme formulation is in form of a gel, which is activated before application on the work of art ('wet form'). In still another embodiment, the activation is performed after the application on the work of art ('dry form').

[0032] Advantageously, the enzymatic bio-cleansing kit and the related method for removing biofilms from substrates, according to the present invention are characterized in that the formulation comprising the enzyme system can be easily removed from the substrate by water washing so that no formulation residues are left on the substrate after the treatment.

Terms and definitions

[0033] For the purpose of understanding the specification and the appended claims, in the following description the chemical elements are defined by means of the respective symbols as reported in a common periodic table of elements. For example, hydrogen is represented by its symbol H; helium is represented by He and so on. Also, it is to be understood that the chemical symbol comprises all isotopes and ions unless stated otherwise. Again, for the sake of brevity, the chemical compounds may be indicated by acronyms widely adopted in the technical field related to the present invention. For example, the acronym EDTA designates ethylene diamine tetraacetic acid.

[0034] For the sake of clarity, the term "particle" as used in the description and in the claims of the present invention shall designate an aggregate of atoms, molecules or other fundamental constituents, such aggregate having sub-micrometric size or supermicrometric and a substantially spherical shape but also a non-symmetrical shape. Particularly, the terms "nanoparticle" or "nanostructure", singular or plural, shall indicate exclusively a particle of size less than about 1 micrometer.

[0035] By the term "biofilm", it is meant any association, simple or complex, of organic and/or inorganic substances with microorganisms such as molds, algae, bacteria, lichens and the like, wherein such association adhere to a substrate, for instance a rock substrate. Also within the present patent specification, the term "bio-cleansing" shall refer to the removal of a pre-existing (i.e. a biofilm covering the substrate before removal) biofilm from a substrate taking advantage of the bio-cleansing system according to the present invention. Unless otherwise stated, by the term "immobilized" it is meant any chemical bound (covalent bond, ionic, hydrogen bond or Van der Waals interaction), physical bound (e.g. electrostatic attraction) or physio-chemical bond that binds or associates permanently or temporarily an enzyme, or more in general a polypeptide, to at least one particle or a nanoparticle. In the description and figures the same reference sign will be used to indicate the "support" (or, more generally, the "means") and the "impregnated support" (or more generally the "impregnated means"), i.e. the support where the formulation containing the enzyme system has been applied thereto. The same reference sign will also be used to identify the corresponding dry or dehydrated form of the "impregnated support".

[0036] In addition, the term "preserving substrate integrity", and similar wordings (e.g. "to preserve substrate integrity"), as used in the description and in the claims, will be referred to a cleansing treatment of a substrate that does not damage nor affect the original (i.e. before biofilm contamination) aesthetic, colors and surface structure of the substrate, according to the best practices of works of art restorations.

[0037] Finally, the term "about" as used herein is intended to include values, particularly within 10% of the stated values. The use of "or" means "and/or" unless stated otherwise. It is to be understood that the detailed description is for the purpose of fully disclosing preferred embodiments of the invention without placing limitations thereon.

Advantageous Effects of the invention

[0038] The kit for enzymatic bio-cleansing according to the present invention and the related methods based on said kit have a number of remarkable advantages over prior-art solutions. These benefits and advantages are described in the following.

[0039] Thanks to the particle immobilization, the kit is stable and presents an improved enzyme cleansing capability, compared to known gel formulations having a greater enzyme content. The inventors experimentally demonstrated that removal of the same biofilm can be achieved with up to 20 times less enzyme, thus reducing enzyme cost and allergenic potential and immunogenicity.

[0040] Furthermore, the kit for bio-cleansing according to the present invention is storable and reusable up to 8

times (single-use is also possible at competitive costs), which represents an outstanding achievement in stone-like biofilm cleaning. In fact, a storable and multiple-use kit allow to both lowering the product cost (due to economies of scale in manufacturing) and the cost for unit of cleaned surface. Furthermore, this feature allows to improve flexibility in management and scheduling of extensive restoration and maintenance programs, particularly in the field of cultural heritage.

[0041] The kit according to the present invention is non-toxic for users and the environment because the silica nanoparticles are bound in a gel or in a liquid formulation, no aerosol containing free nanoparticles and/or enzyme is formed and little enzyme is used. To further increase user and environment safety, formulations based on micro-scale particles can also prepared.

[0042] More importantly, the bio-cleansing kit according to the present invention and the related bio-cleansing method do not damage the substrate (during use, salts formation is minimized on the surface which may affect the art-work) as traditional biocide-based cleansing composition, or known composition based on enzymes bound to polymeric particle. In fact, the formulation residues remained on the substrate after the treatment, can be easily removed from the substrates using water washing. Nevertheless, particles trapped in the pores of the substrate do not affect the surface integrity because, being metal oxide (e.g. silica), they are stable and chemically compatible with many rock compositions.

[0043] Finally, the bio-cleansing kit according to the present invention is simple to prepare in various configurations, as the examples herein provided demonstrate.

[0044] Additional objects and advantages of the invention will be set forth in part in the description which follows, and in part will be obvious from the description, or may be learned by practice of the invention.

[0045] In summary, the advantages of the bio-cleansing kit according to the present invention allow to remove biofilms from various kinds of substrates, particularly stone-like substrates, more effectively, economically and safely than known solutions. It will be apparent to those skilled in the art that the advantages of the present invention cannot be achieved by prior-art cleaning system based on enzymatic systems.

DESCRIPTION OF DRAWINGS

[0046] The present invention will be more fully understood by reference to the following drawings which are for illustrative purposes only:

Figure 1 schematically represents a section of the bio-cleansing kit according to the present invention. In (a), the formulation containing the enzyme system is applied to the surface of a support; in (b), this formulation is dispersed in or impregnates the support itself; while in (c), the kit is support-less and the enzyme system is dispersed in a medium comprised in the formulation itself;

Figure 2 illustrates applications of the bio-cleansing kit according to the invention on three different stone-like materials;

Figure 3 schematically represents a section of the bio-cleansing kit, according to the third embodiment of the present invention, characterized by fixing means integrated to said kit;

Figure 4 schematically represents a section of the bio-cleansing kit according to the present invention characterized by additional elements which, in (a), are a temperature controller and a humidifier system applied to the surface of the impregnated support, while in (b) a temperature controller integrated with the support itself;

Figure 5 shows the results of the bio-cleansing treatment on two stone works located in a historic site in Puglia (Italy).

[0047] These figures illustrate and demonstrate various features and embodiments of the present invention, and of the manufacturing method thereof, but they are not to be construed as limiting the invention.

DESCRIPTION OF THE BIO-CLEANSING KIT IN THE BEST MODE

[0048] With reference to the enclosed Figure 1b), the enzymatic bio-cleansing kit according to the present invention is indicated with the number (1) and comprises a formulation (10) containing an enzymatic system (20); a means (30) above which said formulation (10) is applied, or in which said formulation is dispersed; a protective film (40) and temperature control means (50).

[0049] Said enzyme system (20) includes trypsin immobilized to mesoporous silica nanoparticles by means of a process that will be described later. Advantageously, the inventors have found that an effective bio-cleansing action can be achieved by immobilizing a quantity of trypsin between about 3 and about 9 mg/g onto mesoporous silica nanoparticles having diameter between about 100 and about 300 nm and a pore diameter between about 2.1 and about 2.3 nm. However, different values are possible depending on the morphology and chemical composition of the nanoparticles, on the enzyme and the biofilm compositions.

[0050] According to a method that will be explained in detail below, the bio-cleansing treatment by means of the kit (1), requires that the support (30) is placed over the substrate (L) and requires that the formulation (10) comes effectively in contact with this substrate.

[0051] To achieve these purposes, in the best mode of the present invention, said formulation (10) is in the form of an enzyme gel that is applied on a rigid or flexible support (30) so that the gel impregnates effectively this support. In particular, it has proved very useful a hydrophilic non-woven sterile gauze made of a viscose/polyester (67%/33%) fabric (e.g. produced by Dermatess

Mater Aid). However other kind of fabrics can be used, but also hydrophilic or superabsorbent polymers, provided they have suitable wetting properties to ensure a good impregnation of the substrate with the gel (10) containing the enzyme system (20). Actually, the inventors have experimentally found that maintaining the right moisture content of the impregnated support (30) constitutes a fundamental requirement to ensure an effective bio-cleansing action.

[0052] In the best mode, said protective film (40) may be an extensible film such as Parafilm® or Domopak® which is placed over the surface (S') of the bio-cleansing kit (1) opposite to the substrate (L) to be cleaned, as illustrated by way of example but not limitation in the Figure 1. The protective film (40) may be shaped to protect also the active surface (S) of the kit that will be placed in direct contact of the substrate (L) during the bio-cleansing treatment. This configuration is particularly useful when the bio-cleansing kit is not immediately used and must be therefore stored for future use. Alternatively, the protection of the kit active surface (S) can be simply achieved by including an additional protective film (40').

[0053] Finally, in the best mode said bio-cleansing (1) kits comprises temperature control means (50), external to said kit and to the impregnated support (30). Said temperature control means (50) can be a temperature-controlled hair-dryer, an aquarium heater or other equivalent devices, but other advantageous solutions useful to control and maintain the temperature within a predefined range during the bio-cleansing treatment have been identified.

[0054] The bio-cleansing kit described herein in the best mode is suitable to numerous variations, all falling within the more general scope of the present invention. For example, it is possible to act on the composition of the enzyme system, or on the formulation or on the support. Furthermore, the bio-cleansing kit can be optimized for removing biofilms from vertical walls, or from uneven or non-uniform surfaces or from large floorings. Some components of the kit, as the temperature control means, may advantageously be integrated in the support of the formulation to further increase the application flexibility of the bio-cleansing kit according to the present invention. Such alternative solutions will be later described with reference to other embodiments.

[0055] The kit configuration according to the best mode ensure optimal hydration of the formulation without altering the pH value. It also maintains a constant contact between the active surface (S) and the substrate by increasing the cleaning activity of the enzyme.

DESCRIPTION OF THE KIT PRODUCING METHOD IN THE BEST MODE

[0056] With reference to the preferred embodiment of the present invention, the process for preparing the enzyme system and the bio-cleansing kit is described in the following by way of example and not of limitation.

Step 1: Preparation and functionalization of the particles

[0057] Suitable mesoporous silica nanoparticles can be easily synthesized by known procedures; alternatively, various types are available commercially, for example from Sigma-Aldrich SBA-15 (777242), or MCM-41 (643 645). The silica nanoparticles may be functionalized using various known techniques, for example making them react at room temperature for about 2 hours in a solution of cyclohexane with 3-aminopropyltriethoxysilane (APTES) and n-propylamine, both in the proportion of between about 1.5 and about 3.0% v/v, so as to obtain a first precipitate.

[0058] By using techniques well-known to the skilled in the art, said first precipitate is filtered and washed with cyclohexane, and finally dried in an oven at about 40°C for a sufficient time to remove solvent residues in order to obtain functionalized silica particles.

Step 2: Enzyme Immobilization

[0059] The functionalized silica particles are then dispersed in a buffer solution containing a predefined amount of trypsin. Preferably, a good enzyme immobilization is achieved with an amount of trypsin comprised between about 3 and about 9 mg per gram of nanoparticles and a phosphate buffer having a pH of about 6 in a concentration of between about 10 and about 20 microMol.

[0060] This dispersion is continuously stirred for about 2 hours at room temperature to obtain a second precipitate. After filtering and drying said second precipitate, for example in air within a protected environment, it is obtained the enzyme system in the form of a white powder composed of silica nanoparticles where trypsin molecules are immobilized thereto.

[0061] Following the procedure described above, stable bonds between the immobilized enzymes and the particles are created, as the skilled in the art can verify by evaluating the UV absorption spectrum and the DRIFT-IR spectrum of the enzyme system. The enzyme system thus synthetized is stable and it is only minimally affected by autolysis. In fact, due to the immobilization on the nanoparticles, the enzyme molecules have a scarce mobility which limits or prevents mutual interactions even in an aqueous solution or in a humid environment.

[0062] The synthesis process is herein described as a non limitative example and it is susceptible of numerous variations, all included within the general scope of the present invention. In fact, depending on the requirements, it is possible to prepare compositions that remove specific biofilms from specific substrates by a proper selection of the particles and of the enzyme. In particular, customization of the enzyme system can be achieved by acting on one or more of the following parameters: particles composition, size or surface morphology; enzyme type or amount in the formulation. For example, silica

particles with dimensions greater than about 1000 nm can be usefully used, as well as particles of other metal oxides such as zirconia (ZrO_2), titania (TiO_2), or mixed oxides such as zirconia/titania. Furthermore, spherical nanoparticles characterized by a uniform distribution of immobilized enzyme, are particularly useful for removing biofilms from macroporous or non-homogeneous substrates. In other working conditions, it is preferable using particles having an oblong shape to improve the contact surface of the immobilized enzyme system with the biofilm on the substrate.

[0063] In addition to trypsin, other hydrolytic and non-hydrolytic enzymes can be used, e.g. pepsin, lipase, papain, amylase, chymotrypsin, elastase, xylanase, cellulase, ligninase, ficine, bromelain. In addition, the immobilization process may not require nanoparticle functionalization, but only the creation of a non-covalent bound between the enzyme and the substrate. Still, the process parameters (e.g. the pH of the precursor solution, the temperature, concentration, dripping speed of the precursor) can be altered to obtain changes to the morphology, shape or average size of the particles.

Step 3: Bio-cleansing kit preparation and conservation

[0064] The process continues with the preparation of the formulation (10), after having synthesized the enzyme system according to steps 1 and 2 herein described, or after having obtained a commercially available suitable enzyme system.

[0065] Initially, the enzyme system (20) is dispersed in a buffer solution, preferably having a pH between about 7.2 and about 8.0. This solution may be, for example, a phosphate buffer $NaH_2\ PO_4 \bullet H_2O$, but other equivalent solutions may be used. A gelling agent is then added so as to obtain the formulation (10) in the form of a homogeneous gel of proper consistency that can be coated on the support (30). Preferably, said gelling agent is methylcellulose (e.g.Tylose® MH300P), added in a ratio of about 1:10 w/w, or another compound equivalent conveniently chosen to obtain a suitable viscosity and to maintain the most suitable pH value for an effective trypsin (or other enzyme) action. Even if a formulation in the form of a gel is preferred, other formulations containing the enzyme system are however possible.

[0066] Then, a uniform layer of gel formulation (10) is formed on the support (30) by dip-coating a pre-cut fabric into a vessel containing the formulation (10) for a time long enough to ensure a good impregnation (e.g. 5 minutes or more). In addition to dip-coating, other known coating techniques (e.g. roll-coating or spraying) may be used depending on the nature of the support (rigid or flexible) and of the formulation (gel, liquid, aerosols) containing the enzyme system. Moreover, to meet specific application needs, these known techniques allow the formulation (10) deposition on specific areas of the support (30) according to a predefined pattern.

[0067] Application on the impregnated fabric (30) surface (S') of the protective film (40), preferably a stretchable film (e.g. Parafilm®, Domopak® or other extensible films), concludes the preparation of the bio-cleansing kit according to the best mode of the present invention. When the kit is not used immediately and protection of the formulation from external agents is required, the protective film (40) can be shaped to cover also the active surface (S). Similarly, a second protective film (40') can be applied on the active surface (S') in direct contact with the biofilm-contaminated substrate (L). In practice, using well-known techniques (e.g. in the packaging industry), one or more protective films can be shaped and applied to protect the surfaces S, and/or S' of the kit according to the various conditions of use and conservation of the kit itself. The bio-cleansing kits thus prepared is ready to be applied to the substrate (L) according to a procedure that will be described below.

[0068] Advantageously, the bio-cleansing kit can be stored and used several days or months after its preparation. In this case, drying of the impregnated support (30) must be included in the kit preparation procedure. This additional step can be performed by placing the kit in a protected environment such as a vented thermostat dryer. In fact, the inventors have surprisingly found that after drying the support (30) impregnated with the gel formulation (10) can be stored even for months without losing its bio-cleansing action properties.

[0069] Preferably, the impregnated support (30) is kept in a plastic container, or wrapped in the protective films (40,40'), and stored in a standard refrigerator at temperature of about 4°C. In this way, the enzymatic system (20) is trapped in the dried support and is not dispersed. Furthermore, in the absence of the right moisture content, the enzyme autolysis is prevented or minimized. Restoration of the cleaning properties before use requires bringing the kit to room temperature and dripping a few drops of buffer solution on the support to re-activate the formulation. The inventors have found that the formulation (10) in the form of a gel should not be dried and preserve well for a long time without losing its cleaning properties.

[0070] Numerous variations of the process for producing the kit herein disclosed are possible, depending on the configuration of the enzymatic bio-cleansing kit. Such variations, all falling within the scope of the present invention, will be subsequently described with reference to other embodiments of the bio-cleansing kit.

[0071] It will be apparent to those skilled in the art, that the process described herein, advantageously allows to separate the preparation of the bio-cleansing kit from its use, thanks to the remarkable feature of maintaining over time the cleaning properties of the enzyme system (20). This feature represents a very significant improvement that allows to overcome the limitations of the solutions known to the state of the art with important practical implications, particularly costs reduction and improved flexibility in scheduling extensive restoration interventions. Other advantages will be obvious to those skilled in the

art.

DESCRIPTION OF THE BIOFILM REMOVING METHOD

[0072] The bio-cleansing kit (1) thus prepared is useful for removing pre-existing biofilms from substrates, preferably stone-like substrates, by means of a method comprising the following steps. Said method is described hereinafter with reference to the best mode of the present invention.

Step 1: Analysis of the substrate and the environmental conditions

[0073] The method for removing biofilm starts with a preliminary step which includes an analysis of the characteristics of the surface to be treated (material, substrate porosity, biofilm type and severity of attack, etc.) as well as the local environmental conditions (temperature, humidity, presence of wind and pollutants, etc.) which may affect the bio-cleansing treatment. Care must be taken, in case the substrate is a valuable work of art such as an ancient mosaic. This preliminary step allows the selection of the most suitable bio-cleansing kit (particularly the enzyme system) according to the working conditions and other requirements.

Step 2: Bio-cleansing kit pre-application preparation

[0074] Once the most suitable enzymatic system is selected in step 1, the method for removing biofilms proceeds with the preparation of the bio-cleansing kit. The support (30), which in the best mode is a hydrophilic fabric (e.g. Dermatess Mater Aid), is first cut to size according to the shape and surface extension of the stone-work contaminated by the biofilm.

[0075] If the formulation has been freshly prepared, then the enzyme system is already active and the bio-cleansing kit is ready for application on the surface (L) of the stone-work to be cleaned. In case the bio-cleansing kit was previously made and stored (see the kit producing method in the best mode described hereinabove), then the bio-cleansing method requires the enzymatic system (20) activation, i.e. the restoration of the proper formulation moisture content, temperature and pH. According to the present invention, the activation of the enzymatic formulation (10) can occur both before and after the application of the bio-cleansing kits on the surface (L) of the stone-work.

[0076] In the first case, a buffer solution (preferably a phosphate buffer) is sprayed to properly wet the impregnated hydrophilic fabric support (30). The bio-cleansing kit is thus ready for the next application step. Otherwise, the enzyme formulation (10) is activated after the application of the bio-cleansing kits on the surface (L) of the object to be treated. In this second case, the buffer solution is sprayed directly on the impregnated support (30). In both cases, it is important to wet properly the impregnated support (30) taking into account, however, the substrate porosity according to the analysis of the previous step 1. In fact, salts formation on the pore should be avoided or limited to a minimum.

Step 3: Application of the bio-cleansing kit on the surface

[0077] The restorer, or other professionals, applies the bio-cleansing kit on the substrate (L), taking care that the entire surface (L), flat or curved, is in contact with the formulation (10) containing the enzyme system (20), which in the best mode is in the form of a gel. As previously mentioned, enzyme system activation can also be made in the present step 3. With reference to the best mode of the invention, herein described by way of example and not of limitation, the inventors have surprisingly found that an optimal bio-cleansing kit activation can achieved by, first, applying a phosphate buffer in form of a gel (and not in liquid form) directly on the substrate (L), and then applying the impregnated fabric (30) with the enzyme gel (10) over the phosphate buffer layer. Advantageously, this arrangement limits salts formation which may deteriorate the stone surface and must be avoided (especially in treatment of historic art-works). Furthermore, it prevents migration of particles-immobilized enzyme from the support, and finally, it limits the use of buffer solution to humidify the kit, further simplifying the bio-cleansing process and reducing costs.

[0078] To maintain the proper degree of moisture and prevent contamination of the formulation, it is useful applying a protective film, for example Domopak® (or other extensible films such as Parafilm®) in the case this component is not already part of the of bio-cleansing kit.

[0079] At this stage, the method according to the present invention requires waiting the necessary time to allow a selective and satisfactory cleaning of the substrate by the enzyme included in the kit. Depending on substrate type and morphology, as well as the biofilm composition, cleaning times between about 30 minutes to about 6 hours are sufficient for effective bio-cleansing of stone-works, provided that the suitable temperature, pH and moisture content are maintained. In order to maintain optimal process condition during treatment, it is possible to adjust the temperature control means (50), according to the external environmental conditions. Moreover, during substrate treatment, it may be needed to restore the moisture content or the pH formulation by spraying or delivering a suitable amount of buffer solution.

[0080] In the best mode of the present invention, as already described, the temperature control unit (50) is an aquarium heater which is a component external to the support (20). Nevertheless, the inventors have identified other advantageous solutions, which will be described with reference to other embodiments.

Step 4: Removal of the kit from the substrate

[0081] When a satisfactory bio-cleansing is achieved, the kit is removed from the substrate. The excess formulation (10) is removed by rinsing the cleaned surface with demineralized water or by dabbing the surface with a sponge or tissue soaked with demineralized water. Noticeably, the biofilm removing method according to the present invention did not alter the original colors nor the substrate of the stone works, as will described in the following examples.

Step 5: Kit conservation and storage

[0082] Since the enzyme system is advantageously reusable several times, the bio-cleansing method according to the present invention concludes with the storage of the bio-cleansing kit, in a home or laboratory refrigerator at a temperature of about 4 °C. This step includes de-hydration of the impregnated support (30) (as described previously) to de-activate the enzyme system, and finally the protection of the kit (1) by means of a protective film (40) or other technically equivalent means.

[0083] Many variations to the removal biofilm process herein disclosed are possible, all falling within the more general scope of this invention. Such variations depend on the specific bio-cleansing kit configuration, the intrinsic characteristics and the external environmental conditions of the substrate. Other factors will be obvious to the expert in the art by the patent disclosure provided or by invention practice. For example, the steps described above can be carried out in whole or only in part. Depending on the needs, the individual steps can be altered or simplified, as well as the step sequence can be modified.

[0084] It will be apparent to those in the art, as the bio-cleansing procedure based on the kit according to the present invention, represents a remarkable achievement over solutions already known to the state of the art, in particular with reference to the bio-cleansing kit reusability.

Example 1: Bio-cleansing treatment of stone-like substrate

[0085] The alleged Figure 2 shows stone substrates that were used for testing the bio-cleansing kit and the biofilm removal process herein disclosed. In particular, the inventors have experimentally verified the efficacy of the kit and the cleaning process on two works of art from the Baths of Caracalla in Rome: a marble capital and a black and white mosaic floor tiles. With reference to Figure 2(b), both works were contaminated by a biofilm consisting of a heterogeneous microbial/fungal consortium comprising, inter alia, invertebrates (Macrobiotus Tardigrade), protozoa (Aspidisia) microalgae, fungi (Zygomycetes and Ascomycetes) and by a multitude of bacteria.

[0086] The kit was prepared according to the teachings of the best mode of the present invention. Supports made of hydrophilic gauze (from Dermatess) 5x5 cm^2 in size were dip-coated with a gel formulation of methylcellulose (Tylose MH300P) comprising trypsin immobilized on mesoporous silica nanoparticles (synthetized by the inventors via standard techniques) in a percentage comprised between about 5 and about 10%. During the treatment of the two stone pieces, the temperature was maintained within a range between about 30°C and about 40°C using an aquarium heater as temperature control means. The proper moisture content of the formulation and a pH between about 7.2 and about 8.0 was maintained by gently wetting the hydrophilic fabric with buffer solution. To keep steady process conditions and avoid contamination of the formulation, the kit was protected with a Parafilm® film.

[0087] Under these process conditions, a single bio-cleansing treatment ranging from about 30 minutes to 90 minutes was able to completely remove the biofilm (greenish-colored in the alleged Figure 2) in the areas of the stone works where the fabric impregnated with the enzyme gel formulation was applied. In particular, it was proved an effective and selective action of the bio-cleansing kit against arginine and lysine (an amino acid produced by the same species constituting the biofilm). Moreover, the application of the kit and the method according to the present invention did not result in damage to the works of art. The residues of the gel formulation left on the substrates after that the impregnated fabric was taken off, were completely removed with ease by using demineralized water.

[0088] After air drying, the impregnated fabric with the bio-cleansing dehydrated gel was stored for 5 months in a home refrigerator. Once reactivated with a phosphate buffer solution, it was possible to reuse the same kit up to 8 times thus demonstrating that bio-cleansing efficacy is maintained over time according to the teachings of the present invention. Surprisingly, the inventors found that the combination of trypsin and silica mesoporous nanoparticles enhanced the biofilm removal capability and reusability compared to prior-art cleansing compositions (having the same enzyme content), particularly formulations based on enzyme immobilized on fumed silica or formulations based on free enzyme. According to the best knowledge of the present inventors, this achievement is not obvious and although further research and experiments are required to understand better this phenomenon, it may depend on the peculiar enzyme distribution or charge distribution around the nanoparticle induced by the mesoporous structure.

Example 2: Bio-cleansing treatment of other substrates

[0089] Bio-cleansing kits substantially similar to that of Example 1 were applied on different substrates: rock (basalt, marble, colored marble), terracotta, plastered and painted walls (with tempera paint or washable paint). The

surfaces of the samples suffered a biofilm attack consisting principally of mold.

[0090] Following the procedure described above, the kits were applied on the substrates both in wet form (i.e. gel enzyme activation before application) and in dry form (i.e. gel enzyme activation after application). The combination of the hydrophilic fabric support and the gel formulation showed an optimal adaptation to the uneven surface profile of the different sample materials. The bio-cleansing treatment conducted for about 90 minutes was effective and easy to perform. The same results were achieved on a wooden surface (natural, polished oak) and a plastic surface (transparent methacrylate) both suffering a mold attack.

[0091] In this way, it has been shown that the bio-cleansing kit herein disclosed can effectively remove biofilms from substrates different than stone-like materials, or from substrates characterized by a non-uniform surface morphology, thus achieving a further object of the present invention.

Example 3: Treatment of substrates having complex shape

[0092] Some configurations of the bio-cleansing kit facilitate removal of biofilm from the surface of stone-work having complex shape. For example, by impregnating a suitable elastic pre-shaped hydrophilic fabric (30) with the enzyme formulation (10), it is advantageously possible to remove biofilm from curved or complex surfaces such as columns or statues heads. For this purpose, the inventors have found useful the impregnation of standard hydrophilic pre-shaped elastic gauzes and tubular nets used in wound treatment (e.g. produced by 3M®).

[0093] Also, known die-cutting techniques are useful when there is the need to perform the bio-cleansing treatment only on specific areas of the surface the fabric or other support (30).

[0094] Alternatively, the formulation (10) can be deposited on specific areas of the support, in this case using standard printing techniques such as ink-jet, or screen printing frames, depending on the viscosity of the formulation.

Mode for Invention

[0095] Hereinafter other embodiments of the invention according to the present invention are provided by way of example and not of limitation. Changes to the kit manufacturing method according to the best mode are provided where such changes do not appear obvious.

[0096] In the second embodiment according to the present invention, here illustrated by way of example and not of limitation, the bio-cleansing kit is "supportless" i.e. it present a configuration which does not comprise any support (30). Unlike the best mode, in this case the formulation (10) is directly applied on the biofilm-contaminated substrate (L) and includes a dispersion of the enzymatic system (20) in a suitable medium (30') which is part of the formulation (10) itself. Said means (30') can be a gel (e.g. a suitable gel buffer solution); in this case, formulation application is performed with a spatula or brush, a technique, which is also suitable for application on vertical substrates. The formulation and the detached biofilm can be removed by simply washing the substrate (L) at the end of the bio-cleansing treatment.

Example 4: Treatment of stone substrate with supportless bio-cleansing kit

[0097] The alleged Figure 5 shows the results of the bio-cleansing treatment on two stone works located in Palazzo Marchesale in the town of Arnesano, Lecce (Italy). This historic site was selected for testing the bio-cleansing kit in the "supportless" configuration according to the second embodiment of the present invention. In fact, the stone works were heavily contaminated by a variegated association of lichens and other microorganism which other cleansing technique failed to remove. The same formulation of Example 1 was freshly prepared, applied to the substrates and maintained in contact of the substrates for about 30, 40 minutes. The residues of the gel formulation on the substrates were completely removed with ease by using demineralized water. As the Figure 5 clearly demonstrate, the bio-cleansing kit and the related method removed completely lichens, thus revealing the original substrate without affecting its integrity and without leaving residues of the formulation after the treatment.

[0098] In a third embodiment, here described by way of example and not of limitation, the enzymatic bio-cleansing kit according to the best mode of the present invention comprehends fixing means (70) to promote adhesion between the substrate and the active surface (S) of the kit and thus enzyme activity especially when removing biofilms from vertical surfaces such as a wall. For this purpose, the inventors have identified several useful solutions.

[0099] In a first solution, said fixing means (70) is a component integrated to the bio-cleansing kit, as schematically depicted in the attached Figure 3, and it is preferably a reversible adhesive system applied along the peripheral edges of the support (30). Depending on the features of the wall to be treated several known adhesive systems can be advantageously used: double-sided adhesive tape for moist and delicate surfaces (e.g. 3M™ Active™ Strips or other tapes for medical tape), velcro-based fastening products, micro-suction systems, but also water glue or other physical or chemical reversible adhesives. Alternatively, said fixing means (70) is an external non-integrated component of the bio-cleansing kit, which can retain the bio-cleansing kit in contact with the wall or floor to be cleaned, such as a frame fixed to a scaffold or a temporary platform, or other technically equivalent known solution.

[0100] Bio-cleansing kits according to the third embod-

iment advantageously allows an effective biofilm removal from vertical walls (L), thus achieving a further object of the present invention. In this case, the inventors have found useful impregnating a hydrophilic fabric (30) with the same gel formulation (10) of the best mode. In fact, the gel presents a good tackiness which limits fall by gravity and the fabric structure has proved a good wettability, because dripping by gravity of the buffer solution is effectively balanced by the capillary rise of the same solution. Nevertheless, if needed, the correct impregnation of the upper portions of the impregnated fabric (30) can be adjusted by providing further buffer solution.

[0101] The temperature control unit (50) may be an external component, such as an aquarium heater or a standard IR lamp, or it can be integrated to the bio-cleansing kit as in the second embodiment.

[0102] In a fourth embodiment, depicted schematically in Figure 4, by way of example and not of limitation, the bio-cleansing kit includes two additional elements consisting of temperature control means (50) integrated to the bio-cleansing of enzymatic kits (which is not an external component as in the best mode), and a humidifier (80). According to this embodiment, two further configurations are possible. In the first configuration, the temperature control unit (e.g. printed resistive heating elements manufactured by GSI) is placed directly on a surface (internal/external) of the support (30) impregnated with the formulation, while in the second configuration the temperature control unit (e.g. heating microwires such as Gerbing MICROWIRE® Heat Technology) is inserted inside the impregnated support (30). A humidifier for home use or a precision humidifier for lab are both suitable. The process conditions are managed by a control system (90). The formulation and the support can be the same by the preferred embodiment.

[0103] In the fifth embodiment according to the present invention, herein described by way of example and not of limitation, the formulation containing the enzymatic system (20) comprises a complexing agent capable of forming a complex with the substances of the substrate (or biofilm) amplifying the enzymatic activity of trypsin. For this purpose, the inventors have found useful EDTA (ethylenediaminetetraacetic acid), a chelating agent inhibiting the action of divalent ions. However, other known chelating agents can be advantageously used.

[0104] In conclusion, the expert in the art will appreciate how the alternative embodiments described above represent a significant improvement compared to the solutions known to the state of the art.

Example 5: Bio-cleansing of vertical surfaces.

[0105] To remove a biofilm consisting of cyanobacteria and mold, which covered a standard household wall (plastered and painted with washable paint), the inventors applied a gelled phosphate buffer solution (pH 7.2-8) directly on the wall and over it a hydrophilic fabric impregnated with a formulation (10) having the same composition of best mode. The hydrophilic fabric was maintained in position on the wall by fixing the edges with a scotch paper. During treatment, the temperature on the wall was constantly maintained at about 37°C (using a standard IR lamp), while the fabric was periodically sprayed with buffer solution for maintaining the proper hydration and a pH value between about 7.2 and about 8. By acting on the viscosity of the gelled phosphate buffer and on the amount of sprayed buffer solution, there were no significant dripping of buffer solution and formulation along the wall.

[0106] This example demonstrates the applicability of the bio-cleansing kit according to the present invention on vertical surfaces, thus achieving a further important object of the present invention.

[0107] In conclusion, it is apparent to those skilled in the art that the present invention fully achieved the intended aim and objects by means of the new enzymatic bio-cleansing kit and the related method for removing pre-existing biofilms from substrates disclosed herein. The invention thus conceived is susceptible of numerous modifications and variations, without departing from the basic concepts as disclosed herein. Moreover, all the details may be replaced with other technically equivalent elements. Furthermore, the order of the process steps described above is shown by way of example, but not limitation and can be changed according to convenience.

[0108] The above description and drawings are only illustrative of preferred embodiments which achieve the objects, features and advantages of the present invention, and it is not intended that the present invention be limited thereto. All structural, chemical, and functional equivalents to the elements of the above-described preferred embodiment that are known to those of ordinary skill in the art are expressly incorporated herein by reference and are intended to be encompassed by the present claims. It will be appreciated that the scope of the present invention fully encompasses other embodiments which may become obvious to those skilled in the art.

[0109] Although the description and examples above contains many details, these should not be construed as limiting the scope of the invention but as merely providing illustrations of some of the presently preferred embodiments of this invention. Therefore, any modification of the present invention which comes within the spirit and scope of the following claims is considered part of the present invention.

[0110] In the appended claims, reference to an element in the singular is not intended to mean 'one and only one' unless explicitly so stated, but rather 'one or more.' Where the characteristics and techniques mentioned in any claim are followed by reference signs, those reference signs have been included for the sole purpose of increasing the intelligibility of the claims and accordingly, such reference signs do not have any limiting effect on the interpretation of each element identified by way of example, but not limitation by such reference signs.

Industrial Applicability

[0111] The kit for enzymatic bio-cleansing and the related cleaning method according to the present invention find application in many professional and consumer fields where biofilm removal from a substrate is required, or desired, for aesthetic, structural, sanitary or conservative purposes. Particularly, the advantages exhibited by the present invention make it most suitable in the field of cultural heritage, as an alternative to traditional biocides enzymatic gel formulations, particularly in the restoration of delicate and valuable works of art e.g. archeologic sites, mosaics, ceramics and ancient frescoes. In fact, the bio-cleansing kit has an excellent ability, not found in the known solutions, to remove biofilm from stone-like surfaces without altering colors or damaging the substrate. However, the present invention has demonstrated to be useful and effective in the removal of treatment of molds from domestic walls or floors. Finally, applications in other fields are also possible, e.g. industrial plant cleaning in the food industry.

Claims

1. A bio-cleansing enzymatic kit suitable for removal of pre-existing biofilm made by biological entities and by chemical entities from a substrate (L), comprising:

- a formulation (10) comprising at least one enzyme system (20),
- wherein said enzyme system (20) comprises a plurality of immobilized enzymes, linked or associated in a stable or temporary manner, to one or more inorganic particles;
- said kit further comprising a support for said formulation comprising means (30) wo which said formulation (10) is applied, or means (30) impregnated with said formulation (10), or
- said kit comprising a medium (30') wherein said enzyme system (20) is dispersed, said medium being included in said formulation (10);
- said means (30, 30') being selected from the group consisting of: a gel, a hydrophilic fabric, a flexible support, a rigid support, a plastic film, or a combination thereof,
- said formulation (10) can be removed with ease from said substrate (L) to preserve substrate integrity.

2. The bio-cleansing enzymatic kit according to 1, wherein said enzyme system (20) comprises trypsin immobilized on mesoporous silica particles.

3. The bio-cleansing enzymatic kit according to 2, wherein said trypsin is immobilized on said particles in a quantity between 3 and 9 mg/g and wherein said mesoporous silica particles have a diameter between 100 and 300 nm with a pore diameter between 2.1 and 2.3 nm.

4. The bio-cleansing enzymatic kit according to any one of the preceding claims, wherein:

- said biological and chemical entities are selected from the group consisting of: molds, algae, bacteria, lichens, waxes, oils, protein compositions, lipid compositions, polysaccharides, keratinous compositions, or a combination thereof; and
- said substrate (L) is made of a material selected from the group consisting of: stone, wood, paper, ceramics, metal, masonry, plaster, cellulose substrate, polymer, plastics, tissue, human skin, animal skin, or a combination thereof.

5. The bio-cleansing enzymatic kit according to claim 1, wherein:

- said enzymes are selected from the group consisting of trypsin, pepsin, lipase, papain, amylase, chymotrypsin, elastase, xylanase, cellulase, ligninase, ficin, bromelain, or a combination thereof; and
- said inorganic particles are selected from the group consisting of: silica particles, silica nanoparticles, zirconia particles, zirconia nanoparticles, metal oxides particles, magnetic particles, mesoporous particles, a nanostructured material, or a combination thereof.

6. The bio-cleansing enzymatic kit according to one or more of the preceding claims, wherein said formulation (10) is in the form of an enzyme gel that is applied on a rigid or flexible support (30) so that the gel impregnates effectively said support (30), wherein said support (30) is a hydrophilic non-woven sterile gauze made of a viscose/polyester (67%/33%) fabric, or hydrophilic or superabsorbent polymers, provided they have suitable wetting properties to ensure a good impregnation of said substrate with said formulation (10) in the form of an enzyme gel containing the enzyme system (20).

7. The bio-cleansing enzymatic kit according to claim 1, wherein:

- said inorganic particles have a substantially spherical shape with a diameter in the range between about 100 to about 300 nm and are mesoporous with a pore diameter in the range between about 2.0 to about 2.5 nm;
- said enzymatic system (20) contains trypsin, in an amount between about 2.5 to about 20 mg/g;
- said medium is a methylcellulose gel or a hy-

drophilic fabric.

8. The bio-cleansing enzymatic kit according to claim 1, comprising a buffer solution selected from the group consisting of: phosphate buffer solution, sodium citrate buffer solution, or a combination thereof.

9. The bio-cleansing enzymatic kit according to one or more of the preceding claims, wherein said enzyme system (20) comprises a chelating agent useful for amplifying the activity of said enzyme, said chelating agent being selected from the group consisting of: EDTA (ethylene diamine tetra-acetic acid), NTA (nitrilotriacetic acid), MGDA (methyl glycine di-acetate), phosphates and polyphosphates, phosphonates (DTPMP, ATMP), IDS (imino di-succinate), or a combination thereof.

10. The bio-cleansing enzymatic kit according to one or more of the preceding claims, **characterized by** the fact of being reusable for two or more times and/or being storable in dried form.

11. The bio-cleansing enzymatic kit according to one or more of the preceding claims, wherein it further comprises one or more of the following additional means:

- temperature control means (50), suitable to maintain the temperature in said kit within a given temperature range;
- a protective film (40, 40'), suitable to prevent contamination of said kit and/or to limit the loss of fluid inside said formulation (10);
- fixing means (70) suitable to maintain said kit in contact with the substrate (L) to be cleansed;
- a control system (90) of the process parameters, preferably temperature, pH, humidity content;
- a humidifier system (80), suitable to add or remove a fluid to said kit so as to maintain a suitable degree of hydration, said fluid being preferably a buffer solution contained in a tank.

12. The bio-cleansing enzymatic kit according to the preceding claim, wherein said protective film (40, 40') is an extensible film which is placed over a surface (S') of the bio-cleansing kit (1) opposite to the substrate (L) to be cleaned or to protect also an active surface (S) of said kit (1) that will be placed in direct contact of said substrate (L) during the bio-cleansing treatment.

13. The bio-cleansing enzymatic kit according to claim 11, wherein said temperature control means (50) is an active or passive element selected from the group consisting of: IR lamps, hot air system, heating fabrics or films, a heating or cooling system based on Peltier cells, a heating or cooling system based on a heat pump, an evaporative cooling system, a cooling system with phase change, a temperature-controlled hair-dryer, an aquarium heater, or a combination thereof.

14. The bio-cleansing enzymatic kit according to claim 11 or 13, wherein one or more of said additional means are integrated in said medium (30) or in said protective film (40).

15. A method for the preparation of the bio-cleansing enzymatic kit according to one or more of the preceding claims, **characterized in that** it comprises the following steps:

- obtaining such inorganic particles,;
- functionalizing said particles, according to known techniques, so as to obtain functionalized inorganic particles;
- dispersing said functionalized inorganic particles in a solution containing said enzyme, and stirring the solution thus obtained until the end of the reaction so as to obtain a precipitate;
- filtering and drying said precipitate so as to obtain said enzymatic system (20) in the form of a powder;
- dispersing said enzyme system (20) in a buffer solution having a suitable pH selected according to said enzyme;
- preparing a formulation (10) comprising said enzymatic system (20), wherein said formulation is in the form of a gel, said gel being obtained by adding to said solution a gelling agent;
- applying said formulation (10) onto said means (30) or impregnating said means (30) with said formulation (10);
- including in said formulation (10) a medium (30') for dispersing said enzyme system (20) therein.

16. Method according to claim 15, wherein said inorganic particles are mesoporous silica nanoparticles and wherein said functionalizing step comprises:

- making said silica nanoparticles to react at room temperature for about 2 hours in a solution of cyclohexane with 3-aminopropyltriethoxysilane (APTES) and n-propylamine, both in the proportion of between about 1.5 and about 3.0% v/v, so as to obtain a first precipitate,
- filtering said first precipitate and washing it with cyclohexane, and
- drying it in an oven at about 40°C for a sufficient time to remove solvent residues in order to obtain said functionalized silica particles.

17. Method according to claim 15 and 16, wherein said dispersing step comprises dispersing said function-

alized silica particles in a buffer solution containing a predefined amount of trypsin and wherein said stirring step comprises stirring said dispersion continuously for about 2 hours at room temperature to obtain a second precipitate, wherein said obtained enzyme system (20) is in the form of a white powder composed of silica nanoparticles where trypsin molecules are immobilized thereto.

18. Method according to claim 17, wherein said enzyme immobilization is achieved with an amount of trypsin comprised between about 3 and about 9 mg per gram of nanoparticles and a buffer having a pH between 7.0 and 8 or of about 6 in a concentration of between about 10 and about 20 microMol, wherein said buffer solution is a phosphate buffer solution or a sodium citrate buffer solution, or a combination of the same.

19. Method according to claim 15, wherein said gelling agent is methylcellulose added in a ratio of about 1:10 w/w.

20. Method according to claim 15, comprising a step of applying a protective film (40,40') to prevent contamination of the film and/or to maintain chemical and physical conditions suitable for the bio-cleansing treatment or storage of said kit and/or drying of the impregnated support (30).

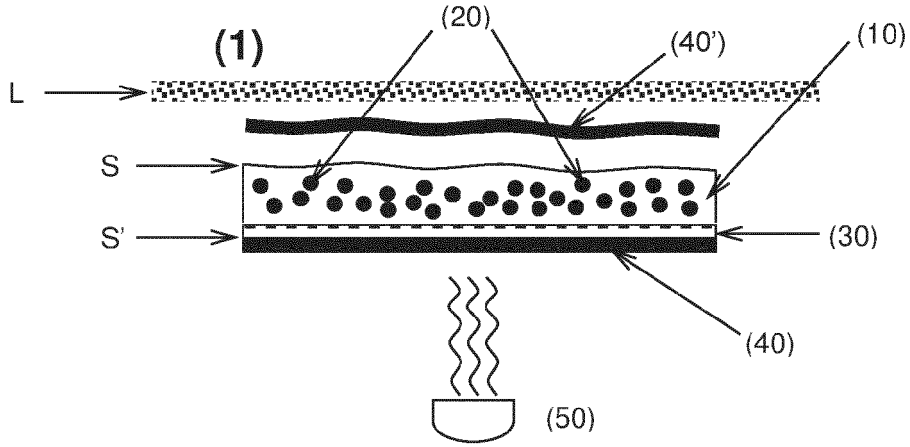
21. Method according to claim 15, wherein said applying step comprises applying said formulation (10) in the form of gel on a support consisting of a hydrophilic fabric by dip-coating, roll-coating or spraying.

22. A method of enzymatic bio-cleansing suitable for removal of pre-existing biofilm made by biological entities and by chemical entities from a substrate (L), by means of the bio-cleansing enzymatic kit according to one or more of the claims 1 to 14, **characterized in that** comprises the following steps:

- performing a study for analyzing the characteristics of said substrate (L), of said pre-existing biofilm made by chemical and biological entities, and of the environmental conditions in which the bio-cleansing treatment is performed;
- providing a formulation (10) comprising at least one enzyme system (20), wherein said enzyme system (20) comprises a plurality of immobilized enzymes, linked or associated in a stable or temporary manner, to one or more inorganic particles, wherein said formulation (10) can be removed with ease from said substrate (L) to preserve substrate integrity;
- according to the results obtained from said study, optimizing said formulation (10);
- according to the results obtained from said study, selecting and providing a support for said formulation comprising:
  - suitable means (30) to which said formulation (10) is applied or means (30) impregnated with said formulation (10), or
  - a suitable medium (30') for dispersing said enzyme system (20) wherein and including said medium (30') in said formulation (10);
- preparing said bio-cleansing enzymatic kit (1), comprising said formulation (10) and said means (30,30'), wherein said means (30, 30') being selected from the group consisting of: a gel, a hydrophilic fabric, a flexible support, a rigid support, a plastic film, or a combination thereof;
- applying said bio-cleansing enzymatic kit (1) on said substrate (L) to be subjected to the bio-cleansing treatment;
- placing the entire surface of said substrate (L) to be treated in contact with said formulation (10),
- waiting the necessary time to allow a selective and satisfactory cleaning of said substrate (L) by the enzyme included in said kit (1),
- removing said bio-cleansing enzymatic kit (1) from said substrate (L).

23. Method according to the preceding claim, comprising at least one of the following steps:

- activating said enzyme system (20) by hydration of said formulation (10), preferably with a buffer solution, or a composition suitable to limit the formation of salts on the surface of the substrate,
- protecting said bio-cleansing enzymatic kit (1) by means of said protective film (40, 40');
- controlling by means of a control system (90) the process parameters, preferably temperature, pH and humidity content of said formulation (10);
- washing said substrate (L), preferably by means of demineralized water to remove residues of said formulation (10) from said substrate or by dabbing the surface with a sponge or tissue soaked with demineralized water;
- deactivating said enzymatic system (20) by drying, so as to enable storage and future use of said bio-cleansing enzymatic kit;
- reusing or storing said bio-cleansing enzymatic kit in a suitable environment to allow future use after drying.



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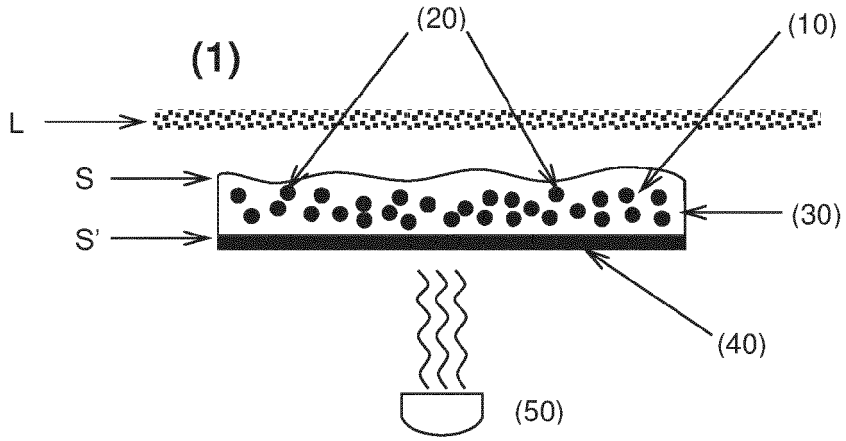

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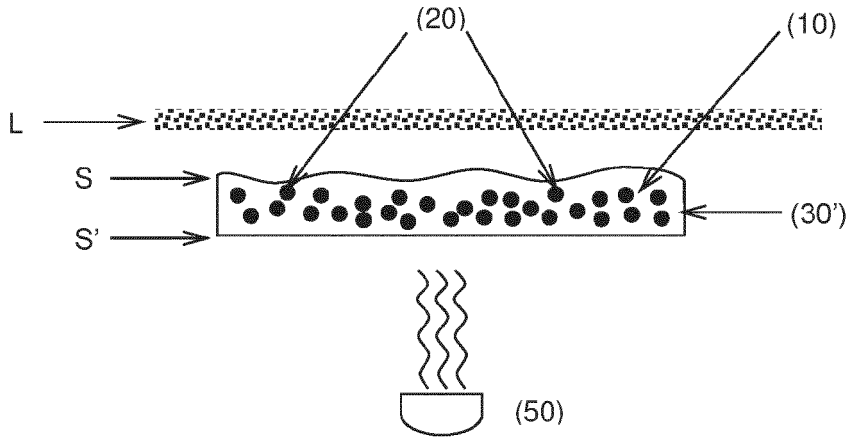
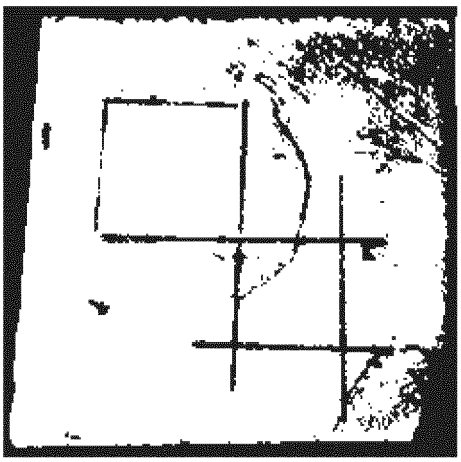


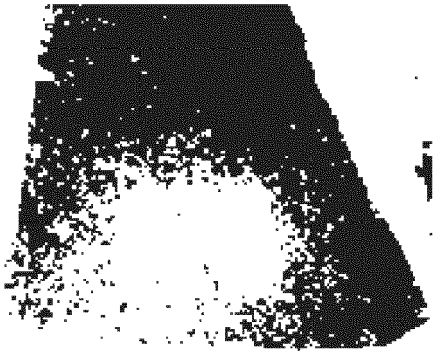
(c)

Figure 1



(a)

(b)



(c)

Figure 2

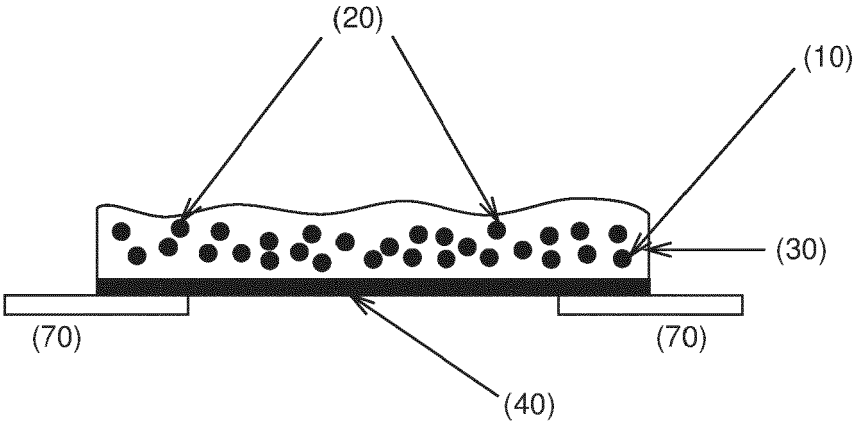


Figure 3

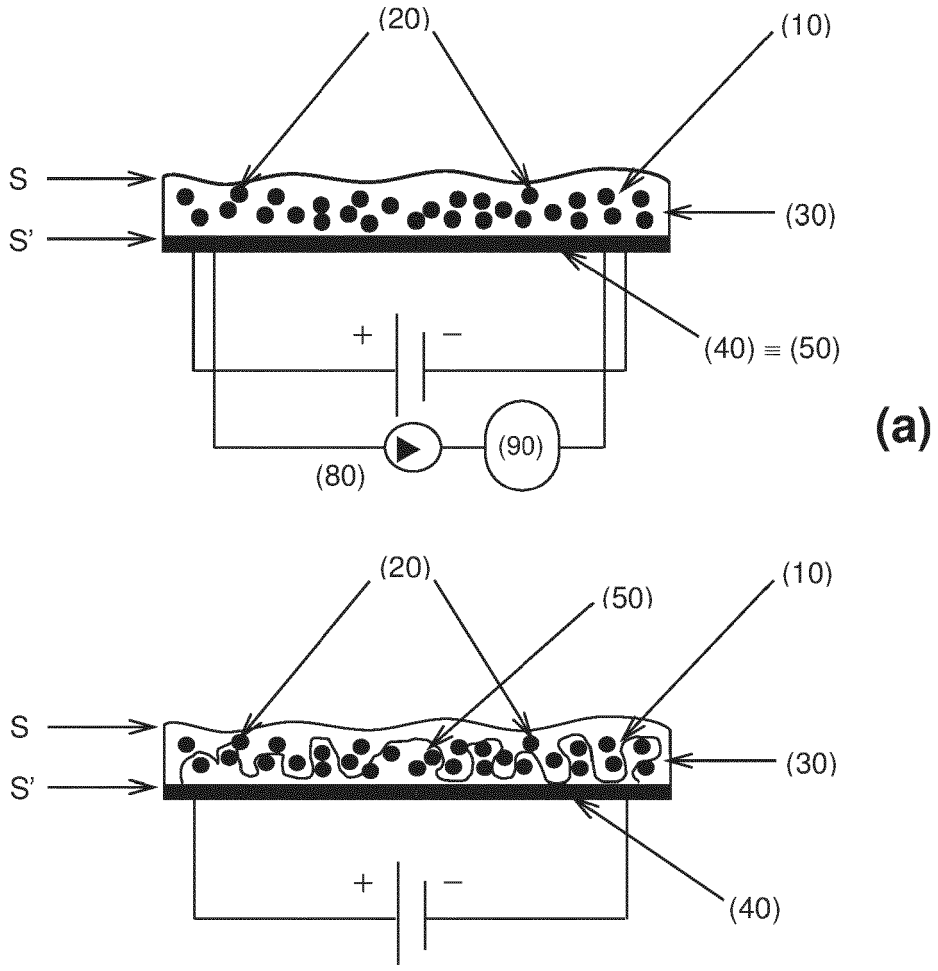
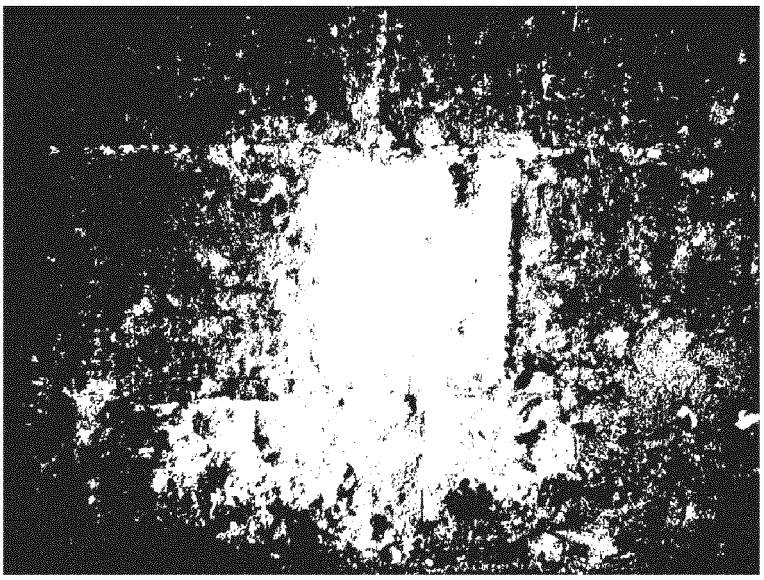


Figure 4



(a)

(b)

Figure 5

Main figure

REFERENCES CITED IN THE DESCRIPTION

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