SYNERGISTIC COMPOSITION OF BIOCIDES

FIELD OF THE INVENTION

This invention is directed to a biocidal composition containing tetrachloroisophthalonitrile and 3-iodo-2-propynylbutylcarbamate in the form of a liquid which serves to inhibit fungal, algae and bacterial growth.

DESCRIPTION OF RELATED ART

The compound 3-iodo-2-propynyl butylcarbamate (hereinafter referred to as "IPBC") is a well-known biocide, having good fungicidal activity. It also has activity against bacterial and unicellular green algae, but has been found to be much less effective against unicellular blue green algae.

IPBC has been mixed with various biocides in combination form for various applications. U.S. Pat. No. 5,162,343 discloses IPBC with sodium 2-pyridinethiol-1-oxide in a biocidal composition. U.S. Pat. No. 5,328,926 discloses IPBC with 1,2-Benzisothiazolin-3-one for use in controlling the growth of fungi and bacteria in fluids. U.S. Pat. No. 5,591,760 discloses IPBC with 4,5-dichloro-2-octyl-3-isothiazolone in various applications. U.S. Pat. No. 5,707,929 discloses IPBC with N-Cyclopropyl-N' (1,1-dimethyl)-6-(methylthio)-1,3,5-triazine-2,4-dismine as a fungicide and algaecide.

Tetrachloroisophthalonitrile (hereinafter referred to as "CTL") has been used as a fungicide in agriculture and in architectural coating applications i.e., paints, stains, and other related coatings. It provides very good, long-term fungicidal protection in the cured coating.

using such organic tin biocides at their present commercial levels as an anti-foulant active ingredient in coating compositions for aquatic (marine) applications. It has been shown that, due to the wide-spread use of tributyltin-type compounds in particular, at concentrations as high as 20 wt.% in paints for ship bottoms, the pollution of surrounding water due to leaching has reached such a level as to cause the degradation of mussel and shell organisms. These effects have been detected along the French-British coastline and a similar effect has been confirmed in U.S. and Far East waters. Under the most recent regulatory restrictions, with limited exceptions, pleasure boats up to 25 meters long are no longer permitted to use anti-foulant paint containing high levels of tributyltin compounds.

Research has shown that as long as the leaching rate of tin can be maintained at or below about 4 $\mu g/cm^2$ per day, aquatic life does not appear to be affected over the long term. However, it has also been found that to be effective for controlling marine algae, as well as higher developed marine organisms, from the painted surface of ship bottoms, a certain minimum leaching rate of tin of about 9 to 16 $\mu g/cm^2$/day is required. Usually, this higher leaching rate is achieved with a concentration of tributyltin compound at about 15% to 20% by weight of paint.

In view of the effectiveness of TBT regulatory authorities have reluctantly agreed that as long as there is no satisfactory substitute for the anti-foulant organic tin active ingredients, larger ships, i.e., those above a length of 25 meters, are still permitted to use such compounds to minimize fouling. There is therefore a desire to provide alternative biocides to TBT based compounds.

US-A-4297137 discloses that the effects of an anti-fouling composition can be lengthened by moderating the release of the anti-fouling constituents. This document discloses anti-fouling paints comprising at least one substance toxic to marine organism uniformly incorporated into a discontinuous solid matrix which is insoluble in sea water and is dispersed in the paint. The matrix is at least partially formed from at least one substance which becomes soluble in sea water under the action of enzymes liberated by the marine organisms to be inhibited and/or by the bacterial film in contact with the paint. Thus when marine organism become associated with the painted surface, the

toxic substance is released and the organisms inhibited. Similar to prior art disclosures, the toxic substances envisaged by US-A-4297137 include only the well known copper and tin based compounds, such as TBT.

Abarzua *et al.*, Mar. Ecol. Prog. Ser., Vol. 123: 301-312, 1995 "*Biotechnological investigation for the prevention of biofouling. I. Biological and biochemical principles for the prevention of biofouling*" propose the extraction of biogenic agents having antibacterial, anti-algal, anti-protozoan and anti-macrofouling properties from algae and marine invertebrates. It is proposed that the structure of the extracted agents may be determined, subsequently synthesised and the synthesised agent used in the prevention of biofouling. No teaching of the extraction or of the synthesis is provided.

EP-A-0866103 discloses a method for controlled release of compounds having antimicrobial activity and coating compositions utilising this system. The method comprises the incorporation of an enzyme and a substrate into a matrix. The enzyme acts of the substrate to provide a compound. In an envisaged embodiment the compound may be acted on by a further enzyme. The substrate and enzyme(s) produce a compound having antimicrobial activity.

US-A-5747078 relates to food products. The document teaches that microbial contamination of food and feed, which can cause severe health problems, may be inhibited by a composition comprising a lactoperoxidase system which provides for the sustained release of hydrogen peroxide. The hydrogen peroxide is prepared by the reaction of an oxidoreductase with an oxidisable substrate. The hydrogen peroxide then reacts with thiocyanate, under catalysis by lactoperoxidase, to produce hypothiocyanate. The hypothiocyanate may then act as an anti-microbial agent. This document provides a background teaching of immobilised enzyme systems. The document is silent concerning anti-foulants or any micro-organism which results in fouling properties.

The present invention alleviates the problem of the prior art

Aspects of the present invention are defined in the appended claims. These and other preferred aspects are discussed below.

This Sheet Has Been Replaced

It has been found that the provision of an integrated system for the generation of an anti-fouling compound utilising an enzyme from a marine organism provides a stable system which

- has long term effectiveness in harsh environment such as marine environments
- requires less substrate than prior art systems to provide a given anti-microbial effect.

  Enzymes from marine organisms, such as algal hexose oxidase (HOX), have low Km values for glucose, namely 2.7 mM. This low Km means that the enzyme has a very high affinity for glucose. In contrast non-marine enzymes such as non-marine glucose oxidase (GOX) may have at least ten fold higher Km value for glucose. In other words prior enzyme systems have a much lower affinity for glucose than that of the present invention. In antifouling applications, this will give a significant difference, since an enzyme with high glucose affinity will be able to convert all the glucose present. On the other hand an enzyme with lower glucose affinity is anticipated to allow leaching of glucose to the surrounding environment. Leaching of glucose will counter the desired antifouling activity because glucose will be a substrate for fouling organisms. Leaching of glucose will therefore lead to increased fouling.
- requires less enzyme than prior art systems to provide a given anti-microbial effect.

  Enzymes from marine organisms, such as algal HOX, also have low Km value for oxygen, again lower than the Km value for oxygen of prior art systems such as GOX. Again this higher affinity for the substrate gives algal HOX an advantage. This is because in antifouling applications the worst fouling will be in "closed" environments like harbours with low waterexchange and high growth of algae and other fouling organisms. At exactly these places were the fouling is worst, the oxygen content of the water will also be the lowest compared to open sea. Enzymes from marine organisms with high affinity for oxygen will therefore be advantageous.
- provides improved activity at likely operational temperatures.

  In surface coating compositions such as antifouling paint the anti-microbial (antifouling) compound typically has to be active between 15 and 30°C. For anti-fouling compositions this is the seawater temperature where fouling gives a problem. Contrary to prior art systems, enzymes from marine organisms, such as

algal HOX, have optimum temperature of activity exactly at the optimum temperature for fouling and these enzymes are therefore perfectly suited as an antifouling agent. The optimum temperature is shown in Example 9

- utilises safe and readily available substrates.
- has improved salt tolerance which leads to further improved activity in marine environments.
- is resistant to degradation by fouling organisms

enzymes from marine organisms, such as algal HOX, are remarkably protease resistant enzymes. They will survive treatment with pronase (a broad spectrum protease preparation) without any loss of activity. This protease resistance is considered especially important in the antifouling application since the enzyme therefore will be resistant to degradation by proteases from the antifouling organisms which are trying to attach themselves onto the coated surface.

In the present specification "foulants" referred to by the terms "anti-foul(s)", "anti-fouling", and "anti-foulants" include organisms which may reside and/or grow on the surface to be treated with the present composition. The organisms include micro-organisms such as bacteria, fungi and protozoa, and algae and organisms such as algae, plants and animals. The organism may be marine organisms.

The composition of the present invention comprises a precursor enzyme and a precursor substrate, wherein the precursor enzyme and the precursor substrate generate a substrate for the enzyme of the present invention by action of the precursor enzyme on the precursor substrate. This combination of precursor enzyme and precursor substrate is herein after referred to as "substrate generator".

The enzyme of the present system may be obtained or may be obtainable from a marine micro-organism

Preferably the enzyme of the present system is obtained or is obtainable from a marine alga. Preferably the enzyme of the present system is obtained or is obtainable from *Chondrus cripus*.

Preferably, the anti-fouling compound is hydrogen peroxide.

Preferably, the enzyme is an oxidase. Preferably, the enzyme is selected from glucose oxidase, L amino acid oxidase, D amino oxidase, galactose oxidase, hexose oxidase, pyranose oxidase, malate oxidase, cholesterol oxidase, arylalcohol oxidase, alcohol oxidase, lathosterol oxidase, aspartate oxidase, amine oxidase, D glutamate oxidase, ethanolamine oxidase, NADH oxidase, urate oxidase (uricase) and mixtures thereof. Preferably, the enzyme is hexose oxidase.

HEXOSE OXIDASE (HOX) ENZYME

Hexose oxidase (D-hexose: O_2-oxidoreductase, EC 1.1.3.5) (also referred to as HOX) is an enzyme that in the presence of oxygen is capable of oxidising D-glucose and several other reducing sugars including maltose, lactose and cellobiose to their corresponding lactones with subsequent hydrolysis to the respective aldobionic acids. Accordingly, HOX differs from another oxidoreductase, glucose oxidase, which can only convert D-glucose, in that the enzyme can utilise a broader range of sugar substrates. The oxidation catalysed by HOX can be illustrated as follows:

D-glucose + O_2 -----> γ-D-gluconolactone + H_2O_2, or

D-galactose + O_2 -----> γ-D-galactonolactone + H_2O_2

HOX is produced naturally by several marine algal species. Such species are found *inter alia* in the family *Gigartinaceae*. As used herein, the term "HOX" denotes an enzyme which is capable of oxidising the substrates selected from the group consisting of D-glucose, D-galactose, D-mannose, maltose, lactose and cellobiose.

Preferably, the hexose oxidase is obtainable or is obtained from marine algae *Chondrus cripus*.

In one aspect the hexose oxidase enzyme is an enzyme covered by the disclosure of EP-A-0832245

HEXOSE OXIDASE (HOX) PRODUCTION

The gene encoding the HOX enzyme has been cloned from the marine algae *Chondrus crispus* (Stougaard and Hansen 1996, Hansen and Stougaard, 1997). The methylotrophic yeast *Hansenula polymorpha* (developed at Rhein Biotech, Dusseldorf/Germany as an expression system for heterologous proteins) has also been used to produce the HOX enzyme (the native protein was purified from marine algae (Poulsen and Høstrup, 1998)). WO 96/40935 and WO 98/13478 also disclose the cloning and expression in recombinant host organisms of a gene encoding a protein with HOX activity.

In a preferred embodiment, the hexose oxidase enzyme comprises the amino acid sequence set out in SEQ ID No 1 or a variant, homologue, derivative or fragment thereof. In a preferred embodiment, the hexose oxidase enzyme comprises the amino acid sequence set out in SEQ ID No 1.

In a preferred embodiment, the hexose oxidase enzyme is encoded by a nucleotide sequence set out in SEQ ID No 1 or a variant, homologue, derivative or fragment thereof. In a preferred embodiment, the hexose oxidase enzyme is encoded by a nucleotide sequence set out in SEQ ID No 1.

In a preferred embodiment, the hexose oxidase enzyme is encoded by a nucleotide sequence capable of hybridising to the nucleotide sequence set out in SEQ ID No 1 or a variant, homologue, derivative or fragment thereof or a sequence complementary to the hybridisable sequence. In a preferred embodiment, the hexose oxidase enzyme is encoded by a nucleotide sequence capable of hybridising to the nucleotide sequence set out in SEQ ID No 1 or a sequence complementary to the hybridisable sequence.

The enzyme, preferably the hexose oxidase enzyme may be prepared in a manner described in British Patent Application No. 9927801.2

VARIANTS/HOMOLOGUES/DERIVATIVES (AMINO ACID SEQUENCE)

Preferred amino acid sequences of the present invention are set out in SEQ ID No 1 or are sequences obtainable from the HOX enzyme of the present invention but also include homologous sequences obtained from any source, for example related viral/bacterial proteins, cellular homologues and synthetic peptides, as well as variants or derivatives thereof.

Thus, the present invention covers variants, homologues or derivatives of the amino acid sequences presented herein, as well as variants, homologues or derivatives of the nucleotide sequence coding for those amino acid sequences.

In the context of the present invention, a homologous sequence is taken to include an amino acid sequence which is at least 75, 85 or 90% identical, preferably at least 95 or 98% identical at the amino acid level over at least, for example, the amino acid sequence as set out in SEQ ID No 1 of the sequence listing herein. In particular, homology should typically be considered with respect to those regions of the sequence known to be essential for enzyme activity rather than non-essential neighbouring sequences. These regions include but are not limited to the putative FAD binding domains in HOX such as $SGGH_{79}C$, $LGGH_{146}I$ and $LGGH_{320}A$. Although homology can also be considered in terms of similarity (i.e. amino acid residues having similar chemical properties/functions), in the context of the present invention it is preferred to express homology in terms of sequence identity.

Homology comparisons can be conducted by eye, or more usually, with the aid of readily available sequence comparison programs. These commercially available computer programs can calculate % homology between two or more sequences.

% homology may be calculated over contiguous sequences, i.e. one sequence is aligned with the other sequence and each amino acid in one sequence is directly compared with the corresponding amino acid in the other sequence, one residue at a time. This is called an "ungapped" alignment. Typically, such ungapped alignments are performed only over a relatively short number of residues.

Although this is a very simple and consistent method, it fails to take into consideration that, for example, in an otherwise identical pair of sequences, one insertion or deletion will cause the following amino acid residues to be put out of alignment, thus potentially resulting in a large reduction in % homology when a global alignment is performed. Consequently, most sequence comparison methods are designed to produce optimal alignments that take into consideration possible insertions and deletions without penalising unduly the overall homology score. This is achieved by inserting "gaps" in the sequence alignment to try to maximise local homology.

However, these more complex methods assign "gap penalties" to each gap that occurs in the alignment so that, for the same number of identical amino acids, a sequence alignment with as few gaps as possible - reflecting higher relatedness between the two compared sequences - will achieve a higher score than one with many gaps. "Affine gap costs" are typically used that charge a relatively high cost for the existence of a gap and a smaller penalty for each subsequent residue in the gap. This is the most commonly used gap scoring system. High gap penalties will of course produce optimised alignments with fewer gaps. Most alignment programs allow the gap penalties to be modified. However, it is preferred to use the default values when using such software for sequence comparisons. For example when using the GCG Wisconsin Bestfit package (see below) the default gap penalty for amino acid sequences is -12 for a gap and -4 for each extension.

Calculation of maximum % homology therefore firstly requires the production of an optimal alignment, taking into consideration gap penalties. A suitable computer program for carrying out such an alignment is the GCG Wisconsin Bestfit package (University of Wisconsin, U.S.A.; Devereux *et al.*, 1984, Nucleic Acids Research 12:387). Examples of other software than can perform sequence comparisons include, but are not limited to, the BLAST package (see Ausubel *et al.*, 1999 *ibid* – Chapter 18), FASTA (Atschul *et al.*, 1990, J. Mol. Biol., 403-410) and the GENEWORKS suite of comparison tools. Both BLAST and FASTA are available for offline and online searching (see Ausubel *et al.*, 1999 *ibid*, pages 7-58 to 7-60). However it is preferred to use the GCG Bestfit program.

Although the final % homology can be measured in terms of identity, the alignment process itself is typically not based on an all-or-nothing pair comparison. Instead, a scaled similarity score matrix is generally used that assigns scores to each pairwise comparison based on chemical similarity or evolutionary distance. An example of such a matrix commonly used is the BLOSUM62 matrix - the default matrix for the BLAST suite of programs. GCG Wisconsin programs generally use either the public default values or a custom symbol comparison table if supplied (see user manual for further details). It is preferred to use the public default values for the GCG package, or in the case of other software, the default matrix, such as BLOSUM62.

Once the software has produced an optimal alignment, it is possible to calculate % homology, preferably % sequence identity. The software typically does this as part of the sequence comparison and generates a numerical result.

The terms "variant" or "derivative" in relation to the amino acid sequences of the present invention includes any substitution of, variation of, modification of, replacement of, deletion of or addition of one (or more) amino acids from or to the sequence providing the resultant amino acid sequence has an enzyme activity, preferably having at least the same enzyme activity as the amino acid sequence set out in SEQ ID No 1.

SEQ ID No 1 may be modified for use in the present invention. Typically, modifications are made that maintain the enzyme activity of the sequence. Amino acid substitutions may be made, for example from 1, 2 or 3 to 10 or 20 substitutions provided that the modified sequence retains the required enzyme activity. Amino acid substitutions may include the use of non-naturally occurring analogues.

SEQ ID No 1 of the present invention may also have deletions, insertions or substitutions of amino acid residues which produce a silent change and result in a functionally equivalent enzyme. Deliberate amino acid substitutions may be made on the basis of similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity, and/or the amphipathic nature of the residues as long as the enzyme activity of the HOX enzyme is retained. For example, negatively charged amino acids include aspartic acid

and glutamic acid; positively charged amino acids include lysine and arginine; and amino acids with uncharged polar head groups having similar hydrophilicity values include leucine, isoleucine, valine, glycine, alanine, asparagine, glutamine, serine, threonine, phenylalanine, and tyrosine.

Conservative substitutions may be made, for example according to the Table below. Amino acids in the same block in the second column and preferably in the same line in the third column may be substituted for each other:

ALIPHATIC	Non-polar	G A P
		I L V
	Polar - uncharged	C S T M
		N Q
	Polar - charged	D E
		K R
AROMATIC		H F W Y

VARIANTS/HOMOLOGUES/DERIVATIVES (NUCLEOTIDE SEQUENCE)

It will be understood by a skilled person that numerous different nucleotide sequences can encode the same HOX enzyme as a result of the degeneracy of the genetic code. In addition, it is to be understood that skilled persons may, using routine techniques, make nucleotide substitutions that do not affect the HOX enzyme encoded by the nucleotide sequence of the invention to reflect the codon usage of any particular host organism in which the HOX enzyme of the present invention is to be expressed.

The terms "variant", "homologue" or "derivative" in relation to the nucleotide sequence set out in SEQ ID No 1 of the present invention includes any substitution of, variation of, modification of, replacement of, deletion of or addition of one (or more) nucleic acid from or to the sequence providing the resultant nucleotide sequence codes for a HOX enzyme

having an enzyme activity, preferably having at least the same activity as the nucleotide sequence set out in SEQ ID No 1 of the sequence listings.

As indicated above, with respect to sequence homology, preferably there is at least 75%, more preferably at least 85%, more preferably at least 90% homology to the sequences shown in the sequence listing herein. More preferably there is at least 95%, more preferably at least 98%, homology. Nucleotide homology comparisons may be conducted as described above. A preferred sequence comparison program is the GCG Wisconsin Bestfit program described above. The default scoring matrix has a match value of 10 for each identical nucleotide and -9 for each mismatch. The default gap creation penalty is -50 and the default gap extension penalty is -3 for each nucleotide.

The present invention also encompasses nucleotide sequences that are capable of hybridising selectively to the sequences presented herein, or any variant, fragment or derivative thereof, or to the complement of any of the above. Nucleotide sequences are preferably at least 15 nucleotides in length, more preferably at least 20, 30, 40 or 50 nucleotides in length.

SUBSTRATE

Preferably, the substrate is selected from peptides, L amino acid, and carbohydrates/sugars, including hexoses, preferably glucose, galactose, lactose, 2-deoxyglucose, pyranose, xylan, cellulose, inulin, starch, dextran, pectin, and mixtures thereof.

In a highly preferred embodiment the enzyme/substrate combination is selected from glucose/hexose oxidase, glucose/glucose oxidase, L amino acid/L amino acid oxidase, galactose/galactose oxidase, lactose/β-galactosidase/hexose oxidase, lactose/β-galactosidase/glucose oxidase, 2-deoxyglucose/glucose oxidase, pyranose/pyranose oxidase, and mixtures thereof.

In one aspect the anti-foulant compound is generated by action of the enzyme on the substrate which is present in the composition. Thus the anti-foulant compound is

generated by a "one-step" process. In some cases the substrate may be prepared in situ. In these cases, the composition further comprises a precursor enzyme and a precursor substrate wherein the precursor enzyme and the precursor substrate are selected such that the precursor enzyme generates the substrate. In this latter aspect the anti-foulant compound is generated by a "two-step" process

In the one-step process preferably the enzyme is selected from hexose oxidase, glucose oxidase, L amino acid oxidase, galactose oxidase, pyranose oxidase, and mixtures thereof.

In the one-step process preferably the substrate is selected from a hexose, preferably glucose, L amino acid, galactose, 2-deoxyglucose, pyranose, and mixtures thereof.

In the one-step process preferably the enzyme/substrate combination is selected from glucose/hexose oxidase, glucose/glucose oxidase, L amino acid/L amino acid oxidase, galactose/galactose oxidase, 2-deoxyglucose/glucose oxidase, pyranose/pyranose oxidase, and mixtures thereof.

In the two-step process preferably the enzyme is hexose oxidase.

In the two-step process preferably the substrate is glucose.

In the two-step process preferably the precursor enzyme is amyloglucosidase.

In the two-step process preferably the precursor substrate is starch.

Thus in the two-step process preferably the precursor substrate/precursor enzyme/enzyme combination is starch/amyloglucosidase/hexose oxidase.

Preferably, the precursor substrate of the two-step process is selected from oligomers and polymers of substrates for oxidative enzymes, starch, lactose, cellulose, dextrose, peptide, inulin, and mixtures thereof.

The provision of precursor substrates are particularly preferred because they provide for sustained and/or prolonged release of substrate by action of the precursor enzyme on the precursor substrate.

Native starch is particularly preferred as a precursor substrate. Native starch provides densely packed crystals which can be readily applied in a surface coatings. Moreover, Native starch is water insoluble.

Cellulose is also a particularly preferred as a precursor substrate. Cellulose is a common component in paint and use of cellulose a precursor substrate reduces the number of additional components which must be added to a paint composition.

Preferably, the precursor enzyme of the two-step process is selected from exo-acting enzymes capable of degrading oligomeric or polymeric substrates to monomeric units, e.g. β-galactosidase, peptidase; amyloglucosidase, and mixtures thereof.

Optionally, the composition further comprises a binder to immobilise at least one of the constituents, optionally to immobilise the enzymes.

The compositions of the present invention may be formulated as coatings, lacquers, stains, enamels and the like, hereinafter referred to generically as "coating(s)".

Thus, in one aspect the present invention provides a coating consisting of a composition as defined above.

Preferably, the coating is formulated for treatment of a surface selected from outdoor wood work, external surface of a central heating system, and a hull of a marine vessel.

The coating may include a liquid vehicle (solvent) for dissolving or suspending the composition.

The liquid vehicle may be selected from any liquid which does not interfere with the activities of any essential components of the composition. In particular, the liquid

vehicle should not interfere with the activity of the essential enzyme(s) and/or anti-foulant compound. Suitable liquid vehicles are disclosed in US-A-5071479 and include water and organic solvents including aliphatic hydrocarbons, aromatic hydrocarbons, such as xylene, toluene, mixtures of aliphatic and aromatic hydrocarbons having boiling points between 100 and 320°C, preferably between 150 and 230°C; high aromatic petroleum distillates, e.g., solvent naptha, distilled tar oil and mixtures thereof; alcohols such as butanol, octanol and glycols; vegetable and mineral oils; ketones such as acetone; petroleum fractions such as mineral spirits and kerosene, chlorinated hydrocarbons, glycol esters, glycol ester ethers, derivatives and mixtures thereof.

The liquid vehicle may contain at least one polar solvent, such as water, in admixture with an oily or oil-like low-volatility organic solvent, such as the mixture of aromatic and aliphatic solvents found in white spirits, also commonly called mineral spirits.

The vehicle may typically contain at least one of a diluent, an emulsifier, a wetting agent, a dispersing agent or other surface active agent. Examples of suitable emulsifiers are disclosed in US-A-5071479 and include nonylphenol-ethylene oxide ethers, polyoxyethylene sorbitol esters or polyoxyethylene sorbitan esters of fatty acids, derivatives and mixtures thereof.

Any suitable surface coating material may be incorporated in the composition and/or coating of the present invention. Examples of trade-recognized coating materials are polyvinyl chloride resins in a solvent based system, chlorinated rubbers in a solvent based system, acrylic resins and methacrylate resins in solvent based or aqueous systems, vinyl chloride-vinyl acetate copolymer systems as aqueous dispersions or solvent based systems, butadiene copolymers such as butadiene-styrene rubbers, butadiene-acrylonitrile rubbers, and butadiene-styrene-acrylonitrile rubbers, drying oils such as linseed oil, alkyd resins, asphalt, epoxy resins, urethane resins, polyester resins, phenolic resins, derivatives and mixtures thereof.

The composition and/or coating of the present invention may contain pigments selected from inorganic pigments, such as titanium dioxide, ferric oxide, silica, talc, or china clay, organic pigments such as carbon black or dyes insoluble in sea water, derivatives

and mixtures thereof.

The composition and/or coating of the present invention may contain materials such as rosin to provide controlled release of the anti-foulant compound, rosin being to a very slight extent soluble in sea water.

The composition and/or coating of the present invention may contain plasticisers, rheology characteristic modifiers, other conventional ingredients and mixtures thereof.

The composition and/or coating of the present invention, particularly the coating, further comprise an adjuvant conventionally employed in compositions used for protecting materials exposed to an aquatic environment. These adjuvants may be selected from additional fungicides, auxiliary solvents, processing additives such as defoamers, fixatives, plasticisers, UV-stabilizers or stability enhancers, water soluble or water insoluble dyes, color pigments, siccatives, corrosion inhibitors, thickeners or anti-settlement agents such as carboxymethyl cellulose, polyacrylic acid or polymethacrylic acid, anti-skinning agents, derivatives and mixtures thereof.

The additional fungicide(s) used in the composition and/or coating of the present invention is preferably soluble in the liquid vehicle.

In one aspect the present invention provides a marine anti-foulant consisting of a composition as defined above.

Preferably, the anti-foulant is self-polishable.

In one aspect of the present invention, the substrate or substrate generator and/or the enzyme are encapsulated. Preferably, the substrate/substrate generator and/or enzyme are encapsulated by a semi-permeable membrane.

The substrate/substrate generator and enzyme may be encapsulated individually independently of each other or may be encapsulated together. In the former embodiment, the substrate/substrate generator or enzyme may be activated by the

foulant. For example, the encapsulating material may be selected such that on contact with a foulant, the substrate/substrate generator or enzyme may be released to contact the other of the substrate/substrate generator or enzyme. In this way, a composition may be provided which only provides an anti-foulant compound or increases provision of an anti-foulant compound when contacted with a foulant.

The composition of the present invention can be provided as a ready-for-use product or as a concentrate. The ready-for-use product may be in the form of an aqueous solution, aqueous dispersion, oil solution, oil dispersion, emulsion, or an aerosol preparation. The concentrate can be used, for example, as an additive for coating, or can be diluted prior to use with additional solvents or suspending agents.

An aerosol preparation according to the invention may be obtained in the usual manner by incorporating the composition of the present invention comprising or dissolved or suspended in, a suitable solvent, in a volatile liquid suitable for use as a propellant, for example the mixture of chlorine and fluorine derivatives of methane and ethane commercially available under the trademark "Freon", or compressed air.

As discussed in US-A-5071479 the composition and/or coating of the present invention may include additional ingredients known to be useful in preservatives and/or coatings. Such ingredients include fixatives such as carboxymethylcellulose, polyvinyl alcohol, paraffin, co-solvents, such as ethylglycol acetate and methoxypropyl acetate, plasticisers such as benzoic acid esters and phthlates, e.g., dibutyl phthalate, dioctyl phthalate and didodecyl phthalate, derivatives and mixtures thereof. Optionally dyes, color pigments, corrosion inhibitors, chemical stabilizers or siccatives (dryers) such as cobalt octate and cobalt naphthenate, may also be included depending on specific applications.

The composition and/or coating of the present invention can be applied by any of the techniques known in the art including brushing, spraying, roll coating, dipping and combinations thereof.

Compositions of the present invention can be prepared simply by mixing the various ingredients at a temperature at which they are not adversely affected. Preparation

conditions are not critical. Equipment and methods conventionally employed in the manufacture of coating and similar compositions can be advantageously employed.

The invention will now be described, by way of example only, in the following Examples.

EXAMPLES

The anti-fouling effect of an anti-fouling composition of the present invention is tested according to the following examples. These Examples show the effectiveness of the present composition at preventing fouling. The Examples also provide for the optimisation of the anti-fouling properties of the present composition.

The hexose oxidase (HOX) used in each of the present examples is available from DaniscoCultor. The HOX is a fermented product is from yeast *Hansenula polymorpha* expressing the gene encoding the HOX enzyme cloned from the marine algae *Chondrus crispus*.

EXAMPLE 1 - Preparation Of An Anti-fouling Composition ("One-Step")

Soluble or immobilised hexose oxidase or another hydrogen peroxide generating enzyme such as glucose oxidase is tested as a anti-foulant compound generating enzyme in an anti-fouling composition. The hexose oxidase may be immobilised for example by binding to an anion exchanger, Q Sepharose FF™ (available from Pharmacia) using 20 mM triethanolamine buffer, pH 7.3. Alternatively, hexose oxidase or alternative hydrogen peroxide generating enzymes is covalently linked to a suitable carrier such as epoxy activated Sepharose™ (Pharmacia, Sweden), carbodiimide activated agarose (Bio-Rad, USA). Other conventional procedures known in the art for immobilisation may also be utilised

The range of concentrations used is 0.0001 to 1000 U of hexose oxidase activity/hydrogen peroxide generating enzyme per ml of anti-fouling composition. One unit of enzyme activity is defined as the amount of enzyme which produces 1 μmol of H_2O_2 per

min at 25 °C.

To ascertain its suitability for use in the present invention the activity of the enzyme may be assayed as follows. Hexose oxidase (HOX) activity is measured in accordance with the following procedure.

The HOX assay is based on the measurement of hydrogen peroxide generated in the oxidation of glucose. The hydrogen peroxide oxidises *o*-dianisidine in the presence of peroxidase to form a dye.

$$\beta\text{-D-glucose} + H_2O + O_2 \xrightarrow{\text{HOX}} \text{D-glucono -delta-lactone} + H_2O_2$$

$$H_2O_2 + o\text{-dianisidine}_{red.} \xrightarrow{\text{POD}} 2\,H_2O + o\text{-dianisidine}_{ox.}$$

Reagents

1. 100 mM phosphate buffer, pH 6.3
2. 100 mM D-glucose (SIGMA, G-8270) in 100 mM phosphate buffer, pH 6.3
3. *o*-Dianisidine (SIGMA, D-3252), 3.0 mg/ml in distilled water
4. Peroxidase (SIGMA, P-8125), 0.10 mg/ml in 100 mM phosphate buffer, pH 6.3

Assay

120 µl reagent 1
150 µl reagent 2
10 µl reagent 3
10 µl reagent 4 and
10 µl enzyme solution

The assay is performed in a microtiter plate. The reaction is initiated by the addition of enzyme solution. The mixture is incubated at 25°C for 15 min with shaking. The blank run contains all the components with water instead of enzyme solution. The formation

of the dye is measured in a microtiter plate reader at 405 nm. The linearity of the reaction can be checked by using a kinetics programme on the microplate reader.

A hydrogen peroxide standard curve can be constructed by using varying concentrations of fresh H_2O_2 (MERCK).

EXAMPLE 2 - Preparation Of An Anti-fouling Composition ("Two-Step")

Glucose and galactose in concentrations of 0.01 to 100 μg per ml of anti-fouling composition are tested as substrates to generate a substrate for hexose oxidase in the systems described in Example 1. In order to provide a continuous substrate generating system, starch, preferably intact starch granules from wheat, maize or potato, in a concentration of from 0.01 ng to 100 μg per ml of anti-fouling composition, are used together with amyloglucosidase (GRINDAMYL™ AG 1500 Bakery Enzyme from DaniscoCultor or another commercial amyloglucosidase product). The components are present in concentrations providing from 0.000001 to 10 AGU per ml of anti-fouling composition.

1 AGU is defined as the amyloglucosidase activity which releases 1 μmol of glucose per minute from maltose (0.5% w/v) in 50 mM sodium acetate, pH 5.0 (adjusted with concentrated acetic acid) at 40°C. The assay is stopped by transferring 200 μl of assay mix to 100 μl of 0.1 M hydrochloric acid chloride and the amount of glucose released is measured using glucose dehydrogenase reagent (Merck no. 12193) or another glucose detection system.

EXAMPLE 3 - Generation of hydrogen peroxide by paint containing HOX

In order to test the ability of hexose oxidase (HOX) to generate hydrogen peroxide the following experiment was performed.

To 11.0 g of paint (water-based wall painting Sadolin Glans 7 and oil based Histor 9010, respectively) were added 0.2, 0.5 and 1g, respectively, of HOX (DaniscoCultor fermented product from *Hansenula polymorpha*) spraydried on starch (10 U/g). To the

water based paint was also added 5g of water per treatment.

Disposable plastic transfer pipettes (Sarstedt) were dipped (head part) in the paint. The transfer pipettes were left to air dry for 3 hours.

Hexose oxidase (HOX) activity was then measured by immersion of the paint covered pipette head into a glass tube with 2 mL of HOX assay reagent, see below, the only HOX activity coming from the HOX in the paint.

The tubes were incubated at room temperature.

As a blank was used paint without added HOX.

The result of the experiment is shown in table 1. HOX is homogeneously distributed in the paint, since the whole surface of the paint immediately turns red when it gets in contact with the HOX assay reagent. The colour development is observed immediately indicating that the paint does not have any inhibiting effect on the HOX activity. This experiment proves that HOX is able to generate hydrogen peroxide from exogenous added substrate (here glucose) even when immobilised in a paint matrix after drying.

Table 1

	Activity
Water based paint, blank	0
0.2g HOX	+
0.5g HOX	++
1.0g HOX	+++
Oil based paint, blank	0
0.2g HOX	+
0.5g HOX	++
1.0g HOX	+++
Control, assay reagent plus free HOX	+++

The range of concentrations used is 0.0001 to 1000 U of hexose oxidase activity or of an

alternative hydrogen peroxide generating enzyme per ml of antifouling composition.

EXAMPLE 4 - Model System For Coating

Dialysis tubing containing an anti-fouling composition is used as a model system for a coating to prevent fouling on the surface of a coated material.

An anti-fouling composition within the dialysis tubing is used to generate an concentration of hydrogen peroxide on the surface of the dialysis tubing effective to prevent fouling. The dialysis tubing used has a cut off value of about 10000 Da. The dialysis tubing is either dialysis tubing or a dialysis cassette (such as Slide-A-Lyzer™ available from Pierce; IL, USA).

The dialysis tubing is immersed in a glass beaker with 1 to 5 litre of lake or sea water collected as described above. The glass beaker is stirred slowly with a magnetic stirrer and incubated at room temperature in proximity to a window to allow daylight to fall thereon. Fouling on the dialysis tubing is monitored visually for up to 4 weeks based on the appearance of a microbial growth layer on the dialysis tubing and rated on a scale of 1 to 5 as described above. As negative control a dialysis tube containing tap water is used.

Optionally, catalase immobilised onto nitrocellulose membrane pieces, which have subsequently been blocked with 0.1% Tween 20, are added to the lake or sea water in order to avoid accumulation of hydrogen peroxide in the water surrounding the dialysis tubing. The concentration of catalase used is in the range of 0.000001 to 100 CU, where 1 CU is defined as the catalase activity degrading 1 μmol of hydrogen peroxide per minute at 30°C in 50 mM sodium phosphate buffer, pH 7.0, as described for catalase in the Sigma catalogue: Biochemicals Organic Compounds for Research and Diagnostic Reagents, Sigma Chemical Company 1995, page 221.

The compositions of the present invention are effective at preventing fouling.

EXAMPLE 5 - Stability of HOX in paint

The painted heads of transfer pipettes described in example 1 were kept at room temperature for 2 month and were then "assayed" in reagent mix as described in example 2.

Table 2

	Activity
Water based paint, blank	0
0.2g HOX	+
0.5g HOX	++
1.0g HOX	not determined
Oil based paint, blank	0
0.2g HOX	+++
0.5g HOX	+++
1.0g HOX	+++
Control, assay reagent plus free HOX	not determined

From the results in table 2 it is clear that HOX was stable for two month at room temperature in a dry paint matrix.

EXAMPLE 6 -Testing Of Coating

Set Up Of A Test System For An Anti-fouling Composition

0.5 to 5 ml. samples of lake or sea water were collected in test tubes from the lake Brabrandsøen near Aarhus, Denmark, and from the Baltic sea off Aarhus. On the day of collection of the water samples the anti-fouling composition to be tested is added to the test tubes and they are sealed with Parafilm™.

The test tubes are incubated at room temperature in proximity to a window to allow daylight to fall thereon. Fouling is monitored visually for up to 4 weeks based on the appearance of a microbial growth layer on the walls of the test tube. For comparison a

test tube with 0.1% of sodium azide and a test tube without anti-fouling composition are used as positive and negative controls, respectively.

These test tubes are rated 1 and 5, respectively, on a scale of 1 to 5 for highly efficient to no anti-fouling activity, respectively.

Commercial marine anti-fouling coating material without added anti-fouling biocide is used. Anti-fouling compositions according to the present invention are mixed into the coating material and applied to the surface of metal, glass and plastic plates according to the instructions of the manufacturer of the coating material.

Coated plates are immersed into water in a lake or in sea water. Fouling on the plates is monitored visually for up to 2 years based on the appearance of a microbial growth layer on the plates and rated on a scale of 1 to 5 as described above. As negative control a coating without anti-fouling composition is used.

The compositions of the present invention are effective at preventing fouling.

EXAMPLE 7 - Stability of antifouling composition in aquarium water

To 10.0 g of paint (oil based Histor 9010) were added 500 mg of starch (Merck 1253), 50 mg of HOX (DaniscoCultor fermented product from *Hansenula polymorpha*) spraydryed on starch (10 U/g).

A disposable plastic transfer pipette (Sarstedt) was dipped (head part) in the paint. The transfer pipette was left to air dry for 24 hours. It was then kept in 250 mL of water from an aquarium in a Kautex bottle for two month. The bottle was standing in a window in full daylight. After two month the pipette head was washed, airdried and then "assayed" in complete HOX reagent mix. The HOX still showed full activity.

EXAMPLE 8 - Proof of substrate generating concept

In order to provide a continuous substrate generating system starch, preferably intact

starch granules from wheat, maize or potato in a concentration of from 0.01 ng to 100 mg per ml of antifouling composition, as well as amyloglucosidase (AMG)(GRINDAMYL™ AG 10000 Bakery Enzyme from DaniscoCultor or another commercial amyloglucosidase product) in concentrations providing from 0.000001 to 100 AGU per ml of antifouling composition are used together with HOX.

To 10.0 g of paint (oil-based Histor 9010) were added 500 mg of starch (Merck), HOX (DaniscoCultor fermented product from *Hansenula polymorpha*) spraydried on starch (10 U/g) and AMG (10000 AGU/g) as indicated in the table.

Disposable plastic transfer pipettes (Sarstedt) were dipped (head part) in the paint. The transfer pipettes were left to air dry for 3 hours.

Hexose oxidase (HOX) activity was then measured by immersion of the paint covered pipette head into a glasstube with 2 mL of HOX assay reagent without glucose, for assay reagent see example 1, the only HOX activity coming from the HOX in the paint and the only substrate for HOX generated by AMG in the paint by hydrolysing starch in the paint to glucose.

The tubes were incubated at room temperature for 48 hours.

As a blank was used paint without HOX and AMG added.

Table 3

	Activity
50 mg HOX + 10 mg AMG	+
50 mg HOX + 20 mg AMG	+
50 mg HOX	-
Blank (no enzymes)	-

The results given in table 3 shows that the combination of HOX and AMG works as intended. AMG is generating glucose from the co-immobilised starch in the paint and HOX is generating hydrogen peroxide from the generated glucose.

EXAMPLE 9 – Temperature Activity

Hexose oxidase (purified HOX) was evaluated with regard to activity as a function of temperature and compared with a commercial glucose oxidase (Amano 081443/00018)

Procedure:

Sample: The enzyme sample is dissolved in water and desalted on a PD10 column using 20mM phosphate buffer pH 6.3 and diluted to 0.4 U/ml

To an Elisa well is added:

150 µl 100 mM glucose in 100 mM phosphate buffer, pH 6.3

120 µl 100 mM phosphate buffer, pH 6.3

10 µl o-dianisidine (3 mg/ml in water)

10 µl peroxidase (0,10 mg/ml in 100 mM phosphate buffer, pH 6.3)

10 µl Sample

Assayed 10 min at 30 °C and measured at 405 nm.

Results

The results from the activity measurement as a function of temperature is shown in table 4 and fig. 1

Table 4

Temperature,	Hexose oxidase	Commercial glucose oxidase
°C	Relative activity, %	Relative activity, %
10	70	67
25	98	67
27.5	100	72
32.5	98	78
37.5	94	78
42	83	85
45	81	100

50	59	94

The results from the activity versus temperature clearly illustrate a difference in the activity profile.

Hexose oxidase has its optimum temperature between 25-35°C, which is almost coinciding with the optimum for the maximum fouling temperature. On the contrary it is seen that GOX has an optimum temperature at 50°C which is far above the temperatures that can ever be reached at sea.

All publications mentioned in the above specification are herein incorporated by reference. Various modifications and variations of the described methods and system of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention which are obvious to those skilled in chemistry or related fields are intended to be within the scope of the following claims.

CLAIMS

1. An anti-fouling composition comprising

(i) a surface coating material;

(ii) an enzyme obtained or obtainable from a marine organism; and

(iii) (a) a substrate for the enzyme; and/or

(b) a precursor enzyme and a precursor substrate, wherein the precursor enzyme and the precursor substrate are selected such that a substrate for the enzyme is generatable by action of the precursor enzyme on the precursor substrate;

wherein the enzyme and the substrate are selected such that an anti-foulant compound is generatable by action of the enzyme on the substrate.

2. A composition according to claim 1 wherein the enzyme is obtained or is obtainable from a marine alga.

3. A composition according to claim 1 or 2 wherein the enzyme is obtained or is obtainable from *Chondrus cripus*.

4. A composition according to any one of the preceding claims wherein the enzyme is hexose oxidase.

5. A composition according to claim 4 wherein the hexose oxidase enzyme comprises the amino acid sequence set out in SEQ ID No 1 or a variant, homologue, derivative or fragment thereof.

6. A composition according to any one of the preceding claims wherein the substrate is a sugar.

7. A composition according to claim 6 wherein the sugar is glucose.

8. A composition according to any one of the preceding claims wherein the composition comprises a precursor enzyme and a precursor substrate, wherein the precursor enzyme and the precursor substrate are selected such that the precursor

substrate generates a substrate for the enzyme by action of the precursor enzyme on the precursor substrate.

9. A composition according to claim 8 wherein the precursor enzyme is amyloglucosidase.

10. A composition according to claim 8 or 9 wherein the precursor substrate is starch.

11. A composition according to any one of the preceding claims wherein the composition further comprises a binder to immobilise at least one of the constituents of the composition, preferably to immobilise the enzyme.

12. A coating consisting of a composition according to any one of the preceding claims.

13. A coating according to claim 12 formulated for treatment of a surface selected from outdoor wood work, external surface of a central heating system, and a hull of a marine vessel.

14 A marine anti-foul consisting of a composition according to any one of the preceding claims.

15. A marine anti-foul according to claim 14 wherein the anti-foulant is self-polishable.

16. A method for releasing an anti-fouling compound from a surface coating, which method comprises incorporating in a surface coating

(i) an enzyme obtained or obtainable from a marine organism; and

(ii) (a) a substrate for the enzyme; and/or

(b) a precursor enzyme and a precursor substrate, wherein a substrate for the enzyme is generated by action of the precursor enzyme on the precursor substrate;

* C L M S *