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(54) Titre : UTILISATION D'HYDROPHOBINE COMME STABILISATEUR DE PHASES
(54) Title: USE OF HYDROPHOBIN AS A PHASE STABILISER

(57) **Abrégé/Abstract:**

The invention relates to hydrophobins that are especially suitable for stabilising two-phase liquid systems.



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(54) Title: USE OF HYDROPHOBIN AS A PHASE STABILISER

(54) Bezeichnung: VERWENDUNG VON HYDROPHOBIN ALS PHASEN-STABILISATOR

(57) Abstract: The invention relates to hydrophobins that are especially suitable for stabilising two-phase liquid systems.

(57) Zusammenfassung: Hydrophobine sind in besonderer Weise geeignet, zweiphasige flüssige Systeme zu stabilisieren.



WO 2006/103252 A3

Use of hydrophobin as a phase stabiliser

5 Description

The present invention relates to the use of hydrophobin and/or one of its derivatives for stabilizing phases in compositions comprising at least two liquid phases, especially oil and water.

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Hydrophobins are small proteins of from about 100 to 150 amino acids, which are characteristic of filamentous fungi, for example of *Schizophyllum commune*. They generally have 8 cysteine units.

15 Hydrophobins have a marked affinity for interfaces and are therefore suitable for coating surfaces, for example in order to alter the properties of the interfaces by forming amphiphatic membranes. For example, Teflon can be coated by means of hydrophobins to obtain a hydrophilic surface.

20 Hydrophobins can be isolated from natural sources. Moreover, production processes for hydrophobins and their derivatives are known. For example, German patent application DE 10 2005 007 480 discloses a production process for hydrophobins and derivatives thereof.

25 The prior art has already proposed the use of hydrophobins for various applications.

WO 96/41882 proposes the use of hydrophobins as emulsifiers, thickeners, surface-active substances, for hydrophilizing hydrophobic surfaces, for improving the water resistance of hydrophilic substrates, for producing oil-in-water emulsions or water-in-oil emulsions. Also proposed are pharmaceutical applications, such as the production of ointments or creams, and cosmetic applications, such as skin protection or the production of shampoos or hair rinses. WO 96/41882 additionally describes compositions, especially compositions for pharmaceutical applications, comprising hydrophobins.

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EP-A 1 252 516 discloses the coating of windows, contact lenses, biosensors, medical devices, vessels for performing tests or for storage, ships' hulls, solid particles or frames or chassis of passenger vehicles with a hydrophobin-comprising solution at a temperature of from 30 to 80°C.

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WO 03/53383 describes the use of hydrophobin for treating keratin materials in cosmetic applications.

WO 03/10331 discloses that hydrophobins have surface-active properties. For instance, a hydrophobin-coated sensor is disclosed, for example a test electrode to which further substances, for example electroactive substances, antibodies or enzymes, are bonded non-covalently.

WO 2004/000880 presents the coating of surfaces with hydrophobin or hydrophobin-like substances.

10

WO 01/74864, which relates to hydrophobin-like proteins, also states that they can be used to stabilize dispersions and emulsions.

The use of proteins for phase separation is also known in principle.

15

For instance, EP-A 05 016 962 describes the use of proteins to improve phase separation of, for example, oil/water or fuel/water mixtures. It is known to those skilled in the art that amphiphilic molecules, depending on the use concentration and surrounding medium, can have either stabilizing or destabilizing effects on phase interfaces.

20

GB 195,876 discloses a process for breaking water-in-oil emulsions using colloids. The colloids mentioned are, by way of example, proteins such as gelatins, casein, albumin or polysaccharides such as gum arabic or gum tragacanth.

25

JP-A 11-169177 describes the use of proteins with lipase activity for breaking emulsions.

WO 01/60916 discloses the use of surfactant-free mixtures of at least one water-soluble protein, at least one water-soluble polysaccharide and at least one water-soluble polymer, for example polyethylene oxide, for various uses, also including for the demulsification of crude oil.

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However, none of the documents cited discloses the use of hydrophobins for preventing re-emulsification.

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The use of proteins has the general advantage that they are naturally occurring substances which are biodegradable and hence do not lead to lasting pollution of the environment.

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In many applications on the industrial scale, for example in the separation of crude oil-water emulsions, one important factor is very rapid phase separation and another is the avoidance or prevention of a re-emulsification of the phases. It was an object of the invention to provide an improved process for stabilizing the phases by use of proteins.

5 The present invention is also directed to a process for stabilizing liquid phases in a composition comprising at least two liquid phases, comprising the addition of at least one hydrophobin in an amount of from 0.001 to 100 ppm to the composition.

10 The present invention is also directed to a formulation comprising at least one organic phase consisting of fuels and/or crude oils and an aqueous phase comprising at least one hydrophobin in an amount of from 0.001 to 80 ppm based on the overall formulation.

15 According to the invention, this object is achieved by the use of at least one hydrophobin in compositions comprising at least two liquid phases, especially oil and water.

In accordance with the invention, the hydrophobin can in principle be used in any amount, provided that it is ensured that the phase stabilization in the compositions comprising at least two liquid phases is improved.

20 In the context of the present invention, "improvement in the phase stabilization" is understood to mean that the re-emulsification of two liquid phases in the case of addition of a substance to a mixture proceeds more slowly than in the same mixture without the addition of the substance, or that the addition of the substance prevents the re-emulsification of two liquid phases.

25 In the context of the present invention, a hydrophobin is also understood to mean derivatives thereof or modified hydrophobins. Modified or derivatized hydrophobins may, for example, be hydrophobin fusion proteins or proteins which have an amino acid sequence which has at least 60%, for example at least 70%, in particular at least 80%, more preferably at least 90%, especially preferably at least 95% identity with the sequence of a hydrophobin, and which also satisfy the biological properties of a

3a

5 hydrophobin to an extent of 50%, for example to an extent of 60%, in particular to an extent of 70%, more preferably to an extent of 80%, especially the property that the surface properties are altered by coating with these proteins such that the contact angle of a water droplet before and after the coating of a glass surface with the protein is increased by at least 20°, preferably by at least 25°, in particular by at least 30°.

10 It has been found that, surprisingly, hydrophobins or derivatives thereof reduce or prevent the new formation of emulsions after phase separation has been completed. This is especially advantageous when there is prolonged existence of two phases alongside one another or the occurrence of new emulsions is to be prevented. In this context, even small amounts of hydrophobin are extremely effective.

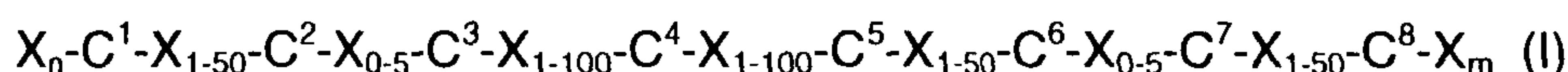
For the definition of hydrophobins, what is crucial is the structural specificity and not the sequence specificity of the hydrophobins. The amino acid sequence of the natural hydrophobins is very diverse, but they all have a highly characteristic pattern of 8 conserved cysteine residues. These residues form four intramolecular disulfide bridges.

The N terminus and C terminus are variable over a relatively wide range. It is possible here to add on fusion partner proteins having a length of from 10 to 500 amino acids by means of molecular biology techniques known to those skilled in the art.

- 5 Moreover, hydrophobins and derivatives thereof are understood in the context of the present invention to mean proteins with a similar structure and functional equivalence.

In the context of the present invention, the term "hydrophobins" should be understood hereinafter to mean polypeptides of the general structural formula (I)

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where X may be any of the 20 naturally occurring amino acids (Phe, Leu, Ser, Tyr, Cys, Trp, Pro, His, Gln, Arg, Ile Met, Thr, Asn, Lys, Val, Ala, Asp, Glu, Gly). In the formula, X may be the same or different in each case. The indices beside X are each the number of amino acids, C is cysteine, alanine, serine, glycine, methionine or threonine, where at least four of the residues designated with C are cysteine, and the indices n and m are each independently natural numbers between 0 and 500, preferably between 15 and 300.

20

The polypeptides of the formula (I) are also characterized by the property that, at room temperature, after coating a glass surface, they bring about an increase in the contact angle of a water droplet of at least 20°, preferably at least 25° and more preferably 30°, compared in each case with the contact angle of an equally large water droplet with the uncoated glass surface.

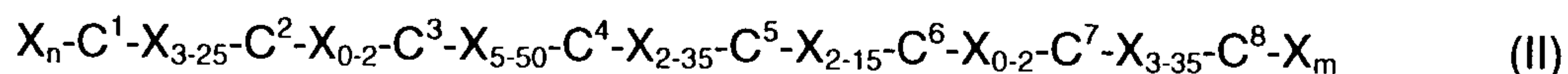
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- The amino acids designated with C¹ to C⁸ are preferably cysteines; however, they may also be replaced by other amino acids with similar space-filling, preferably by alanine, serine, threonine, methionine or glycine. However, at least four, preferably at least 5, more preferably at least 6 and in particular at least 7 of positions C¹ to C⁸ should consist of cysteines. In the inventive proteins, cysteines may either be present in reduced form or form disulfide bridges with one another. Particular preference is given to the intramolecular formation of C-C bridges, especially that with at least one intramolecular disulfide bridge, preferably 2, more preferably 3 and most preferably 4 intramolecular disulfide bridges. In the case of the above-described exchange of cysteines for amino acids with similar space-filling, such C positions are advantageously exchanged in pairs which can form intramolecular disulfide bridges with one another.

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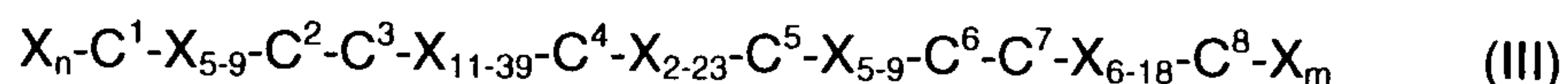
If cysteines, serines, alanines, glycines, methionines or threonines are also used in the positions designated with X, the numbering of the individual C positions in the general formulae can change correspondingly.

- 5 Preference is given to using hydrophobins of the general formula (II)



- 10 to perform the present invention, where X, C and the indices beside X and C are each as defined above, the indices n and m are each numbers between 0 and 300, and the proteins additionally feature the above-illustrated change in contact angle, and, furthermore, at least 6 of the residues designated with C are cysteine. More preferably, all C residues are cysteine.

- 15 Particular preference is given to using hydrophobins of the general formula (III)



- 20 where X, C and the indices besides X are each as defined above, the indices n and m are each numbers between 0 and 200, and the proteins additionally feature the above-illustrated change in contact angle, and at least 6 of the residues designated with C are cysteine. More preferably, all C residues are cysteine.

- 25 The X_n and X_m residues may be peptide sequences which naturally are also joined to a hydrophobin. However, one or both residues may also be peptide sequences which are naturally not joined to a hydrophobin. This is also understood to mean those X_n and/or X_m residues in which a peptide sequence which occurs naturally in a hydrophobin is lengthened by a peptide sequence which does not occur naturally in a hydrophobin.

- 30 If X_n and/or X_m are peptide sequences which are not naturally bonded into hydrophobins, such sequences are generally at least 20, preferably at least 35, more preferably at least 50 and most preferably at least 100 amino acids in length. Such a residue which is not joined naturally to a hydrophobin will also be referred to hereinafter as a fusion partner. This is intended to express that the proteins may consist of at least
35 one hydrophobin moiety and a fusion partner moiety which do not occur together in this form in nature.

- The fusion partner moiety may be selected from a multitude of proteins. It is also possible for a plurality of fusion partners to be joined to one hydrophobin moiety, for
40 example on the amino terminus (X_n) and on the carboxyl terminus (X_m) of the

hydrophobin moiety. However, it is also possible, for example, for two fusion partners to be joined to one position (X_n or X_m) of the inventive protein.

Particularly suitable fusion partners are proteins which naturally occur in microorganisms, especially in *E. coli* or *Bacillus subtilis*. Examples of such fusion partners are the sequences yaad (SEQ ID NO: 15 and 16), yaae (SEQ ID NO: 17 and 18), and thioredoxin. Also very suitable are fragments or derivatives of these sequences which comprise only some, for example from 70 to 99%, preferentially from 5 to 50% and more preferably from 10 to 40% of the sequences mentioned, or in which individual amino acids or nucleotides have been changed compared to the sequence mentioned, in which case the percentages are each based on the number of amino acids.

In a further preferred embodiment, the fusion hydrophobin, as well as the fusion partner as an X_n or X_m group, also has a so-called affinity domain (affinity tag / affinity tail). In a manner known in principle, this comprises anchor groups which can interact with particular complementary groups and can serve for easier workup and purification of the proteins. Examples of such affinity domains comprise $(\text{His})_k$, $(\text{Arg})_k$, $(\text{Asp})_k$, $(\text{Phe})_k$ or $(\text{Cys})_k$ groups, where k is generally a natural number from 1 to 10. It may preferably be a $(\text{His})_k$ group, where k is from 4 to 6.

The proteins used in accordance with the invention as hydrophobins or derivatives thereof may also be modified in their polypeptide sequence, for example by glycosylation, acetylation or else by chemical crosslinking, for example with glutaraldehyde.

One property of the hydrophobins or derivatives thereof used in accordance with the invention is the change in surface properties when the surfaces are coated with the proteins. The change in the surface properties can be determined experimentally, for example, by measuring the contact angle of a water droplet before and after the coating of the surface with the protein and determining the difference of the two measurements.

The performance of contact angle measurements is known in principle to those skilled in the art. The measurements are based on room temperature and water droplets of 5 μl and the use of glass plates as substrates. The exact experimental conditions for an example of a suitable method for measuring the contact angle are given in the experimental section. Under the conditions mentioned there, the fusion proteins used in accordance with the invention have the property of increasing the contact angle by at least 20° , preferably at least 25° , more preferably at least 30° , compared in each case with the contact angle of an equally large water droplet with the uncoated glass surface.

Particularly preferred hydrophobins for performing the present invention are the hydrophobins of the dewA, rodA, hypA, hypB, sc3, basf1, basf2 type, which are characterized structurally in the sequence listing which follows. They may also only be parts or derivatives thereof. It is also possible for a plurality of hydrophobin moieties, preferably 2 or 3, of identical or different structure to be bonded to one another and to be bonded to a corresponding suitable polypeptide sequence which is not bonded to a hydrophobin in nature.

Also particularly suitable in accordance with the invention are the fusion proteins yaad-Xa-dewA-his (SEQ ID NO: 20), yaad-Xa-rodA-his (SEQ ID NO: 22) or yaad-Xa-basf1-his (SEQ ID NO: 24), with the polypeptide sequences specified in brackets and the nucleic acid sequences which code therefor, especially the sequences according to SEQ ID NO: 19, 21, 23. Proteins which, proceeding from the polypeptide sequences shown in SEQ ID NO. 20, 22 or 24, arise through exchange, insertion or deletion of from at least one up to 10, preferably 5 amino acids, more preferably 5% of all amino acids, and which still have the biological property of the starting proteins to an extent of at least 50%, are also particularly preferred embodiments. A biological property of the proteins is understood here to mean the change in the contact angle by at least 20°, which has already been described.

Derivatives particularly suitable for performing the invention are residues derived from yaad-Xa-dewA-his (SEQ ID NO: 20), yaad-Xa-rodA-his (SEQ ID NO: 22) or yaad-Xa-basf1-his (SEQ ID NO: 24) by truncating the yaad fusion partner. Instead of the complete yaad fusion partner (SEQ ID NO: 16) with 294 amino acids, it may be advantageous to use a truncated yaad residue. The truncated residue should, though, comprise at least 20, more preferably at least 35 amino acids. For example, a truncated radical having from 20 to 293, preferably from 25 to 250, more preferably from 35 to 150 and, for example, from 35 to 100 amino acids may be used.

A cleavage site between the hydrophobin and the fusion partner or the fusion partners can be utilized to release the pure hydrophobin in underivatized form (for example by BrCN cleavage at methionin, factor Xa cleavage, enterokinase cleavage, thrombin cleavage, TEV cleavage, etc.).

It is also possible to generate fusion proteins in succession from one fusion partner, for example yaad or yaae, and a plurality of hydrophobins, even of different sequence, for example DewA-RodA or Sc3-DewA, Sc3-RodA. It is equally possible to use hydrophobin fragments (for example N- or C-terminal truncations) or mutein which have up to 70% homology. The optimal constructs are in each case selected in relation to the particular use, i.e. the liquid phase to be separated.

The hydrophobins used in accordance with the invention or present in the inventive compositions can be prepared chemically by known methods of peptide synthesis, for example by Merrifield solid-phase synthesis.

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Naturally occurring hydrophobins can be isolated from natural sources by means of suitable methods. Reference is made by way of example to Wösten et. al., Eur. J Cell Bio. 63, 122-129 (1994) or WO 96/41882.

- 10 Fusion proteins can be prepared preferably by genetic engineering methods, in which one nucleic acid sequence, especially DNA sequence, encoding the fusion partner and one encoding the hydrophobin moiety are combined in such a way that the desired protein is generated in a host organism as a result of gene expression of the combined nucleic acid sequence. Such a preparation process is disclosed, for example, in
- 15 German patent application DE 102005007480.4.

- Suitable host organisms (production organisms) for the preparation method mentioned may be prokaryotes (including the Archaea) or eukaryotes, particularly bacteria including halobacteria and methanococcia, fungi, insect cells, plant cells and
- 20 mammalian cells, more preferably *Escherichia coli*, *Bacillus subtilis*, *Bacillus megaterium*, *Aspergillus oryzae*, *Aspergillus nidulans*, *Aspergillus niger*, *Pichia pastoris*, *Pseudomonas spec.*, lactobacilli, *Hansenula polymorpha*, *Trichoderma reesei*, SF9 (or related cells), among others.

- 25 The studies also relate to the use of expression constructs comprising, under the genetic control of regulatory nucleic acid sequences, a nucleic acid sequence which encodes a polypeptide used in accordance with the invention, and also vectors comprising at least one of these expression constructs.

- 30 Constructs used preferably comprise, 5' upstream from the particular encoding sequence, a promoter and, 3'downstream, a terminator sequence and if appropriate further customary regulatory elements, in each case linked operatively to the encoding sequence.

- 35 In the context of the present invention, an "operative linkage" is understood to mean the sequential arrangement of promoter, encoding sequence, terminator and if appropriate further regulatory elements such that each of the regulatory elements can fulfill its function as intended in the expression of the encoding sequence.

- 40 Examples of operatively linkable sequences are targeting sequences, and also enhancers, polyadenylation signals and the like. Further regulatory elements comprise

selectable markers, amplification signals, replication origins and the like. Suitable regulatory sequences are, for example, described in Goeddel, Gene Expression Technology : Methods in Enzymology 185, Academic Press, San Diego, CA (1990).

- 5 In addition to these regulation sequences, the natural regulation of these sequences may still be present upstream of the actual structural genes and, if appropriate, have been genetically modified so as to switch off the natural regulation and increase the expression of the genes.
- 10 A preferred nucleic acid construct also advantageously comprises one or more so-called "enhancer" sequences, joined functionally to the promoter, which enable increased expression of the nucleic acid sequence. Also at the 3' end of the DNA sequences, it is possible for additional advantageous sequences to be inserted, such as further regulatory elements or terminators.
- 15 The nucleic acids may be present in the construct in one or more copies. It is also possible for further markers such as antibiotic resistances or genes which complement auxotrophies to be present in the construct, if appropriate for selection on the construct.
- 20 Advantageous regulation sequences for the preparation are present, for example, in promoters such as the cos, tac, trp, tet, trp-tet, lpp, lac, lpp-lac, lacIq-T7, T5, T3, gal, trc, ara, rhaP(rhaPBAD) SP6, lambda-PR or imlambda-P promoter, which advantageously find use in Gram-negative bacteria. Further advantageous regulation sequences are present, for example, in the Gram-positive promoters amy and SP02,
- 25 and in the yeast or fungal promoters ADC1, MFalpha, AC, P-60, CYC1, GAPDH, TEF, rp28, ADH.

It is also possible to use synthetic promoters for the regulation.

- 30 For expression in a host organism, the nucleic acid construct is advantageously inserted into a vector, for example a plasmid or a phage which enables optimal expression of the genes in the host. Apart from plasmids and phages, vectors are also understood to mean all other vectors known to those skilled in the art, for example viruses such as SV40, CMV, baculovirus and adenovirus, transposons, IS elements,
- 35 phasmids, cosmids, and linear or circular DNA, and also the Agrobacterium system.

- These vectors can be replicated autonomously in the host organism or replicated chromosomally. Suitable plasmids are, for example, in E. coli pLG338, pACYC184, pBR322, pUC18, pUC19, pKC30, pRep4, pHS1, pKK223-3, pDHE19.2, pHS2,
- 40 pPLc236, pMBL24, pLG200, pUR290, pIN-III"3-B1, tgt11 or pBdCl, in Streptomyces pIJ101, pIJ364, pIJ702 or pIJ361, in Bacillus pUB110, pC194 or pBD214, in

Corynebacterium pSA77 or pAJ667, in fungi pALS1, pIL2 or pBB116, in yeasts 2alpha, pAG-1, YEp6, YEp13 or pEMBLYe23 or in plants pLGV23, pGHIac+, pBIN19, pAK2004 or pDH51. The plasmids mentioned constitute a small selection of the possible plasmids. Further plasmids are known to those skilled in the art and can be
5 taken, for example, from the book Cloning Vectors (Eds. Pouwels P. H. et al. Elsevier, Amsterdam-New York-Oxford, 1985, ISBN 0 444 904018).

Advantageously, the nucleic acid construct, for the expression of the further genes present, additionally also comprises 3'- and/or 5'-terminal regulatory sequences for enhancing the expression, which are selected for optimal expression depending upon
10 the host organism and gene or genes selected.

These regulatory sequences are intended to enable the controlled expression of the genes and of the protein expression. Depending on the host organism, this can mean, for example, that the gene is expressed or overexpressed only after induction, or that it
15 is expressed and/or overexpressed immediately.

The regulatory sequences or factors can preferably positively influence and thus increase the gene expression of the genes introduced. Thus, an amplification of the regulatory elements can advantageously be effected at the transcription level by using
20 strong transcription signals such as promoters and/or enhancers. In addition, it is also possible to enhance the translation by, for example, improving the stability of the mRNA.

In a further embodiment of the vector, the vector comprising the nucleic acid construct or the nucleic acid can also be introduced into the microorganisms advantageously in the form of a linear DNA and be integrated into the genome of the host organism by means of heterologous or homologous recombination. This linear DNA can consist of a linearized vector such as a plasmid or only of the nucleic acid construct or the nucleic acid.
25

For an optimal expression of heterologous genes in organisms, it is advantageous to alter the nucleic acid sequences in accordance with the specific "codon usage" used in the organism. The "codon usage" can be determined easily with reference to computer evaluations of other, known genes of the organism in question.
30

An expression cassette is prepared by fusion of a suitable promoter with a suitable coding nucleotide sequence and a terminator signal or polyadenylation signal. To this end, common recombination and cloning techniques are used, as described, for example, in T. Maniatis, E. F. Fritsch and J. Sambrook, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1989) and in T. J. Silhavy, M. L. Berman and L. W. Enquist, Experiments with Gene Fusions,
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Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1984) and in Ausubel, F. M. et al., Current Protocols in Molecular Biology, Greene Publishing Assoc. and Wiley Interscience (1987).

- 5 For expression in a suitable host organism, the recombinant nucleic acid construct or gene construct is advantageously inserted into a host-specific vector which enables an optimal expression of the genes in the host. Vectors are well known to those skilled in the art and can be taken, for example, from "Cloning Vectors" (Pouwels P. H. et al., eds., Elsevier, Amsterdam-New York-Oxford, 1985).

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- With the aid of vectors, it is possible to prepare recombinant microorganisms which have been transformed, for example, with at least one vector and can be used for the production of the hydrophobins or derivatives thereof used in accordance with the invention. Advantageously, the above-described recombinant constructs are introduced
15 into a suitable host system and expressed. Preference is given to using the cloning and transfection methods familiar to those skilled in the art, for example coprecipitation, protoplast fusion, electroporation, retroviral transfection and the like, in order to bring about the expression of the nucleic acids mentioned in the particular expression system. Suitable systems are described, for example, in Current Protocols in Molecular
20 Biology, F. Ausubel et al., ed., Wiley Interscience, New York 1997, or Sambrook et al. Molecular Cloning: A Laboratory Manual, 2nd edition, Cold Spring Harbor Laboratory, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989.

- It is also possible to prepare homologously recombined microorganisms. To this end, a
25 vector is prepared which comprises at least a section of a gene to be used or a coding sequence, in which, if appropriate, at least one amino acid deletion, addition or substitution has been introduced in order to change, for example to functionally disrupt, the sequence ("knockout" vector). The sequence introduced may, for example, also be a homolog from a related microorganism or be derived from a mammalian, yeast or
30 insect source. The vector used for the homologous recombination may alternatively be configured such that the endogenous gene in the case of homologous recombination has been mutated or altered in another way, but still encodes the functional protein (for example, the upstream regulatory region can be changed such that the expression of the endogenous protein is changed). The changed section of the gene used in
35 accordance with the invention is in the homologous recombination vector. The construction of suitable vectors for homologous recombination is described, for example, in Thomas, K. R. and Capecchi, M. R. (1987) Cell 51: 503.

- In principle, all prokaryotic or eukaryotic organisms are useful as recombinant host
40 organisms for such nucleic acids or such nucleic acid constructs. Advantageously, the host organisms used are microorganisms such as bacteria, fungi or yeasts.

Advantageously, Gram-positive or Gram-negative bacteria are used, preferably bacteria from the families Enterobacteriaceae, Pseudomonadaceae, Rhizobiaceae, Streptomycetaceae or Nocardiaceae, more preferably bacteria of the genera Escherichia, Pseudomonas, Streptomyces, Nocardia, Burkholderia, Salmonella, Agrobacterium or Rhodococcus.

The organisms used in the above-described preparation processes for fusion proteins are, depending on the host organism, grown or cultured in a manner known to those skilled in the art. Microorganisms are generally grown in a liquid medium which comprises a carbon source, usually in the form of sugars, a nitrogen source, usually in the form of organic nitrogen sources such as yeast extract or salts such as ammonium sulfate, trace elements such as iron, manganese and magnesium salts, and also, if appropriate, vitamins, at temperatures between 0 and 100°C, preferably between 10 to 60°C, with oxygen sparging. The pH of the nutrient liquid can be kept at a fixed value, i.e. is regulated or not during the growth. The growth can be effected batchwise, semibatchwise or continuously. Nutrients can be introduced at the start of the fermentation or be replenished semicontinuously or continuously. The enzymes can be isolated from the organisms by the process described in the examples or be used for the reaction as a crude extract.

The hydrophobins used in accordance with the invention, or functional, biologically active fragments thereof, can be prepared by means of a process for recombinant preparation, in which a polypeptide-producing microorganism is cultivated, the expression of the proteins is induced if appropriate and they are isolated from the culture. The proteins can also be produced in this way on an industrial scale if this is desired. The recombinant microorganism can be cultivated and fermented by known processes. Bacteria can be propagated, for example, in TB or LB medium and at a temperature of from 20 to 40°C and a pH of from 6 to 9. Suitable cultivation conditions are described specifically, for example, in T. Maniatis, E. F. Fritsch and J. Sambrook, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1989).

If the proteins are not secreted into the culture medium, the cells are then disrupted and the product is obtained from the lysate by known protein isolation processes. As desired, the cells can be disrupted by high-frequency ultrasound, by high pressure, for example in a French pressure cell, by osmolysis, by the action of detergents, lytic enzymes or organic solvents, by homogenizers or by combination of a plurality of the processes listed.

The proteins can be purified by known chromatographic processes, such as molecular sieve chromatography (gel filtration) such as Q Sepharose chromatography, ion

exchange chromatography and hydrophobic chromatography, and also with other customary processes such as ultrafiltration, crystallization, salting-out, dialysis and native gel electrophoresis. Suitable processes are described, for example, in Cooper, F. G., Biochemische Arbeitsmethoden [Biochemical Techniques], Verlag Walter de Gruyter, Berlin, New York, or in Scopes, R., Protein Purification, Springer Verlag, New York, Heidelberg, Berlin.

It may be particularly advantageous to ease the isolation and purification of the fusion hydrophobins by providing them with specific anchor groups which can bind to corresponding complementary groups on solid supports, especially suitable polymers. Such solid supports may, for example, be used as a filling for chromatography columns, and the efficiency of the separation can generally be increased significantly in this manner. Such separation processes are also known as affinity chromatography. For the incorporation of the anchor groups, it is possible to use, in the preparation of the proteins, vector systems or oligonucleotides which extend the cDNA by particular nucleotide sequences and hence encode altered proteins or fusion proteins. For easier purification, modified proteins comprise so-called "tags" which function as anchors, for example the modification known as the hexa-histidine anchor. Fusion hydrophobins modified with histidine anchors can be purified chromatographically, for example, using nickel-Sepharose as the column filling. The fusion hydrophobin can subsequently be eluted again from the column by means of suitable agents for elution, for example an imidazole solution.

In a simplified purification process, it is possible to dispense with the chromatographic purification. To this end, the cells are first removed from the fermentation broth by means of a suitable method, for example by microfiltration or by centrifugation. Subsequently, the cells can be disrupted by means of suitable methods, for example by means of the methods already mentioned above, and the cell debris can be separated from the inclusion bodies. The latter can advantageously be effected by centrifugation. Finally, the inclusion bodies can be disrupted in a manner known in principle in order to release the fusion hydrophobins. This can be done, for example, by means of acids, bases, and/or detergents. The inclusion bodies with the fusion hydrophobins used in accordance with the invention can generally be dissolved completely even using 0.1 M NaOH within approx. 1 h. The purity of the fusion hydrophobins obtained by this simplified process is generally from 60 to 80% by weight based on the amount of all proteins. The solutions obtained by the simplified purification process described can be used to perform this invention without further purification.

The hydrophobins prepared as described may be used either directly as fusion proteins or, after detachment and removal of the fusion partner, as "pure" hydrophobins.

When a removal of the fusion partner is intended, it is advisable to incorporate a potential cleavage site (specific recognition site for proteases) into the fusion protein between hydrophobin moiety and fusion partner moiety. Suitable cleavage sites are especially those peptide sequences which otherwise occur neither in the hydrophobin moiety nor in the fusion partner moiety, which can be determined easily with bioinformatic tools. Particularly suitable examples are BrCN cleavage at methionine, or protease-mediated cleavage with factor Xa cleavage, enterokinase cleavage, thrombin cleavage, TEV (tobacco etch virus protease) cleavage.

10 According to the invention, the hydrophobins or derivatives thereof can be used to stabilize the already separated phases in compositions comprising at least two liquid phases. The compositions may in principle be any compositions, provided that they have at least two liquid phases.

15 In particular, the compositions may also be compositions which, before the addition of the at least one hydrophobin or derivative thereof, were present in the form of an emulsion, were then separated into two phases in an prolonged process (preferably more than 1 minute, especially more than 5 minutes) and were only then admixed with hydrophobin.

20

In the context of the present invention, the composition may, as well as the at least two liquid phases, in principle also comprise further phases.

25 The at least two liquid phases are two liquid phases of different density, preferably an oil and water, two organic solutions of different density, a fuel and water or a solvent and water. In the context of the present invention, aqueous solutions are solutions which comprise water, optionally in combination with a further solvent. Each of the liquid phases may, in the context of the present invention, comprise further substances.

30 According to the invention, an oil is preferably a crude oil.

Suitable solvents are all liquids which form biphasic mixtures with water, especially organic solvents, for example ethers, aromatic compounds such as toluene or benzene, alcohols, alkanes, alkenes, cycloalkanes, cycloalkenes, esters, ketones, naphthenes or halogenated hydrocarbons.

35

In a further embodiment, the present invention therefore relates to a use as described above of at least one hydrophobin or of at least one derivative thereof, said composition comprising oil, preferably crude oil, and water, or else fuel and water.

40

In the context of the present invention, the composition may also comprise further phases, for example a solid or liquid phase, especially a solid phase.

5 The hydrophobins or derivatives thereof may be used for all uses known to those skilled in the art. In particular, in the context of the present invention, mention should be made of the use as a phase stabilizer in gasoline fuel/water mixtures, in other fuel/water mixtures, crude oil and water phases in crude oil extraction or crude oil transport, and the desalting of crude oil by extraction of crude oil with water and subsequent further conduction of the resulting phases.

10

It is possible to break emulsions by adding demulsifiers. For example, extracted crude oil is generally present at a relatively stable water-in-oil emulsion which, according to the type of deposit, may comprise up to 90% by weight of water. In the workup and purification of the crude oil, after the removal of a majority of the water, a crude oil
15 which still comprises from approx. 2 to 3% by weight of water is obtained. This forms a stable emulsion with the oil, which cannot be removed completely even by centrifuging and adding conventional demulsifiers. This is problematic in that the water firstly has a high salt content and hence corrosive action, and the residual water secondly increases the volume to be transported and to be stored, which leads to increased
20 costs. It has been found that hydrophobins or derivatives thereof can be used in order to improve the phase separation in these compositions. A very rapid separation is achieved.

In this case, the demulsifier has to be adjusted to the type of emulsified oils and fats
25 and to any emulsifiers and surfactants present in order to achieve an optimal action. The breaking of emulsions can additionally be promoted by an elevated temperature, for example a temperature of from 0 to 100°C, for example from 10 to 80°C, especially from 20 to 60°C.

30 A further inventive use is phase stabilization in oil-in-water or water-in-oil mixtures, for example biphasic systems which have been used as cooling lubricants and should be recycled. Water/oil mixtures are also obtained, for example, on ships as bilge water. In this case, the separation of emulsions and maintenance of the separated phases is necessary in order to be able to reliably remove the water.

35

The amount of the hydrophobin or derivative used may vary within wide ranges, the amount advantageously being adjusted to the composition itself and any further components present in the composition.

40 When, for example, the composition comprises substances which delay or worsen phase separation of the at least two liquid phases, for example surfactants or

emulsifiers, a larger amount of a hydrophobin or of a derivative is advantageously used.

5 Since oils, especially crude oils, consist of a mixture of many chemical compounds, it is necessary, owing to the different chemical composition of the oil, the water and salt contents and the specific conditions of the emulsion splitting, such as temperature, duration of the emulsion splitting, type of metered addition and interactions with further components of the mixture, to adjust the demulsifier to the specific conditions.

10 It has been found that, surprisingly, even small amounts of a hydrophobin or derivative thereof lead to an improvement in the phase stabilization.

The hydrophobin or derivative thereof may, in accordance with the invention, be used in any suitable amount. In general, the at least one hydrophobin or derivative thereof is
15 used in an amount of from 0.001 to 100 ppm based on the overall composition; preferably in an amount of from 0.001 to 80 ppm, more preferably from 0.001 to 20 ppm and most preferably from 0.01 to 10 ppm.

In the context of the present invention, the unit ppm means mg per kg.

20

In a further embodiment, the present invention therefore relates to a use as described above, wherein the hydrophobin or the at least one derivative thereof is used in an amount of from 0.001 to 100 ppm based on the overall composition. The concentration used is determined by the person skilled in the art depending on the type of phase
25 composition to be stabilized.

When the composition is a composition comprising fuels and water, the hydrophobin or derivative thereof is used generally in an amount of from 0.001 to 20 ppm, preferably from 0.005 to 2 ppm, especially from 0.01 to 1 ppm, more preferably from 0.05 to
30 1 ppm.

When the composition is a composition comprising crude oil and water, the hydrophobin or derivative thereof is used generally in an amount of from 0.01 to 100 ppm, preferably from 0.1 to 80 ppm, especially from 0.1 to 50 ppm, more preferably
35 from 0.1 to 20 ppm.

According to the invention, it is also possible that the composition, as well as the at least one hydrophobin or derivative thereof, comprises further compounds which improve the phase stabilization. The compounds may be all compounds known to
40 those skilled in the art for such applications. Examples of suitable further compounds for improving the phase stabilization are, especially for the application as emulsion

breakers in crude oil production, oxyalkylated phenol-formaldehyde resins, EO/PO block copolymers, crosslinked diepoxides, polyamides or alkoxylates thereof, salts of sulfonic acids, ethoxylated fatty amines, succinates and the compounds specified in DE 10 2005 006 030.7 for such uses.

5

In a further embodiment, the present invention relates to a use as described above, wherein at least one further compound which improves the phase stabilization is used as well as at least one hydrophobin or the at least one derivative thereof.

10 In a further aspect, the present invention also relates to a process for stabilizing liquid phases in a composition comprising at least two liquid phases, comprising the addition of at least one hydrophobin or at least one derivative thereof to the composition.

15 The composition may be a composition as described above, comprising at least two liquid phases, for example compositions comprising oil, preferably crude oil, and water, or else compositions comprising fuel and water.

20 The process according to the invention may comprise further steps, for example first the performance of a phase separation or the breakage of emulsions and subsequent addition of hydrophobins to the aqueous phase.

25 According to the invention, hydrophobins or derivatives thereof can be added to the aqueous phase of a 2-phase system, or else to formulations comprising fuels. On contact of the formulation with water, this enables stabilization of the phases or prevents re-emulsification.

30 It is equally advantageous to add hydrophobins or derivatives thereof to crude oil-water phases in order, for example, to prevent the reformation of emulsions in the course of transport.

The formulation comprising fuels may, in the context of the present invention, comprise further additives which are typically present in such formulations. Suitable additives are, for example, specified in WO 2004/087808.

35 In the context of the present invention, fuels are understood to mean light, medium or heavy heating oils.

In the context of the present invention, fuels are understood to mean gasoline fuels, diesel fuels or turbine fuels. The fuels are more preferably gasoline fuels.

40

The additives mentioned are used in the amounts which appear to be suitable to the person skilled in the art for the particular application.

5 The inventive formulations may additionally be combined with further customary components and additives. Mention should be made here, for example, of carrier oils without pronounced detergent action.

10 Suitable mineral carrier oils are fractions obtained in crude oil processing, such as brightstock or base oils having viscosities, for example, from the SN 500-2000 class; but also aromatic hydrocarbons, paraffinic hydrocarbons and alkoxyalkanols. Likewise suitable in accordance with the invention is a fraction which is known as "hydrocrack oil" and is obtained in the refining of mineral oil (vacuum distillate cut having a boiling range from about 360 to 500°C, obtainable from natural mineral oil catalytically hydrogenated and isomerized and also deparaffinized under high pressure). Likewise
15 suitable are mixtures of abovementioned mineral carrier oils.

Examples of synthetic carrier oils usable in accordance with the invention are selected from: polyolefins (polyalphaolefins or polyinternalolefins), (poly)esters, (poly)alkoxylates, polyethers, aliphatic polyetheramines, alkylphenol-started polyethers,
20 alkylphenol-started polyetheramines and carboxylic ester of long-chain alkanols.

Further suitable carrier oil systems are, for example, described in DE-A 38 26 608, DE-A 41 42 241, DE-A 43 09 074, EP-A 0 452 328 and EP-A 0 548 617, which are hereby explicitly incorporated by reference.

25 Further suitable synthetic carrier oils are alkoxyated alkylphenols, as described in DE-A 10 102 913.6.

30 The carrier oils mentioned are used in the amounts which appear to be suitable to the those skilled in the art for the particular application.

Further customary additives are corrosion inhibitors, for example based on ammonium salts of organic carboxylic acids, said ammonium salts tending to form films, or on heterocyclic aromatics in the case of nonferrous metal corrosion protection; antioxidants or stabilizers, for example based on amines such as p-phenylenediamine, dicyclohexylamine or derivatives thereof, or on phenols such as 2,4-di-tert-butylphenol or 3,5-di-tert-butyl-4-hydroxyphenylpropionic acid; further conventional demulsifiers; antistats; metallocenes such as ferrocene; methylcyclopentadienylmanganese tricarbonyl; lubricity improvers (lubricity additives) such as particular fatty acids,
40 alkenylsuccinic esters, bis(hydroxyalkyl) fatty amines, hydroxyacetamides or castor oil; and dyes (markers). If appropriate, amines are also added to lower the pH of the fuel.

The detergent additives mentioned with the polar moieties (a) to (i) are added to the fuel typically in an amount of 10 to 5000 ppm by weight, especially from 50 to 1000 ppm by weight. The other components and the additives mentioned are, if desired, added in amounts customary therefor.

5

According to the invention, suitable fuels are all fuels known to those skilled in the art, for example gasoline fuels, as described, for example, in Ullmann's Encyclopedia of Industrial Chemistry, 5th Ed. 1990, Volume A16, p. 719ff. According to the invention, suitable fuels are also diesel fuel, kerosene and jet fuel.

10

In particular, a gasoline fuel having an aromatics content of not more than 60% by volume, for example not more than 42% by volume, and a sulfur content of not more than 2000 ppm by weight, for example not more than 150 ppm by weight, is suitable.

15

The aromatics content of the gasoline fuel is, for example, from 10 to 50% by volume, for example from 30 to 42% by volume, especially from 32 to 40% by volume. The sulfur content of the gasoline fuel is, for example, from 2 to 500 ppm by weight, for example from 5 to 150 ppm by weight, or from 10 to 100 ppm by weight.

20

Moreover, a suitable gasoline fuel may have, for example, an olefin content up to 50% by volume, for example from 6 to 21% by volume, especially from 7 to 18% by volume; a benzene content of up to 5% by volume, for example from 0.5 to 1.0% by volume, especially from 0.6 to 0.9% by volume, and/or an oxygen content of up to 25% by weight, for example up to 10% by weight, or from 1.0 to 2.7% by weight, especially

25

from 1.2 to 2.0% by weight.

In particular, mention may be made by way of example of those gasoline fuels which simultaneously have an aromatics content of not more than 38% by volume, an olefin content of not more than 21% by volume, a sulfur content of not more than 50 ppm by weight, a benzene content of not more than 1.0% by volume and an oxygen content of

30

The content of alcohols and ethers in the gasoline fuel may vary over a wide range. Examples of typical maximum contents are 15% by volume for methanol, 65% by volume for ethanol, 20% by volume for isopropanol, 15% by volume for tert-butanol, 20% by volume for isobutanol and 30% by volume for ethers having 5 or more carbon atoms in the molecule.

35

The summer vapor pressure of a gasoline fuel suitable in accordance with the invention is typically not more 70 kPa, especially 60 kPa (in each case at 37°C).

40

The RON of the gasoline fuel is generally from 75 to 105. A customary range for the corresponding MON is from 65 to 95.

The specifications mentioned are determined by customary methods (DIN EN 228).

5

The invention is illustrated in detail hereinafter by examples.

Examples

Example 1

10 Preparations for the cloning of yaad-His₆ / yaaE-His₆

A polymerase chain reaction was carried out with the aid of the oligonucleotides Hal570 and Hal571 (Hal 572/ Hal 573). The template DNA used was genomic DNA of the bacterium *Bacillus subtilis*. The resulting PCR fragment comprised the coding
15 sequence of the *Bacillus subtilis* yaaD / yaaE gene, and an NcoI and BglII restriction cleavage site respectively at each end. The PCR fragment was purified and cut with the restriction endonucleases NcoI and BglII. This DNA fragment was used as an insert and cloned into the vector pQE60 from Qiagen, which had been linearized beforehand with the restriction endonucleases NcoI and BglII. The vectors pQE60YAAD#2 /
20 pQE60YaaE#5 thus formed may be used to express proteins consisting of YAAD::HIS₆ or YAAE::HIS₆.

Hal570: gcgcgcccattggctcaaacaggtactga

Hal571: gcagatctccagccgcgttcttgcatatc

25 Hal572: ggccatgggattaacaataggtgtactagg

Hal573: gcagatcttacaagtgccttttgcttatattcc

Example 2

Cloning of yaad hydrophobin DewA-His₆

30

A polymerase chain reaction was carried out with the aid of the oligonucleotides KaM 416 and KaM 417. The template DNA used was genomic DNA of the mold *Aspergillus nidulans*. The resulting PCR fragment comprised the coding sequence of the hydrophobin gene dewA and an N-terminal factor Xa proteinase cleavage site. The
35 PCR fragment was purified and cut with the restriction endonuclease BamHI. This DNA fragment was used as an insert and cloned into the vector pQE60YAAD#2 which had been linearized beforehand with the restriction endonuclease BglII.

The vector #508 thus formed can be used to express a fusion protein consisting of
40 YAAD::Xa::dewA::HIS₆.

KaM416: GCAGCCCATCAGGGATCCCTCAGCCTTGGTACCAGCGC

KaM417:CCCGTAGCTAGTGGATCCATTGAAGGCCGCATGAAGTTCTCCGTCTCCGC

Example 3

5 Cloning of yaad hydrophobin RodA-His₆

The plasmid #513 was cloned analogously to plasmid #508 using the oligonucleotides KaM 434 and KaM 435.

10 KaM434: GCTAAGCGGATCCATTGAAGGCCGCATGAAGTTCTCCATTGCTGC

KaM435: CCAATGGGGATCCGAGGATGGAGCCAAGGG

Example 4

15 Cloning of yaad hydrophobin BASF1-His₆

The plasmid #507 was cloned analogously to plasmid #508 using the oligonucleotides KaM 417 and KaM 418.

20 The template DNA used was a synthetic DNA sequence (hydrophobin BASF1) (see appendix, SEQ ID NO. 11 and 12).

KaM417:CCCGTAGCTAGTGGATCCATTGAAGGCCGCATGAAGTTCTCCGTCTCCGC

KaM418: CTGCCATTCAGGGGATCCCATATGGAGGAGGGAGACAG

25 Example 5

Cloning of yaad hydrophobin BASF2-His₆

The plasmid #506 was cloned analogously to plasmid #508 using the oligonucleotides KaM 417 and KaM 418.

30

The template DNA used was a synthetic DNA sequence (hydrophobin BASF2) (see appendix, SEQ ID NO. 13 and 14).

KaM417:CCCGTAGCTAGTGGATCCATTGAAGGCCGCATGAAGTTCTCCGTCTCCGC

35 KaM418: CTGCCATTCAGGGGATCCCATATGGAGGAGGGAGACAG

Example 6

Cloning of yaad hydrophobin SC3-His₆

40 The plasmid #526 was cloned analogously to plasmid #508 using the oligonucleotides KaM464 and KaM465.

The template DNA used was cDNA from Schyzophyllum commune (see appendix, SEQ ID NO. 9 and 10).

KaM464: CGTTAAGGATCCGAGGATGTTGATGGGGGTGC

5 KaM465: GCTAACAGATCTATGTTGCGCCCGTCTCCCCGTCGT

Example 7

Fermentation of the recombinant E. coli strain yaad hydrophobin DewA-His₆

10 Inoculation of 3 ml of LB liquid medium with a yaad hydrophobin DewA-His₆-expressing E. coli strain in 15 ml Greiner tubes. Inoculation for 8 h at 37°C on a shaker at 200 rpm. In each case two 1 l Erlenmeyer flasks with baffles and 250 ml of LB medium (+ 100 µg/ml of ampicillin) are inoculated with 1 ml in each case of the preliminary culture and incubated for 9 h at 37°C on a shaker at 180 rpm.

15 Inoculate 13.5 l of LB medium (+ 100 µg/ml of ampicillin) with 0.5 l of preliminary culture (OD_{600nm} 1:10, measured against H₂O) in a 20 l fermenter. At an OD_{60nm} of ~3.5, addition of 140 ml of 100 mM IPTG. After 3 h, cool fermenter to 10°C and centrifuge off fermentation broth. Use cell pellet for further purification.

20

Example 8

Purification of the recombinant hydrophobin fusion protein

100 g of cell pellet (100 – 500 mg of hydrophobin) are made up to total volume 200 ml with 50 mM sodium phosphate buffer, pH 7.5, and resuspended. The suspension is treated with an Ultraturrax type T25 (Janke and Kunkel; IKA-Labortechnik) for 10 minutes and subsequently incubated with 500 units of Benzonase (Merck, Darmstadt; order No. 1.01697.0001) at room temperature for 1 hour to degrade the nucleic acids. Before the cell disruption, filtration is effected with a glass cartridge (P1).

30 For cell disruption and for the scission of the remaining genomic DNA, two homogenizer cycles are carried out at 1500 bar (Microfluidizer M-110EH; Microfluidics Corp.). The homogenate is centrifuged (Sorvall RC-5B, GSA rotor, 250 ml centrifuge cup, 60 minutes, 4°C, 12 000 rpm, 23 000 g), the supernatant was placed on ice and the pellet was resuspended in 100 ml of sodium phosphate buffer, pH 7.5.

35

Centrifugation and resuspension are repeated three times, the sodium phosphate buffer comprising 1% SDS at the third repetition. After the resuspension, the mixture is stirred for one hour and a final centrifugation is carried out (Sorvall RC-5B, GSA rotor, 250 ml centrifuge cup, 60 minutes, 4°C, 12 000 rpm, 23 000 g).

40

According to SDS-PAGE analysis, the hydrophobin is present in the supernatant after the final centrifugation (figure 1). The experiments show that the hydrophobin is probably present in the form of inclusion bodies in the corresponding E. coli cells. 50 ml of the hydrophobin-comprising supernatant are applied to a 50 ml nickel Sepharose High Performance 17-5268-02 column (Amersham) which has been equilibrated with 50 mM Tris-Cl pH 8.0 buffer. The column is washed with 50 mM Tris-Cl pH 8.0 buffer and the hydrophobin is subsequently eluted with 50 mM Tris-Cl pH 8.0 buffer which comprises 200 mM imidazole. To remove the imidazole, the solution is dialyzed against 50 mM Tris-Cl pH 8.0 buffer.

Figure 1 shows the purification of the hydrophobin prepared:

Lane A: Application to nickel-Sepharose column (1:10 dilution)

Lane B: Flow-through = washing step eluate

Lanes C - E: OD 280 Maxima of the elution fractions (WP1, WP2, WP3)

Lane F shows the applied marker.

The hydrophobin of figure 1 has a molecular weight of approx. 53 kD. Some of the smaller bands represent degradation products of the hydrophobin.

Example 9

Performance testing; characterization of the hydrophobin by change in contact angle of a water droplet on glass

Substrate:

Glass (window glass, Süddeutsche glass, Mannheim):

The hydrophobin purified according to example 8 was used.

- Concentration of the hydrophobin in the solution: 100 µg/ml, the solution further comprised 50 mM of sodium acetate buffer and 0.1% polyoxyethylene(20)-sorbitan monolaurate (Tween[®] 20), pH of the solution: 4
- Immersion of glass plates into this solution overnight (temperature 80°C)
- The hydrophobin-coated glass plates are then withdrawn from the solution and washed in distilled water,
- Then incubation 10min / 80°C / 1% SDS solution in distilled water
- Washing again in distilled water

The samples are dried under air and the contact angle (in degrees) of a droplet of 5 μ l of water with the coated glass surface is determined at room temperature.

5 The contact angle was measured on a Dataphysics OCA 15+ contact angle system, Software SCA 20.2.0. (November 2002). The measurement was effected according to the manufacturer's instructions.

Untreated glass gave a contact angle of $30 \pm 5^\circ$.

10 The glass plate coated with the hydrophobin according to example 8 (yaad-dewA-his₆) gave a contact angle of $75 \pm 5^\circ$.

==> Increase in the contact angle: 45°

15 Example 10

Experiments on phase stabilization by a hydrophobin

20 In each case 50 ml of an emulsion of crude mineral oil (homogeneous crude mineral oil, Wintershall AG, Emlichheim, probe 60, 64, 83, 87, 301 and 507) and water were introduced into snap-lid glass bottles. The emulsion was prepared by emulsifying 1000 ppm of crude mineral oil in approx. 50 ml of water by means of an Ultraturrax stirrer (stirring time of 4 Minutes at 24000 rpm).

25 Added to this emulsion were:

In case A no demulsifier added,
In case B 10 ppm of hydrophobin from Example 8,
In case C 10 ppm of polyDADMAC (demulsifier with solids content from 28 to 32%
30 (ISO3251), viscosity from 200 to 800 mPas (ISO2595))
In case D 10 ppm of Lupasol SK (polyamidoamine grafted with polyethyleneimine, manufacturer: Nippon Shokubai, Japan)

35 Thereafter, the samples were left to stand for 3 days, in the course of which the emulsions separated (see schematic illustration in figure 2, upper row).

The samples were then shaken gently by hand by a couple of circular motions. In cases A, C and D this again formed high opacity (emulsion formation); in the case of B (comprising hydrophobin), the phase separation was preserved.

40

In individual cases, flakes also formed in sample B comprising 10 ppm of hydrophobin, but they rose immediately to the upper oil phase (see lower row of the schematic illustration in figure 2).

- 5 These experiments demonstrate that hydrophobins stabilize emulsions which have already separated into two separate phases better than commercial demulsifiers.

The hydrophobin can also be added to the aqueous phase after the emulsion has split.

10 Example 11

Comparison of phase stabilization

- 15 5% by weight solutions of the demulsifiers listed in the Table in 3:1 xylene/isopropanol mixture (based on volume) are first prepared.

The hydrophobin from Example 8 was made up as a 1% solution (0.25% active substance) in distilled water 1 h before the addition.

- 20 Demulsifiers used by way of example are:

Pluronic® PE 6800: (ethylene oxide/propylene oxide copolymer)

Basorol® P380: (triol polyol polyether)

Basorol® HP: (tetrol-ethylene oxide/propylene oxide copolymer)

25

A crude oil emulsion (Wintershall AG, Emlichheim, probes 60, 64, 83, 87, 301 and 507 with a water content of 62% by volume, determined by DIN ISO 3733 distillation process) was heated to a temperature of 52°C in a closed vessel in a water bath for approx. 2 h.

30

The crude oil emulsion was homogenized by shaking for approx. 30 sec and in each case 100 ml of the crude oil emulsion were filled into 100 ml shaking cylinders. The oil-filled shaking cylinder was introduced into the water bath.

- 35 An Eppendorf pipette was used in each case to dose 50 µl of the 5% by weight solution of the abovementioned demulsifiers into a shaking cylinder comprising crude oil emulsion, and the cylinder was closed with the glass stopper. Thereafter, the shaking cylinder was taken out of the water bath, shaken 60 x and decompressed. The shaking cylinder was then placed back into the water bath (52°C) and the volume of the water
40 which then separates out was read off after 30 and 240 min. The results are reproduced in the table which follows.

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After 240 minutes, the amounts of hydrophobin stated in the table are introduced by means of a syringe into the water which has separated out in each case by means of a disposable syringe. Subsequently, the mixtures are shaken for 30 sec in each case. Thereafter, the samples are left to stand at 52°C for 1 minute and then the amount of
5 the water which has separated out is determined. The results are compiled in the table.

Table

Demulsifier	Water separated out (ml) after time [min]		Amount of protein added to the water phase	Water separated out (ml)
	30	240		after 1 min
Pluronic® PE6800	2	39	0 ppm	34
Pluronic® PE6800	6	39	1.0 ppm	39
Pluronic® PE6800	3	39	3.0 ppm	39
Basorol® P380	56	60	0 ppm	56
Basorol® P380	55	60	1.0 ppm	58
Basorol® P380	54	60	0.5 ppm	58
Basorol® HP	50	59	0 ppm	57
Basorol® HP	53	59	1.0 ppm	59
Basorol® HP	50	59	0.5 ppm	58

5 It is seen clearly that the addition of the hydrophobin accelerates the re-separation of the phases. Accordingly, the re-emulsification of the water phase into the oil phase appears to be reduced by the protein.

Also astonishing is the low concentration of from 0.5 to 1 ppm of hydrophobin which is sufficient to achieve the result.

Claims

1. A process for stabilizing liquid phases in a composition comprising at least two liquid phases, comprising the addition of at least one hydrophobin in an amount of from 0.001 to 100 ppm to the composition.
2. The process according to claim 1, wherein the hydrophobin is a fusion hydrophobin or a derivative thereof.
3. The process according to claim 2, wherein the fusion hydrophobin is at least one of yaad-Xa-dewA-his (SEQ ID NO: 20), yaad-Xa-rodA-his (SEQ ID NO: 22) or yaad-Xa-basf1-his (SEQ ID NO: 24), where yaad may also be a truncated fusion partner yaad' having from 20 to 293 amino acids.
4. The process according to any one of claims 1 to 3, wherein the composition comprising at least two liquid phases is a composition comprising oil and water or a composition comprising fuel and water.
5. The process according to any one of claims 1 to 4, wherein the hydrophobin is used in an amount of from 0.001 to 80 ppm based on the overall composition.
6. The process according to any one of claims 1 to 5, wherein the composition is a crude oil-water composition and the hydrophobin is used in an amount of from 0.001 to 20 ppm based on the overall composition.
7. The process according to any one of claims 1 to 5, wherein the composition is a fuel-water composition and the hydrophobin is used in an amount of from 0.001 to 20 ppm based on the overall composition.
8. The process according to any one of claims 1 to 7, wherein the phases are first split and then a hydrophobin is added to the aqueous phase.

9. A formulation comprising at least one organic phase consisting of fuels and/or crude oils and an aqueous phase comprising at least one hydrophobin in an amount of from 0.001 to 80 ppm based on the overall formulation.
- 5 10. The formulation according to claim 9, wherein the formulation comprises at least one fuel and at least one hydrophobin or a derivative thereof in an amount of from 0.01 to 1 ppm based on the overall formulation.
11. The formulation according to claim 10, wherein the fuel is gasoline fuels, diesel fuels or turbine fuels.
- 10 12. The formulation according to any one of claims 9 to 11, wherein the hydrophobin is a fusion hydrophobin or a derivative thereof.
13. The formulation according to any one of claims 9 to 12, wherein the fusion hydrophobin is at least one of yaad-Xa-dewA-his (SEQ ID NO: 20), yaad-Xa-rodA-his (SEQ ID NO: 22) or yaad-Xa-basf1-his (SEQ ID NO: 24), where yaad may also be a truncated fusion partner yaad' having from 20 to 293 amino acids.
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Figur 2

