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(71) Applicant: **TEKNOLOGIAN TUTKIMUSKESKUS VTT OY** [FI/FI]; Vuorimiehentie 3, 02150 Espoo (FI).

(72) Inventors: **LAPPALAINEN, Timo**; VTT, PL 1603, 40101 Jyväskylä (FI). **PAANANEN, Arja**; VTT, PL 1000, 02044 VTT (FI). **LIENEMANN, Michael**; VTT, PL 1000, 02044 VTT (FI). **SZILVAY, Geza**; VTT, PL 1000, 02044 VTT (FI).

(74) Agent: **BOCO IP OY AB**; Itämerenkatu 5, 00180 Helsinki (FI).

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(54) Title: A METHOD FOR INCREASING FOAM STABILITY

(57) Abstract: The present invention relates to a method for producing foam, said foam comprising hydrophobin or hydrophobin variants, low-molecular-weight surfactant and fibers. The invention also relates to the foam obtained with said method. Furthermore, the invention relates to uses of the foam in light-weight 3D products, such as insulating materials and package cushion materials.

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A METHOD FOR INCREASING FOAM STABILITY

FIELD OF THE INVENTION

The present invention relates to a method for producing foam, said foam comprising hydrophobin or hydrophobin variants, low-molecular-weight surfactant and fibers. The invention also relates to the foam obtained with said method. The foam-fiber mixture can optionally contain also other additives. Especially, the invention relates to the stabilization of fibre foams in the production of light-weight 3D products. Furthermore, the invention relates to uses of the foam in insulating materials, especially in heat and noise insulating materials, and also in package cushion materials.

BACKGROUND OF THE INVENTION

Foam stability is important in various fields of technology. Enhancement of foam stability by hydrophobins has been reported e.g. for food applications (Cox *et al.* 2009). WO 2008046698 relates to food foams. Hydrophobins have previously been used especially to stabilize the foamy structure of a food foam.

Hydrophobins are amphiphilic proteins produced for example by filamentous fungi. They are important during various growth and development stages during which they, for example, form protective spore coatings (Latge *et al.* 1988 and Boualen *et al.* 2008) and constituents of the hyphal cell wall (Askolin *et al.* 2005). Furthermore, hydrophobins support fungal growth by lowering the water surface tension for hyphal penetration of the air–water interface (Wösten *et al.* 1999 and 2000). The latter process involves self-assembly of spread out hydrophobin monolayers at the air–water interface (Linder 2009 and Szilvay *et al.* 2007) which may be recognized by other proteins at the surface of the fungal cell. Recently the structure-function relationships of class II hydrophobin HFBI have been investigated by means of site-directed mutagenesis to gain an understanding of the role of charged amino acids in the structure of hydrophobins (Lienemann *et al.* 2015).

Hydrophobin proteins have a unique amphiphilic structure and can be called as natural surfactants. They tend to associate with each other and assemble at the air-water interface into highly ordered monolayers that are strong and elastic (Linder 2009). They attach as coating to hydrophobic substrates enabling protein-based surface functionalization.

Surfactants are central to the product performance in the foam forming technology. Low-molecular-weight surfactants, such as sodium dodecyl sulphate (SDS), are commonly used in foaming technology. SDS is an excellent foaming agent (SDS diffusion occurs rapidly), and this is one of the reasons for why it is included in many personal care products, such as toothpaste. For foam to exist there must be a substituent with the bulk of the liquid to lower surface tension.

Foams can be categorized into two different types: open-cell and closed-cell. Open-cell is a type of foam where the small gas pockets are not completely closed. Open-cell foam is a very good air barrier but does not provide any type of water vapor barrier. Closed-cell foam has a smaller, more compact cell structure. It is a very good air barrier as well as a water vapor barrier.

WO 2008/107439 discloses open-cell foam based on a melamine-formaldehyde condensation product, a polyurethane or a polyimide, which has been modified with hydrophobins. In this work hydrophobins are not in focus when producing the foam but they are used for coating the ready-made foam, i.e. hydrophobins are used here for functionalizing the open-cell foam. An open-cell foam modified with hydrophobin is also disclosed in WO 2010072665. The purpose of using hydrophobins in this reference is to help the spinning process. The result is a non-woven material, but it does not contain foam texture at any point during processing steps, and hence, no stabilization of the foam structure is needed.

Foam-forming technology is a central part of producing traditional insulation materials. Traditional insulation materials include rock and slag wool, usually made from rock (basalt, diabase) or iron ore blast furnace slag, fiberglass, made from molten glass, high-density fiberglass and plastic fiber, usually made from recycled plastic. Spray foam insulation is an alternative to traditional building insulation such as fiberglass.

Thermal insulators are nowadays often made from stone and glass wool. Most of the sound insulation products are made from combining plastics, rubber, metals and synthetic fibre-based materials, which makes them not well recyclable. Disadvantage of using, for example, glass fibres, polyester fibres and polyurethane fibres include health issues and that they are not biodegradable.

Package cushioning is used to help protect fragile items during shipment. Several types of polymeric foams are used for cushioning. The most common are: Expanded Polystyrene (also Styrofoam), polypropylene, polyethylene and polyurethane. These can be molded to engineered shapes or sheets which are cut and glued into cushion structures. Some degradable foams are also available.

Control and adjustment of the drainage rate of a foam is a very important topic when manufacturing porous structures. In the production of light-weight 3D products use of long-living foam is recommended. Unfortunately stability of the foam that is based on low-molecular-weight surfactant and fibers is not sufficient even when using high dosages of such low-molecular-weight surfactant.

SUMMARY OF THE INVENTION

The idea of this invention is the use of surface-active proteins, hydrophobins in the stabilization of fibre foams in the production of light-weight 3D products such as heat and sound insulators and package cushions.

Surfactants are central to the product performance in the foam forming technology. Low-molecular-weight surfactants, such as SDS, are the most efficient surfactants in foaming a fibre suspension, but the foam lasts only for a short time. This can be advantageous in the manufacturing of web-like products such as paper and board. However, in the production of light-weight 3D products (such as heat and sound insulators and package cushions) use of long-living foam is recommended. The 3D structure of fibrous network should not collapse during the drainage of water and/or drying of the product.

The solution of the present invention is the use of hydrophobins (surface-active proteins) in stabilization of fibre foams. Hydrophobins are well-known for their capability to form strong interfacial films at the air-water interface, which relates to exceptionally stable foams and foam-based products. Use of a mixture of low-molecular-weight surfactant (such as SDS) and hydrophobins in foam forming brings stability to the foam structure without losses in the foam volume. The commercial potential is for example in the possibility to tune the material properties like the product porosity and thickness, which enables development and design of new products from natural resources.

It is an object of the invention to provide a method for producing an open-cell foam, which comprises the step of introducing gas to the aqueous solution comprising low-molecular-weight surfactant, hydrophobin or a variant thereof.

Another object of this invention is an open-cell foam comprising low-molecular-weight surfactant, hydrophobin or a variant thereof and fibers.

Foam forming according to the present invention will serve as a platform technology to enable manufacturing of totally new biomaterial-based 3D-structured materials.

A further object of this invention is an insulating material which comprises the open-cell foam comprising low-molecular-weight surfactant, hydrophobin or a variant thereof and fibers.

The invention also discloses noise and thermal insulation materials which comprise the open-cell foam comprising low-molecular-weight surfactant, hydrophobin or a variant thereof and fibers.

Use of the open-cell foam comprising low-molecular-weight surfactant, hydrophobin or a variant thereof and fibers in an insulating material is also an object of this invention.

Also use of the open-cell foam comprising low-molecular-weight surfactant, hydrophobin or a variant thereof and fibers in noise or thermal insulation material is an object of the invention.

A package cushioning material comprising the open-cell foam comprising low-molecular-weight surfactant, hydrophobin or a variant thereof and fibers and also use of such foam in package cushioning are further objects of the present invention.

Advantageous embodiments of the invention are set forth in the dependent claims. Other objects, details and advantages of the invention will become apparent from the following drawings, detailed description and examples.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1: **A)** Three-dimensional structure of *Trichoderma reesei* hydrophobin HFBI (PDB-ID 2FZ6). The hydrophobic patch provides attractive interaction during adsorption to hydrophobic substrates and to the gaseous phase during the formation of monolayers at the air-water interface. **B)** Computational model of HFBI monolayer with 6-fold symmetry (referred to as β-structure by Magarkar *et al.* 2014). The side chain atoms of the mutated residues are shown as sticks and the positions of the mutated side residues in the HFBI sequence are indicated next to the corresponding

alpha-carbon atom. Individual HFBI molecules of the pore-forming hexamer complex are highlighted by shading. Pores in the layer are evident that are flanked by the negatively charged side chains of residues D40 and D43 as well as the positively charged side chain of R45.

Figure 2: Zeta potential of HFBI wild-type and variants D30N/K32Q (SEQ ID NO:7), D40Q/D43N (SEQ ID NO:8), D40Q/D43N/R45Q/K50Q (SEQ ID NO:9) and R45Q/K50Q (SEQ ID NO:10) measured in 0.1 mg/ml protein solutions. The isoelectric points were determined from this plot as 6.1, 5.8, 7.0, 6.2 and 3.6, respectively.

Figure 3: Concentration dependence of HFBI variant aggregation. The molecular weight of homodimers is plotted against the HFBI variant concentration.

Figure 4: Interfacial rheology data (storage modulus, G′ = A; loss modulus, G″ = B) of HFBI wild type and HFBI variants D30N/K32Q, D40Q/D43N, R45Q/K50Q and D40Q/D43N/R45Q/K50Q at the air-water interface as a function of adsorption time. The interfacial layers are adsorbed from 0.3 μM protein solutions.

Figure 5: AFM images of LB-films of HFBI that were assembled on the air–water interface and have been transferred to a flat mica substrate, dried and imaged in air using tapping mode. Displayed are typical 200 nm phase images of the HFBI variants D30N/K32Q, D40Q/D43N, R45Q/K50Q and D40Q/D43N/R45Q/K50Q (A–D, respectively).

Figure 6: Culture supernatant of *T. reesei* strain M411 secreting HFBI variant D30N/K32Q. The wrinkles of the air bubble next to fungal mycelium indicate the presence of hydrophobins that assemble as monolayers at the air–water interface.

Figure 7: Sequence alignment of Class II hydrophobin protein sequences. VDHI of *Verticillium dahliae* (Uniprot accession ID Q1PSW0), HCF-5 of *Cladosporium fulvum* O94203), HCF-6 of *Cladosporium fulvum* (Q9C2X0), HYD4 of *Gibberella moniliformis* (Q6YF29), HFBIII of *Trichoderma reesei* (G0RVE5), HYD2 of *Metarhizium brunneum* (F8R8G0), GEO1 of *Geosmithia pallida* (I7B278), SRH1 of *Trichoderma harzianum* (P79072), HFBI of *Trichoderma reesei* (P52754), HFBII of *Trichoderma reesei* (P79073), CU of *Ophiostoma ulmi* (Q06153), QID3 of *Trichoderma harzianum* (P52755), CRP of *Cryphonectria parasitica* (P52753), NC2 of *Neurospora crassa* (Q7S3P5), FcHYD5p of *Fusarium culmorum* (A9NIV5), MHP1 of *Magnaporthe grisea* (AF126872) and FpHYD5 of *Fusarium poae* (D3VN12) were aligned using Clustal

Omega (Sievers *et al.* 2011). The shown sequences were obtained by performing a search in protein databases for class II hydrophobin sequences showing the typical Cys patterning. Sequences where excluded from this alignment if they represented protein fragments, oligomeric hydrophobins or hydrophobins without experimental data on the protein level. The amino termini sequences were found to be highly variable and only sequences after the first Cys were used in the alignments for clarity. The Cys-pattern is shown by alignment of the Cys residues (marked with black background shading). The HFBI sequence is boxed and begins at position eight. Annotations of the secondary structure elements and the residues forming the hydrophobic patch are given according to the published HFBI crystal structure (Hakanpää *et al.* 2006). Similarity among the aligned residues was determined with COBALT (Papadopoulos *et al.* 2007) and shaded accordingly (dark grey = high similarity at three bits relative entropy threshold; light grey = high similarity at two bits relative entropy threshold). Charged residues are shown in bold.

Figure 8: Side view of the modelled HFBI monolayer shown in Figure 1B. The location of residues D30 and K32 inside the layer close to the hydrophobic patch (bottom) is evident.

Figure 9 shows foamability followed by monitoring foam volume as a function of time when SDS only is mixed with fibers (from six independent experiments).

Figure 10 shows foam stability, i.e. collapse rate of foam, followed by monitoring liquid drainage volume as a function of time when SDS only is mixed with fibers (from six independent experiments).

Figure 11 shows foamability followed by monitoring foam volume as a function of time when HFBI-R45Q/K50Q to SDS ratio (w/w) is 1:10 (from two independent experiments).

Figure 12 shows foam stability, i.e. collapse rate of foam, followed by monitoring drainage volume as a function of time when HFBI-R45Q/K50Q to SDS ratio (w/w) is 1:10 (from two independent experiments).

Figure 13 shows foam stability followed by monitoring fibre foam volume as a function of time, SDS alone (square), SDS-HFBI-R45Q/K50Q mixture (circle) and SDS-HFBI-D40Q/D43N/R45Q/K50Q mixture (triangle). The mixtures contain either 1:10 (w/w, open symbols) or 1:3 (w/w, closed symbols) of hydrophobin.

Figure 14 Foam was produced in a mixer, and foaming dynamics was recorded with a CCD camera.

Figure 15 Determination of foamability and foam stability of the foam-fibre mixture. A) Stock in a transparent vessel before mixing. B) Foam-fibre mixture after 60 s mixing C) Foam-fibre mixture 1502 s after the cessation of mixing. The liquid drained (0.2 l) from the foam is clearly visible in the bottom of the transparent vessel.

Figure 16 Foam stability (t½) as a function of retention of hydrophobin in the foam.

SEQUENCE LISTING

SEQ ID NO:1 The amino acid sequence of the proenzyme form of *T. reesei* HFBI hydrophobin. The signal and propeptide are displayed in bold and italics, respectively (removed during secretion). HFBI residues D30, K32, D40, D43, R45 and K50 that were substituted in the corresponding variants are underlined.

MKFFAIAALFAAAAVA*QPLEDR*SNGNGNVCPPGLFSNPQCCATQVLGLIGLDCKVPSQNVY
DGTDFRNVCAKTGAQPLCCVAPVAGQALLCQTAVGA

SEQ ID NO:2 The amino acid sequence of the mature form of *T. reesei* HFBI hydrophobin.

SNGNGNVCPPGLFSNPQCCATQVLGLIGLDCKVPSQNVYDGTDFRNVCAKTGAQPLCCVAPVA
GQALLCQTAVGA

SEQ ID NO:3 Sequence of a probe complementary to *hph* resistance marker. The probe was used in PCR for confirming the *hfb2* gene disruption.

ATTCTTCGCCCTCCGAGAGC

SEQ ID NO:4 Sequence of a probe complementary to the *hfb2* region located upstream of the deletion cassette insertion. The probe was used in PCR for confirming the *hfb2* gene disruption.

GAATCATGCTGGAGTGAAGG

SEQ ID NO:5 Sequence of a primer that binds to the CBHI promoter. The primer was used for analysing acetamide-resistant clones for HFBI variant gene insertion by sequencing.

CAACTCAGATCCTCCAGGAGAC

SEQ ID NO:6 Sequence of a primer that binds to the CBHI terminator. The primer was used for analysing acetamide-resistant clones for HFBI variant gene insertion by sequencing.

TCATGATACGGGCTCACCAAG

SEQ ID NO:7 The amino acid sequence of a variant D30N/K32Q.

SNGNGNVCPPGLFSNPQCCATQVLGLIGLNCQVPSQNVYDGTDFRNVCAKTGAQPLCCVAPV
AGQALLCQTAVGA

SEQ ID NO:8 The amino acid sequence of a variant D40Q/D43N.

SNGNGNVCPPGLFSNPQCCATQVLGLIGLDCKVPSQNVYQGTNFRNVCAKTGAQPLCCVAPVA
GQALLCQTAVGA

SEQ ID NO:9 The amino acid sequence of a variant D40Q/D43N/R45Q/K50Q.

SNGNGNVCPPGLFSNPQCCATQVLGLIGLDCKVPSQNVYQGTNFQNVCAQTGAQPLCCVAPV
AGQALLCQTAVGA

SEQ ID NO:10 The amino acid sequence of a variant R45Q/K50Q.

SNGNGNVCPPGLFSNPQCCATQVLGLIGLDCKVPSQNVYDGTDFQNVCAQTGAQPLCCVAPV
AGQALLCQTAVGA

DEPOSITIONS

The obtained HFBI-variant secreting *Trichoderma reesei* strains were deposited at the VTT Culture collection with the IDs D-121448 (-D40Q/D43N/R45Q/K50Q), D-121449 (-D40Q/D43N), D-121450 (-R45Q/K50Q) and D-121451(-D30N/K32Q). The *T. reesei* *hfb2::hph* strain was deposited at the VTT culture collection (VTT Technical Research Centre of Finland P.O. Box 1000, FI-02044 VTT, Finland) under the ID D-121447. All strains were deposited on the 24th of August 2012.

DEFINITIONS

A "foaming agent" is a material that facilitates formation of foam. Foaming agent can be such as a surfactant or a blowing agent. A surfactant, when present in small amounts, reduces surface tension of a liquid (reduces the work needed to create the foam). A blowing agent is a gas that forms the gaseous part of the foam.

A foam as referred herein is a substance that is formed by trapping pockets of gas in a liquid or solid. In most foams, the volume of gas is large, with thin films of liquid or solid separating the regions of gas. An important division of solid foams is into closed-cell foams and open-cell foams. In a closed-cell foam, the gas forms discrete pockets, each completely surrounded by the solid material. "Open-cell-structured foams" as referred herein contain pores that are connected to each other and form an interconnected network that is relatively soft. Open-cell foams will fill with whatever they are surrounded with. If filled with air, a relatively good insulator is the result, but, if the open cells fill with water, insulation properties would be reduced. Foam rubber is a type of open-cell foam. The solid component of an open-cell foam may be an organic polymer-like polyurethane, or a ceramic or a metal. These materials are used in a wide range of applications where the high porosity and large surface area are needed, including filters, catalyst supports, fuel tank inserts, and loudspeaker covers.

"Hydrophobins" as referred herein are a group of small (~100 amino acids) cysteine-rich proteins that are expressed by filamentous fungi. There exist also bacterial hydrophobins. As shown in Bromley *et al*. 2015, these do not contain disulphide bonds but resemble the fungal hydrophobins in their functional properties. They are known for their ability to form a hydrophobic (water-repellent) coating on the surface of an object. They were first discovered and separated in *Schizophyllum commune* in 1991. Hydrophobins have been identified in ascomycetes and basidiomycetes; whether they exist in other groups is not known. Hydrophobins are generally found on the outer surface of conidia and of the hyphal wall, and may be involved in mediating contact and communication between the fungus and its environment. Some family members contain multiple copies of the domain.

This family of proteins includes the rodlet proteins of *Neurospora crassa* (gene eas) and *Emericella nidulans* (gene rodA), these proteins are the main component of the hydrophobic sheath covering the surface of many fungal spores.

Genomic sequencing of two fungi from dry or salty environments (*Wallemia sebi* and *W. ichthyophaga*) revealed that these species contain predicted hydrophobins with unusually high proportion of acidic amino acids and therefore with potentially novel characteristics. High proportion of acidic amino acids is thought to be an adaptation of proteins to high concentrations of salt.

Based on differences in hydropathy patterns and biophysical properties, hydrophobins can be divided into two categories: class I and class II. HFBI and HFBII are two class II hydrophobins from the fungus *Trichoderma reesei* and are quite similar with a sequence identity of 66%. The published data on class I and II hydrophobins show that there is a functional division between the classes which mainly seems to involve the structure and solubility of their aggregates. Systematic investigations of surface binding of class II hydrophobins have not been reported before, but adsorption of the class I hydrophobin SC3 has been characterized much more in detail. In the case of SC3, the formation of rodlet layers seem to be an essential component of the binding. The isolation and of the gene for *T. reesei* HFBI has been described in (Nakari-Setala *et al.* 1996) and HFBII in (Nakari-Setala *et al.* 1997). The isolation of the *srhI* gene of *T. harzianum* has been described before. Hydrophobins can self-assemble into a monolayer on hydrophobic/hydrophilic interfaces such as a water/air interface. Class I monolayer contains the same core structure as amyloid fibrils, and is positive to Congo red and thioflavin T. The monolayer formed by class I hydrophobins consist of tightly packed rodlet structures, and can only be dissociated by concentrated trifluoroacetate or formic acid. Monolayer assembly involves large structural rearrangements with respect to the monomer. Class II hydrophobins form highly ordered structure at fluid interfaces, and the interfacial layers can be more easily dissociated, for example by ethanol.

"Hydrophobin variants" refer here to genetically modified hydrophobins. Genetically modified hydrophobins comprise for example variants comprising substitutions of one or more amino acids and also hydrophobin fusion proteins that are linked to one or more linker sequence or to other proteins through or without such linker sequences. The variant as disclosed by the invention can comprise an amino acid sequence having a substitution of one or more charged amino acid residues against sterically related non-charged homologues in amino acid sequence of the mature hydrophobin HFBI or HFBII.

That the polypeptide has "surface activity" means here that the molecule is amphiphilic containing both hydrophilic and hydrophobic groups and hence has tendency to accumulate at the interface and lower the surface (or interfacial) tension between air and water, or between oil and water.

"Surfactants" are compounds that lower the surface tension (or interfacial tension) between two liquids or between a liquid and a solid. Surfactants are usually compounds that are amphiphilic, meaning they contain both, hydrophobic groups (their *tails*) and hydrophilic groups (their *heads*). Most commonly, surfactants are classified according to the polar head group. A non-ionic surfactant has no charge groups in its head. The head of an ionic surfactant carries a net charge. If the charge is negative, the surfactant is more specifically called anionic; if the charge is positive, it is called cationic. If a surfactant contains a head with two oppositely charged groups, it is termed zwitterionic. Surfactants may act as detergents, wetting agents, emulsifiers, foaming agents, and dispersants.

As used herein, surfactant refers to molecules and/or polymers that are used to mediate the admixture or dissolution of water into base polymers such as those used in accordance with an exemplary embodiment of the present invention. Some commonly encountered surfactants include ionic surfactants such as anionic surfactants (typically based on sulfate, sulfonate, or carboxylate anions), bis(2-ethylhexyl) sulfosuccinate, sodium salt, sodium dodecyl sulfate (SDS) (or SLS, sodium lauryl sulfate, another name for the compound), ammonium lauryl sulfate, and other alkyl sulfate salts, sodium laureth sulfate, also known as sodium lauryl ether sulfate (SLES), sodium myreth sulfate, alkyl benzene sulfonate, and fatty acid salts. Anionic surfactants comprise also docusates: dioctyl sodium sulfosuccinate, perfluorooctanesulfonate (PFOS), perfluorobutanesulfonate and linear alkylbenzene sulfonates (LABs). The commonly encountered surfactants include also cationic surfactants (typically based on quaternary ammonium cations) such as, but not limited to, cetyl trimethylammonium bromide (CTAB), a.k.a. hexadecyl trimethyl ammonium bromide, and other alkyltrimethylammonium salts, cetylpyridinium chloride (CPC), polyethoxylated tallow amine (POEA), benzalkonium chloride (BAC), benzethonium chloride (BZT), 5-Bromo-5-nitro-1,3-dioxane, dimethyldioctadecylammonium chloride, cetrimonium bromide and dioctadecyldimethylammonium bromide (DODAB). Zwitterionic (amphoteric) surfactants include for example dodecyl betaine, dodecyl dimethylamine oxide, cocamidopropyl betaine, and coco ampho glycinate. Nonionic surfactants include such as, but not limited to, alkyl poly(ethylene oxide), copolymers of poly(ethylene oxide) and poly(propylene oxide) (commercially called poloxamers or poloxamines), and alkyl polyglucosides, including, but not limited to, octyl glucoside, decyl maltoside, fatty alcohols, cetyl alcohol, oleyl alcohol, cocamide MEA, and cocamide DEA.

The term "strong interfacial film" refers to fluid interface between air and water possessing surface elastic modulus over 0.1 N/m (value presented by Cox *et al.* 2009). The value is derived from the theoretical calculations by Kloek *et al.* 2001. The phrase "improved formation of an elastic protein film" means that a formed interfacial film has improved visco-elastic properties showing increased surface elastic modulus values.

At "liquid-air interfaces", surface tension results from the greater attraction of water molecules to each other (due to cohesion) than to the molecules in the air (due to adhesion). The net effect is an inward force at its surface that causes water to behave as if its surface was covered with a stretched elastic membrane. Because of the relatively high attraction of water molecules to each other, water has a high surface tension (72.8 mN/m at 20 °C) compared to that of most other liquids.

"Particle aggregation" refers to formation of clusters in a colloidal suspension and represents the most frequent mechanism leading to destabilization of colloidal systems. During this process, which normally occurs within short periods of time (seconds to hours), particles dispersed in the liquid phase stick to each other, and spontaneously form irregular particle clusters, flocs, or aggregates. This phenomenon is also referred to as *coagulation* or *flocculation* and such a suspension is also called *unstable*. Colloidal particles may also remain dispersed in liquids for long periods of time (days to years). This phenomenon is referred to as *colloidal stability* and such a suspension is said to be *stable*. Stable suspensions are often obtained at low salt concentrations or by addition of chemicals referred to as *stabilizers* or *stabilizing agents*.

"Dewatering of fibre foam" as referred herein is a two-step process comprising the steps of drainage and drying.

"Drainage" as used herein refers to the natural or artificial removal of surface and sub-surface water from an area. Drainage occurs either through gravitation (i.e. water comes out gravimetrically) or is done for example by using suction boxes.

"Drying" as referred herein comprises non-contact drying methods, such as drying in the oven, air impingement drying, microwave drying, through air drying, infrared drying and combination(s) of those.

DETAILED DESCRIPTION OF THE INVENTION

The basic idea of this invention is the use of hydrophobins that are surface-active proteins in stabilization of fibre foams. Hydrophobins are of a great interest in the world currently, especially due to their capability to form strong interfacial films at the air–water interface (Linder 2005; Wösten and Scholtmeijer 2015). This characteristic relates to formation of exceptionally stable foams (Cox *et al.* 2009) and product stability. Use of a mixture of a low-molecular-weight surfactant (like SDS) and hydrophobins in foam forming brings stability to the foam structure without losses in the foam volume.

Hydrophobins are a well-defined class of proteins (Wessels *et al.* 1997; Wosten, *et al.* 2001) capable of self-assembly at a hydrophobic/hydrophilic interface, and having a conserved sequence: X_n-C-X_{5-9}-C-C-X_{11-39}-C-X_{8-23}-C-X_{5-9}-C-C-X_{6-18}-C-X_m (Wessels *et al.* 1997) where X represents any amino acid, and n and m independently represent an integer. Typically, a hydrophobin has a length of up to 125 amino acids. The cysteine residues (C) in the conserved sequence are part of disulphide bridges. Bacterial hydrophobins do not contain disulphide bonds but resemble the fungal hydrophobins in their functional properties (Bromley *et al.* 2015). In the context of the present invention, the term hydrophobin has a wider meaning to include functionally equivalent proteins still displaying the characteristic of self-assembly at a hydrophobic-hydrophilic interface resulting in a protein film, such as proteins comprising the sequence: X_n-C-X_{1-50}-C-X_{0-5}-C-X_{1-100}-C-X_{1-100}-C-X_{1-50}-C-X_{0-5}-C-X_{1-50}-C-X_m (De Vocht *et al.* 1998) or parts thereof still displaying the characteristic of self-assembly at a hydrophobic-hydrophilic interface resulting in a protein film. In accordance with the definition of the present invention, self-assembly can be detected by adsorbing the protein to Teflon and using Circular Dichroism to establish the presence of a secondary structure (in general, a-helix) (De Vocht *et al.*1998).

The formation of a film can be established by incubating a Teflon sheet in the protein solution followed by at least three washes with water or buffer (Wösten *et al.* 1994). The protein film can be visualised by any suitable method, such as labeling with a fluorescent marker or by the use of fluorescent antibodies, as is well established in the art. m and n typically have values ranging from 0 to 2000, but more usually m and n in total are less than 100 or 200. The definition of hydrophobin in the context of the present invention includes fusion proteins of a hydrophobin and another polypeptide as well as conjugates of hydrophobin and other molecules such as polysaccharides.

Hydrophobins identified to date are generally classified as either class I or class II. Both types have been identified in fungi as secreted proteins that self-assemble at hydrophobic/hydrophilic interfaces into amphipathic films. Assemblages of class I hydrophobins are relatively insoluble whereas those of class II hydrophobins readily dissolve in a variety of solvents. Hydrophobin-like proteins have also been identified in filamentous bacteria, such as Actinomycete and *Steptomyces* sp. (WO01/74864). These bacterial proteins, by contrast to fungal hydrophobins, form only up to one disulphide bridge since they have only two cysteine residues. Such proteins are an example of functional equivalents to hydrophobins having the consensus sequences $X_n\text{-}C\text{-}X_{5\text{-}9}\text{-}C\text{-}C\text{-}X_{11\text{-}39}\text{-}C\text{-}X_{8\text{-}23}\text{-}C\text{-}X_{5\text{-}9}\text{-}C\text{-}C\text{-}X_{6\text{-}18}\text{-}C\text{-}X_m$ and $X_n\text{-}C\text{-}X_{1\text{-}50}\text{-}C\text{-}X_{0\text{-}5}\text{-}C\text{-}X_{1\text{-}100}\text{-}C\text{-}X_{1\text{-}100}\text{-}C\text{-}X_{1\text{-}50}\text{-}C\text{-}X_{0\text{-}5}\text{-}C\text{-}X_{1\text{-}50}\text{-}C\text{-}X_m$, and are within the scope of the present invention.

The hydrophobins can be obtained by extraction from native sources, such as filamentous fungi, by any suitable process. For example, hydrophobins can be obtained by culturing filamentous fungi that secrete the hydrophobin into the growth medium or by extraction from fungal mycelia with 60% ethanol. It is particularly preferred to isolate hydrophobins from host organisms that naturally secrete hydrophobins. Preferred hosts are hyphomycetes (e.g. Trichoderma), basidiomycetes and ascomycetes. Traditionally, particularly preferred hosts for food products have been food-grade organisms, such as *Cryphonectria parasitica* which secretes a hydrophobin termed cryparin (MacCabe and Van Alfen 1999).

Alternatively, hydrophobins can be obtained by the use of recombinant technology. For example host cells, typically micro-organisms, may be modified to express hydrophobins and the hydrophobins can then be isolated and used in accordance with the present invention. Techniques for introducing nucleic acid constructs encoding hydrophobins into host cells are well known in the art. More than 34 genes coding for hydrophobins have been cloned, from over 16 fungal species (see for example W096/41882 which gives the sequence of hydrophobins identified in Agaricus bisporus; and Wösten *et al.* 2001). Recombinant technology can also be used to modify hydrophobin sequences or synthesize novel hydrophobins having desired/improved properties.

Typically, an appropriate host cell or organism is transformed by a nucleic acid construct that encodes the desired hydrophobin. The nucleotide sequence coding for the polypeptide can be inserted into a suitable expression vector encoding the necessary elements for transcription and translation and in such a manner that they will be expressed under appropriate conditions (e.g. in proper orientation and correct reading frame and with appropriate targeting and expression sequences). The methods required to construct these expression vectors are well known to those skilled in the art.

A number of expression systems may be used to express the polypeptide coding sequence. These include, but are not limited to, bacteria, fungi (including yeast), insect cell systems, plant cell culture systems and plants all transformed with the appropriate expression vectors. Suitable fungal species, include yeasts such as (but not limited to) those of the genera *Saccharomyces, Kluyveromyces, Pichia, Hansenula, Candida, Schizo saccharomyces* and the like, and filamentous species such as (but not limited to) those of the genera *Aspergillus, Trichoderma, Mucor, Neurospora, Fusarium* and the like.

The sequences encoding the hydrophobins are preferably at least 80% identical at the amino acid level to a hydrophobin identified in nature, more preferably at least 95% or 100% identical. However, persons skilled in the art may make conservative substitutions or other amino acid changes that do not reduce the biological activity of the hydrophobin. For the purpose of the invention these hydrophobins possessing this high level of identity to a hydrophobin that naturally occurs are also embraced within the term "hydrophobins". Hydrophobins can be purified from culture media or cellular extracts by, for example, the procedure described in WO01/57076 which involves adsorbing the hydrophobin present in a hydrophobin-containing solution to surface and then contacting the surface with a surfactant, such as Tween 20, to elute the hydrophobin from the surface. See also Collen *et al.*, 2002; Calonje *et al.*, 2002; Askolin *et al.*, 2001; and De Vries *et al.*, 1999.

Hydrophobins suitable for a particle of the invention are preferably selected from class I and class II hydrophobins. Known hydrophobins include but are not restricted to HFBI, HFBII, SRH1 and SC3. When selecting a hydrophobin the differences between class I and class II assemblages can be exploited to achieve desired properties. Class

I are highly insoluble, the class II hydrophobin assemblages and adsorbed surface layers dissociate more easily. No rodlet-type surface structures have this far been reported for class II hydrophobins, and in many ways the class II hydrophobins seem to be less extreme in their behavior. Without being bound to a theory, class II hydrophobins appear to be more suitable for applications of this invention where high insolubility could even be a hindrance.

The present invention relates to a method for producing an open-cell foam, which comprises the step of introducing gas to an aqueous solution comprising a low-molecular-weight surfactant, hydrophobin or a variant thereof and fibers. The foam produced is actually a foam-fiber mixture. Especially, the invention relates to improving the stability of such open-cell foam.

The present invention relates to a method for producing an open-cell foam, wherein hydrophobin is selected from the group consisting of class I and class II hydrophobins and bacterial hydrophobins. In a preferred embodiment the hydrophobin is class II hydrophobin. In one preferred embodiment the class II hydrophobin is selected from the group consisting of wild-type HFBI, HFBII, SRHI and a variant thereof. In still another preferred embodiment the hydrophobin used is HFBI or HFBII or a variant thereof.

Foam can be generated using various methods of introducing gas to the aqueous solution of surfactants. Mechanical mixing with high shear forces is a common method of foam generation, especially in industry. One of the advantages of mechanical mixing is that it ensures high homogeneity of the solution. According to a preferred embodiment of the invention gas is introduced by mechanical, by blowing gas through a thin nozzle, blowing gas through a porous plug, or by the nucleation of gas bubbles in a liquid which is supersaturated.

Over time, liquid-based foams evolve through a combination of three basic mechanisms: drainage of the continuous phase (due to gravity), coarsening by diffusion of gas from smaller to larger bubbles (internal pressure is larger in the smaller bubbles), and by rupture. A foam filled with nitrogen gas or air will coarsen more slowly than one consisting of CO_2 bubbles, since diffusion through the film is largely determined by the solubility of gas. Instead of air, any inert gas, or a mixture of air and any inert gas, is suitable in the present invention. With the choice of gas the properties of the obtained foam, especially the foam stability can be affected and tuned. Preferably, the gas is air.

Dewatering of fibre foam according to the present invention is a two-step process comprising the steps of drainage and drying. Drainage refers to the natural or artificial removal of surface and sub-surface water from an area. Drainage occurs either through gravitation (i.e. water comes out gravimetrically) or can also be done by using suction boxes. Drying of foam is generally done by non-contact drying methods, such as drying in the oven, air impingement drying, microwave drying, through air drying, infrared drying and combination(s) of those.

The present invention relates to a method of producing an open-cell foam, which comprises the steps of introducing gas to the aqueous solution comprising a low-molecular-weight surfactant, a class I or II hydrophobin or a variant thereof and fibers and dewatering the obtained foam. Preferably, the dewatering step comprises drainage and drying.

A variant of the present invention can be a variant class I or II hydrophobin. There are several class II hydrophobins originating from filamentous fungi e.g. *Trichoderma reesei*, which are suitable in the present invention.

A variant used in the method of the present invention comprises an amino acid sequence having a substitution of one or more charged amino acid residues against sterically related non-charged homologues in amino acid sequence of the mature hydrophobin HFBI of SEQ ID NO:2.

According to a preferred embodiment of the invention the variant comprises an amino acid sequence having a substitution of one or more amino acid residues at positions 30, 32, 40, 43, 45 or 50 of the hydrophobin, wherein the amino acid positions correspond to the amino acid sequence of the mature hydrophobin HFBI of SEQ ID NO:2.

According to another preferred embodiment of the invention the variant comprises an amino acid sequence having a substitution of one or more amino acid residues at positions 40, 43, 45 or 50 of the hydrophobin, wherein the amino acid positions correspond to the amino acid sequence of the mature hydrophobin HFBI of SEQ ID NO:2.

According to a preferred embodiment of the invention the variant comprises an amino acid sequence set forth in SEQ ID NO:2 with one or more substitutions selected from the group consisting of D30N, K32Q, D40Q, D43N, R45Q, and K50Q, wherein the positions of the amino acid substitutions are numbered in reference to the positions in SEQ ID NO:2. The variant can thus be selected from the group consisting of SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9 and SEQ ID NO:10.

According to another preferred embodiment of the invention the variant comprises an amino acid sequence set forth in SEQ ID NO:2 with one or more substitutions selected from the group consisting of D40Q, D43N, R45Q, and K50Q, wherein the positions of the amino acid substitutions are numbered in reference to the positions in SEQ ID NO:2. The variant can thus be selected from the group consisting of SEQ ID NO:9 and SEQ ID NO:10.

According to still another preferred embodiment the variant comprises an amino acid sequence set forth in SEQ ID NO: 7 (D30N/K32Q), SEQ ID NO: 8 (D40Q/D43N), SEQ ID NO:10 (R45Q/K50Q) or SEQ ID NO:9 (D40Q/D43N/ R45Q/K50Q).

The variant D30N/K32Q (SEQ ID NO:7) has a substitution of aspartic acid to asparagine at position 30 and a substitution of lysine to glutamine at position 32 of the mature hydrophobin HFBI (SEQ ID NO:2).

The variant D40Q/D43N (SEQ ID NO:8) has a substitution of aspartic acid to glutamine at position 40 and a substitution of aspartic acid to asparagine at position 43 of the mature hydrophobin HFBI (SEQ ID NO:2).

The variant D40Q/D43N/R45Q/K50Q (SEQ ID NO:9) has a substitution of aspartic acid to glutamine at position 40, a substitution of aspartic acid to asparagine at position 43, a substitution of arginine to glutamine at position 45 and a substitution of lysine to glutamine at position 50 of the mature hydrophobin HFBI (SEQ ID NO:2).

The variant R45Q/K50Q (SEQ ID NO:10) has a substitution of arginine to glutamine at position 45 and a substitution of lysine to glutamine at position 50 of the mature hydrophobin HFBI (SEQ ID NO:2).

The hydrophobins used according to the invention can also be modified further in their polypeptide sequence, for example by glycosylation, acetylation or else by chemical crosslinking, for example with glutardialdehyde.

The present invention relates to a method for increasing foam stability in products, characterized in that the method comprises the step of using a hydrophobin composition according to the present invention, which composition has been defined above.

According to an embodiment of the method of the present invention, the foam stability is increased by forming a strong and stable foam, a strong elastic film, or providing improved visco-elastic properties of interfacial films.

Product stability of foams can be increased by improving properties of either the continuous phase or the air-water, or both continuous phase and interface. By strengthening the visco-elastic properties of the interface, more elastic and stronger interfacial films are formed and the bubbles are more resistant to coalescence and disproportionation.

The present invention relates to use of the hydrophobin composition according to the present invention as a stabilizer in open-cell foams containing low-molecular-weight surfactant and fibers. Especially, the present invention relates to a composition comprising a hydrophobin variant of class II hydrophobin, wherein the variant comprises an amino acid sequence having at least 80%, preferably at least 95% identity to the amino acid sequence of SEQ ID NO:2 and the variant comprises charged residues, wherein the variant has surface activity and capability to form strong interfacial films. In another embodiment of the invention hydrophobin is class II hydrophobin HFBII or a variant thereof.

The present invention relates to a composition comprising a hydrophobin variant of class II hydrophobin characterized in that the variant comprises an amino acid sequence having at least 80%, preferably at least 95% identity to the amino acid sequence of SEQ ID NO:2 and the variant comprises charged residues, wherein the variant has surface activity and capability to form strong interfacial films.

An embodiment of the invention relates to a variant comprising at least 80%, preferably at least 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, or 94% identity, most preferably at least 95%, 96%, 97%, 98%, 99%, or 100% identity to the amino acid sequence of the mature hydrophobin HFBI of SEQ ID NO:2.

In a preferred embodiment the present invention relates to class II hydrophobins which have been modified by any of the following amino acid substitutions:

(i) substitution of a basic amino acid between the conserved cysteine #4 (Position 25 in sequence alignment of Figure 7) and #5 (Position 42 in sequence alignment of Figure 7) against a non-charged homologue;

(ii) substitution of a basic amino acid between the conserved cysteine #5 (Position 42 in sequence alignment of Figure 7) and #6 (Position 52 in sequence alignment of Figure 7) against a non-charged homologue;

(iii) substitution of an acidic amino acid between the conserved cysteine #4 (Position 13 in sequence alignment of Figure 7) and #5 (Position 42 in sequence alignment of Figure 7) against a non-charged homologue.

In the present invention mutations made to a hydrophobin molecule by molecular biology means enhances the formation of a highly elastic protein film at the air-water interface. The high surface elasticity relates to foam stability. The mutations made are shown in Lienemann *et al.* 2015 for HFBI variant proteins.

The film strengths for HFBI variants D30N/K32Q, D40Q/D43N, R45Q/K50Q and D40Q/D43N/R45Q/K50Q were measured by the interfacial shear rheology technique. The surface shear rheology value (storage modulus) at the air-water interface for variant HFBI-R45Q/K50Q was exceptionally high, being appr. 1.4 N/m for 0.3 uM solution. This is the highest value ever measured for proteins, and is 40% better value than for wild-type HFBI and twice as good as HFBII used in applications by Unilever (Cox *et al.* 2007).

According to our results variants R45Q/K50Q and D40Q/D43N/R45Q/K50Q provide a significant improvement in foam stability.

The technical core feature of the present invention is the use of hydrophobins and the variants thereof as stabilizers in different foam compositions comprising low-molecular-weight surfactant and fibers.

One embodiment of the invention is an insulating material which comprises the open-cell foam comprising low-molecular-weight surfactant, hydrophobin or a variant thereof and fibers. Another embodiment of the invention is noise and/or thermal insulation material which comprises the open-cell foam comprising low-molecular-weight surfactant, hydrophobin or a variant thereof and fibers. Still another embodiment of the invention is a package cushioning material which comprises the open-cell foam comprising low-molecular-weight surfactant, hydrophobin or a variant thereof and fibers.

Foam forming is an emerging technology, where surfactants are central to the product performance. Sodium dodecyl sulfate or SDS (also known as Sodium lauryl sulfate), is an organic compound with the formula $CH_3(CH_2)_{11}OSO_3Na$. It is an anionic surfactant used in many cleaning and hygiene products. The salt is of an organosulfate consisting of a 12-carbon tail attached to a sulfate group, giving the material the amphiphilic properties required of a detergent. It is an inexpensive and effective foamer. Any low-molecular-weight surfactant can be used in the present invention. In a preferred embodiment SDS is used as a surfactant. Sodium laureth sulfate, or sodium lauryl ether sulfate (SLES) and ammonium lauryl sulfate (ALS) and sodium alkyl ether sulfonate (SAES) can be used as alternatives to SDS.

Also other commonly encountered surfactants can be used in the present invention. They include ionic surfactants such as anionic surfactants (typically based on sulfate, sulfonate, or carboxylate anions), bis(2-ethylhexyl) sulfosuccinate, sodium salt, sodium dodecyl sulfate (SDS) (or SLS, sodium lauryl sulfate, another name for the compound), ammonium lauryl sulfate, and other alkyl sulfate salts, sodium laureth sulfate, also known as sodium lauryl ether sulfate (SLES), sodium myreth sulfate, alkyl benzene sulfonate, and fatty acid salts. Anionic surfactants comprise also docusates: dioctyl sodium sulfosuccinate, perfluorooctanesulfonate (PFOS), perfluorobutanesulfonate and linear alkylbenzene sulfonates (LABs). The commonly encountered surfactants include also cationic surfactants (typically based on quaternary ammonium cations) such as, but not limited to, cetyl trimethylammonium bromide (CTAB), a.k.a. hexadecyl trimethyl ammonium bromide, and other alkyltrimethylammonium salts, cetylpyridinium chloride (CPC), polyethoxylated tallow amine (POEA), benzalkonium chloride (BAC), benzethonium chloride (BZT), 5-Bromo-5-nitro-1,3-dioxane, dimethyldioctadecylammonium chloride, cetrimonium bromide and dioctadecyldimethylammonium bromide (DODAB). Zwitterionic (amphoteric) surfactants include for example dodecyl betaine, dodecyl dimethylamine oxide, cocamidopropyl betaine, and coco ampho glycinate. Nonionic surfactants include such as, but not limited to, alkyl poly(ethylene oxide), copolymers of poly(ethylene oxide) and poly(propylene oxide) (commercially called poloxamers or poloxamines), and alkyl polyglucosides, including, but not limited to, octyl glucoside, decyl maltoside, fatty alcohols, cetyl alcohol, oleyl alcohol, cocamide MEA, and cocamide DEA.

According to our experiments, SDS is the most efficient surfactant in foaming the fibre suspension, but the foam lasts only for a short time (Figures 9 and 10). In the manufacturing of web-like products such as paper and board, use of short-living foam can be advantageous. However, in the production of light-weight 3D products (such as heat and sound insulators) use of long-living foam is recommended. The 3D-structure of fibrous network should not collapse during the drainage of water (in the beginning the collapse of wet foam is dominated by drainage of liquid) and/or drying of the product. It is also known from previous studies that the increase in the foaminess coincides with faster foam decay and vice versa.

According to one preferred embodiment of the present invention, the low-molecular-weight surfactant is selected from the group consisting of sodium dodecyl sulphate (SDS), sodium laureth sulfate, sodium lauryl ether sulfate (SLES), ammonium lauryl sulfate (ALS) and sodium alkyl ether sulfonate (SAES). In the most preferred embodiment the low-molecular-weight surfactant is SDS.

It is known that at concentrations below CMC of SDS, hydrophobins dominate the interfacial properties (Zhang *et al.* 2011 and Stanimirova *et al.* 2014). In the presence of surfactants hexadecylterimethyl ammonium bromide, CTAB, SDS, and hexaethylene monododecyl ether, HFBII/surfactant complexes are formed. The binding of HFBII decreases the surfactant micelle aggregation number for increasing HFBII concentration in solution, and the number of hydrophobin molecules bound/micelle increases (Xiaoli *et al.* 2011). Thus according to the present invention hydrophobins can enhance the production of thicker sheets / special products by foam formation technique where drying takes time and stronger interfacial properties are required to keep the structure during drying. The present invention comprising hydrophobins in the foam composition thus strengthens the obtained foam (bubbles). One result of this strengthening is that the foam structure can be dried quicker, which makes the present method more time saving and also cost-effective compared to the known methods for obtaining foams.

Based on our data, a hydrophobin–surfactant mixture could result in a foaming product with increased value. In the mixture, SDS (or other low-molecular-weight surfactants) would still be responsible for quick foaming (good foamability), but hydrophobins as nanometer-sized particles would eventually replace the surfactants at the interface and stabilize and strengthen the foam structure resulting in improved foam stability.

Foaming with mixtures of samples containing different ratios of the foaming agents SDS and hydrophobin suggested that the SDS–hydrophobin ratio is important for foam stabilisation. For example, incorporation of 1:3 (w/w) of hydrophobins in the surfactant mixture resulted in foam with a volume comparable to that of the control, and which last for considerably longer time (hours vs minutes) than the foam which was prepared with SDS alone.

Preferably, the hydrophobin to SDS ratio is in the range from 1:2 to 1:10 (w/w). In the most preferred embodiment the hydrophobin to SDS ratio is 1:3 (w/w).

Based on the results obtained with wild-type and variant hydrophobins, foam stabilization can be achieved at least with wild-type hydrophobin class I or II hydrophobin and variants thereof. The concentration of hydrophobins in the supernatant from the protein production bioreactor is already high (>100 mg/l; Lienemann *et al.* 2015), and the hydrophobins in said supernatant can be used for foam formation instead of purified proteins in order to lower the production costs. Generally, the amount of surfactants (one or more different surfactant) in a mixture used for foam formation is about 0.06 % or 0.6 g/l. The amount of hydrophobin in the mixture can be from 0.06 to 2 g/l. More preferably the amount is 0.06 to 0.3 g/l. Most preferably the amount of hydrophobin is 0.2 g/l.

One benefit of this invention is that the hydrophobin–surfactant mixture allows that material properties like the product porosity and thickness can be tuned. More stable interfacial films protect the fibre-foam structure from collapsing during drainage of liquid and during drying. The use of proteins in fibre foaming also opens up a sustainable route for bringing desired functionality to the end product (for example by cross-linking the fibre network), and hence, enables the development/design of new products from natural resources.

Foam forming technology allows the inclusion of a wide variety of raw materials, such as nanoparticles, microfibrillated cellulose and flexible fibres over 25 mm in length. Therefore, different wood fibres and their mixtures can be used in the present invention. Fibres can be also recycled from used paper material. In addition, non-wood plant fibres such as straw, bagasse, bamboo, cotton and hemp can be used. Pulp mixtures can also contain a variable amount of synthetic fibres.

In the present invention surfactants (hydrophobins) and SDS are used in stabilization of fibre foams. Thermal or noise insulation and package cushions may be prepared from a variety fibre based materials. These materials may include glass fibers (fiberglass), polystyrene, polyurethane foam, vermiculite, cellulosic fibers (e.g. wood fibers, cotton fibers, etc.). The preferred fibers according to the present invention are wood fibers.

In a preferred embodiment the fiber is selected from the group consisting of wood, soft wood, linen and cotton. In the most preferred embodiment, bleached never dried soft wood Kraft pulp (pine) is used.

The foam forming mixture can further comprise additives, such as retention aid, filler, hydrophobizing agent, and wet and dry strength chemicals.
In one embodiment of the invention is the method for producing an open-cell foam, wherein the mixture further comprises additives such as retention aid, filler, hydrophobizing agent, and wet and dry strength chemicals.

An open-cell foam comprising low-molecular-weight surfactant, hydrophobin and fibers is one aspect of the invention. The open-cell foam comprising low-molecular-weight surfactant, hydrophobin and fibers, wherein the foam further comprises additives such as such as retention aid, filler, hydrophobizing agent, and wet and dry strength chemicals, is another aspect of the invention. Preferably, the density of said foam is between 5 to 100 kg/m^3, and specific air flow resistance is from 3 to 500 kPas/m^2.

Other objects of the present invention are insulating material, noise and/or thermal insulation material, and a package cushioning material comprising the open-cell foam comprising low-molecular-weight surfactant, hydrophobin and fibers. Optionally, these materials comprise also other above listed additives.

Further object of the invention is the use of the open-cell foam according to the invention in insulating materials – such as noise and thermal – and in package cushioning.

EXAMPLES

EXAMPLE 1

Materials and Methods

Reagents and Chemicals

HFBI variants were produced in *T. reesei* and purified from culture supernatant by reversed-phase chromatography as described below. The plasmid pTNS27 was provided by T. Nakari-Setälä (VTT, Finland; (Askolin et al 2005)). The *T. reesei* protein production strain M219 was obtained from M. Saloheimo (VTT, Finland).

Production of HFBI variants in Trichoderma reesei

HFBI variants were produced in the genetically modified *Trichoderma reesei* strains VTT D-121448 (-D40Q/D43N/R45Q/K50Q), VTT D-121449 (-D40Q/D43N), VTT D-121450 (-R45Q/K50Q) and VTT D-121451. The production was performed in 1 l Trichoderma minimal medium (TrMM), which had the following composition: 0.11 M KH_2PO_4, 38 mM $(NH_4)_2SO_4$, 2.4 mM $MgSO_4$, 4.1 mM $CaCl_2$, 0.18 mM $FeSO_4 \times 7\ H_2O$, 95 µM $MnSO_4 \times H_2O$, 49 µM $ZnSO_4 \times H_2O$, 0.16 mM $CoCl_2 \times 6\ H_2O$ and 2% (w/v) glucose, pH 5.2) supplemented with 4% (w/v) lactose and 2% (w/v) spent grain extract. The fungal cultivation was performed in shake flasks during seven days at 28°C with 200 rpm shaking after which the culture liquid was stored frozen until further processing.

Purification of HFBI variants from Trichoderma reesei culture supernatant

Purification of the HFBI variants was performed using successive purification in an aqueous two-phase system (ATPS) and by reversed-phase chromatography essentially as described in Paananen *et al.* 2003. Initially, the frozen fungal culture liquid was thawed in a water bath at 22°C for 1 h and insoluble material was removed by consecutive centrifugation at 4400×g for 25 min and gravity filtration of the supernatant through grade 597-1/2 quantitative folded filter paper with 4-7 µm pore size and a diameter of 320 mm (Whatman GmbH, Germany). The pH of the filtered solution was adjusted to 5 by addition of 1 M sodium acetate solution. To the supernatant was added Berol 532 detergent (AkzoNobel N.V., The Netherlands) to a final concentration of 2% (w/v). The solution was mixed by gentle shaking and allowed to separate in a separation funnel. The detergent phase was isolated followed by the addition of 20 ml of 50 mM sodium acetate buffer (pH 5.15) containing 40 mM EDTA were and 0.18 ml 2-methylpropan-1-ol. The mixture was mixed gently and the phases were allowed to separate in a separation funnel. The lower phase was purified further by preparative reversed-phase chromatography using a Vydac C4 (1 × 20 cm) column and a gradient elution from an aqueous solution containing 0.1% trifluoroacetic acid to 100% acetonitrile containing 0.1% trifluoroacetic acid. HFBI variant proteins eluting as fractions with a high absorption at λ=230 nm were pooled and lyophilized.

Size-exclusion chromatography (SEC)

HFBI variants dissolved in 100 µL buffer were injected at varying concentrations into a Superdex 75 column (Amersham, Sweden) and eluted with 10 mM Na-acetate buffer at pH 5.5 containing 0.2 M NaCl using an Äkta Explorer (Amersham, Sweden) HPLC system. Protein elution was detected at λ = 230 nm. Reference proteins were injected to correlate the elution time with molecular weight. The references used were vitamin B-12 (1.4 kDa, Sigma, Germany), aprotinin (6.5 kDa, Sigma, Germany), ribonuclease A (13.7 kDa, Amersham Pharmacia Biotech, Sweden), and ovalbumin (43.0 kDa, Amersham Pharmacia Biotech, Sweden).

Measurement of Isoelectric Point

The samples were prepared by dissolving wild-type and variants of HFBI in ddH_2O at a concentration of 0.1 mg/mL, and the pH was adjusted using 100 mM and 1.0 M HCl and 10 and 100 mM NaOH. The zeta-potential was measured using a Zetasizer Nano-ZS instrument (Malvern, UK) and a sample volume of 1.0 ml.

Drop flattening measurements

The time required for plateau formation at a hydrophobin containing drop was measured with an optical contact angle and surface tension meter (CAM 200, KSV NIMA, Finland). This was done by dissolving the studied protein dissolved in ddH_2O and pipetting a 50 µL drop of this solution onto a Parafilm sheet. Pictures were taken with 60 s interval in order to detect initial drop flattening moment, at which the drop started to form a plateau. The reported drop flattening times were determined as the average of three measurements.

Formation and static analysis of HFBI monolayers

Formation of HFBI variant monolayers was analysed in a humidified atmosphere using a Langmuir trough surface pressure sensor and pre-soaked 20.6 mm perimeter Wilhelmy paper plates (KSV NIMA, Finland). Surface pressure was monitored by probing 20 ml of 5 mM Na-acetate (pH 5.5) in a glass beaker that has been equilibrated to room temperature. The sample protein was dissolved at a concentration of 0.85-1.0 µM by short magnetic stirring (1.5–2 min) and the change of surface pressure was monitored until an equilibrium was reached (typically after 30 min to 1 h).

HFBI layer compression assay using the Langmuir trough

Monolayers of HFBI variants were assembled and its structural properties were measured using a KSV Minimicro trough (KSV NIMA, Finland). Protein layers were prepared at the air–water interface by injecting 20 µg dissolved protein into 55 ml of 5 mM Na-acetate buffer (pH 5.5) at 21°C. This solution was left for 45 min in order to allow the hydrophobin proteins to reach an equilibrium between an immersed state and the air–water interface assembly. Compression/expansion cycle isotherms were recorded while moving the barriers at a constant rate of 2 mm/min and switching to expansion at 35 mN/m surface pressure.

Interfacial Rheology

The interfacial shear rheological properties of wild-type HFBI and HFBI variants at the air–water interface were measured at RT using a DHR-2 rheometer (TA Instruments, U.K.) equipped with a Pt–Ir du Noüy ring (13 mm diameter, Krüss, Germany, flamed prior to use) in oscillatory mode. The concentrations of stock solutions of HFBI and HFBI variants were determined by UPLC. A 0.3 μM solution (≈ 2.3μg/ml = 0.00023% (w/v)) of HFBI (or variant) in Milli-Q water was prepared to a 50 ml volumetric flask and let to temper to RT (22°C) for 1 h prior to experiments. The solution was placed in a glass dish of 60 mm diameter and the du Noüy ring was placed onto the surface according to the manufacturer's instructions. The interfacial shear elastic (storage) modulus, G', and viscous (loss) modulus, G'' were monitored at constant frequency of 0.1 Hz and constant strain of 0.1%, during film formation for at least 2 h to reach the equilibrium. The chosen frequency and strain were measured to be in the linear viscoelastic region. Each measurement was replicated at least twice.

Atomic Force Microscopy (AFM)

A NanoScopeV Multimode8 AFM (E scanner, Bruker) and NCS15/AlBS cantilevers (μMasch, USA) were used in all measurements. All images were recorded in tapping mode in air with scan rates 0.7–1 Hz (free amplitude about 0.68V, damping ratio 0.7–0.8). Images were only flattened to remove possible tilt in the image data (NanoScope Analysis), and no further processing was done. A scanning probe image processor (SPIP, Image Metrology, Denmark) was used for image analysis. The topography and phase contrast images were captured simultaneously. The phase contrast image shows the phase difference between the oscillations of the cantilever driving piezo and the detected oscillations. It is thought that image contrast is derived from surface properties such as stiffness and viscoelasticity (hard tapping) or hydrophilicity/hydrophobicity (light tapping), but it also shows enhanced edge structures.

Results

The presented experiments were intended to improve the understanding of the molecular basis for hydrophobin monolayer formation and for the adsorption of secondary proteins to hydrophobin monolayers. In particular, this study addresses the roles of charged hydrophobin residues that are involved in this process. These can be classified in two categories, e.g. the solvent exposed residues D30, K32, D40, D43, R45 and K50 (Figure 1A) and the residues D30 which K32 that are inaccessible to the solvent due to their location inside the protein layer in interaction (Figure 8). The charged residues proximal to the hydrophobic patch (D30 and K32) were also identified in computational studies as potentially important for intermolecular interactions (Magargar et al) (Figure 1B).

Production of HFBI variants with substituted charged amino acids

In order to study the roles of charged HFBI side chains, *Trichoderma reesei* strains were created with modified chromosomal DNA that contained added HFBI genes in which charged residues were substituted with sterically similar but uncharged homologues. Prior to these gene insertions, the gene *hfb2* encoding for the naturally secreted hydrophobin HFBII had to be disrupted because it would interfere with the HFBI variant purification. This was performed by insertion of a hygromycin resistance cassette in the *hfb2* gene of the *T. reesei* protein production strain M219. The cassette was amplified from plasmid pTNS27 essentially as described in (Penttilä *et al.* 1987). Knock-out mutants were isolated by initial plating on hygromycin-containing top agar [150 µg/ml] and consecutive transfer to agar plates containing 0.1% (w/v) Triton X-100 and hygromycin [125 µg/ml]. The *hfb2* gene disruption was confirmed by PCR (Phire Plant Direct PCR kit (Thermo, Finnzymes F-130)) using two probes that were complementary to the *hph* resistance marker (SEQ ID NO:3; hph_sekv 1: 5'-ATTCTTCGCCCTCCGAGAGC-3') and the *hfb2* region located upstream of the deletion cassette insertion (SEQ ID NO:4; Mil73_fw: 5'-GAATCATGCTGGAGTGAAGG-3'). In addition, the absence of HFBII in the culture supernatant was confirmed by Western analysis (4-20% acrylamide Criterion gradient gel, Bio-Rad with HFBII-specific polyclonal antibodies Data not shown). The *T. reesei hfb2::hph* strain was deposited at the VTT culture collection under the ID D-121447.

The strain D-121447 was used for cloning of the HFBI variants using homologous recombination. This technique replaced a part of the highly expressed *cbhI* gene with an insertion cassette containing the acetamide resistance gene *amdS* and a HFBI variant gene downstream of the *cbhI* promotor. Four vectors containing this cassette were produced that differed in the encoded HFBI variant (E.g.: pMil003: -D40Q/D43N/R45Q/K50Q, pMil004: -D40Q/D43N, pMil005: -R45Q/K50Q and pMil006: -D30N/K32Q). The homologous recombination and clone selection was performed as described above with the exception that acetamide [10 mM] was used as a selective antibiotic. Acetamide-resistant clones were analysed for HFBI variant gene insertion by sequencing using primers that bind to the CBHI promoter (SEQ ID NO:5; T010_Cbh1 prom 5′ sekv: 5′-CAACTCAGATCCTCCAGGAGAC-3′) and CBHI terminator (SEQ ID NO:6; T043_Cbh1_term_R sekv: 5′-TCATGATACGGGCTCACCAAG-3′). Secretion of HFBI variants was confirmed by subjecting culture supernatants to Western blot analysis using polyclonal HFBI-specific antibodies. The obtained HFBI-variant secreting *Trichoderma reesei* strains were deposited at the VTT Culture collections with the IDs D-121448 (-D40Q/D43N/R45Q/K50Q), D-121449 (-D40Q/D43N), D-121450 (-R45Q/K50Q) and D-121451(-D30N/K32Q).

T. reesei variants were secreting the HFBI variants to the supernatant (Figure 6) at high concentrations that could be purified by the ATPS method yielding 100–500 mg per litre of shake flask culture (D30N/K32Q: 244.7 mg/l, D40Q/D43N: 98.5 mg/l, R45Q/K50Q: 390 mg/l and D40Q/D43N/R45Q/K50Q: 504 mg/l). Zeta-potential measurements showed that the introduced amino acid substitutions resulted in changes of the isoelectric point as expected according to the type of the modified acid (Figure 2 and Table 1).

Table 1. Experimentally determined molecular weight (MW) according to MALDI-TOF mass spectrometry, isoelectric point (IEP) determined from duplicate measurements with theoretical value based on amino acid sequence given in brackets. The hydrophobically adsorbed mass (HAM) was determined from the presented QCM experiments on hexanethiol coated surfaces. The molecular area (MA) and the surface tension of HFBI variant solutions (γ) were determined from interfacial HFBI protein layers assembled in a Langmuir trough.

	D40Q/D43N	R45Q/K50Q	D30N/K32Q	D40Q/D43N/R45Q/K50Q	wild-type
MW [Da]	7546	7506	7533	7517	7535
IEP	7.0 (8.2)	3.6 (3.9)	5.8 (5.7)	6.2 (5.5)	6.1 (5.7)
HAM [ng/cm^2]	289 ± 45	217 ± 35	247 ± 35	293 ± 56	223 ± 98
MA [Å^2]	323 ± 34	324 ± 55	322 ± 52	343 ± 38	323 ± 51
γ [mN/m]	31.3 ± 7.5	36.0 ± 0.6	36.5 ± 0.0	36.6 ± 0.3	35.5 ± 1.6

Size-exclusion chromatography (SEC)

The concentration-dependent aggregation of the studied HFBI variants was studied by dissolving the protein at concentrations ranging between 3 µM and 1 mM, separation over a SEC column and monitoring their elution. At concentrations below 30 µM, the four HFBI variants and the wild-type were all at around 5 kDa corresponding to the monomer (7.5 kDa). Larger aggregates with a molecular weight corresponding to hexamers were formed when the hydrophobin concentration was increased above 100 µM, (Figure 3). The transition between these two states appeared to resemble the wild-type in the case of all tested variants.

Assembly of hydrophobin variant layers at the air-water interface

The surface activity of hydrophobins can be easily observed when a drop of hydrophobin solution is left to evaporate and a plateau formation can be observed at the top facing part of the drop due to the concentration-dependent formation of a hydrophobin monolayer at the air-water interface. In order to test the ability of the HFBI variants to participate in this process, droplets of hydrophobin solution [0.1 mg/ml in 5.0 mM Na acetate buffer (pH 5.5)] were left to evaporate and the time necessary for plateau formation determined using the CAM apparatus. For all four variants, the time until drop flattening (Table 1) was 30-70% longer than the incubation period required by the wild-type (19 ± 3.5 min).

The molecular area occupied in an HFBI variant layer at the air–water interface was determined with the Langmuir-Blodgett apparatus by measurement of the 2nd compression isotherm. Here, no clear difference in molecular area could be seen to the wild-type HFBI (3.23 ± 0.51 nm^2) (see Table 1).

In an additional Langmuir-Blodgett apparatus experiment, the surface tension reduction upon HFBI variant layer formation at the air-water interface was measured. No significant difference could be seen in surface tension reduction between the variants (D40Q/D43N: 31.3 ± 7.5 mN/m, R45Q/K50Q: 36.0 ± 0.6 mN/m, D40Q/D43N/R45Q/K50Q: 36.6 ± 0.3 mN/m, D30N/K32Q: 36.5 ± 0.0 mN/m) and the wild-type (35.5 ± 1.6 mN/m) (see Table 1).

Interfacial rheology of hydrophobin variant layers

The adsorption of wild-type HFBI and HFBI variants to the air–water interface was monitored and viscoelastic properties determined by interfacial shear rheology measurements. The storage modulus (elastic, G′) and loss modulus (viscous, G″) of the interfacial hydrophobin layers are shown in Figures 4A and 4B, respectively. At equilibrium the shear storage modulus, G′, for wild-type HFBI reached a value of 1.04 ± 0.01 N/m, which is comparable to previously reported values for HFBI (0.7 N/m in Cox *et al.* 2007)). The corresponding values for the HFBI variants D30N/K32Q, D40Q/D43N, R45Q/K50Q and D40Q/D43N/R45Q/K50Q are 0.85 ± 0.10 N/m, 0.62 ± 0.01 N/m, 1.44 ± 0.03 N/m and 1.09 ± 0.01 N/m, respectively. The G′ values are in the similar range or less than for wild-type HFBI, except the high value for R45Q/K50Q variant that showed ~40% increase in the elastic modulus. The equilibrium shear loss modulus values, G″ (Figure 4B) for all hydrophobins were less than 0.06 N/m. Tanδ values at equilibrium were very low, less than 0.1, for all hydrophobins showing dominance of the G′ values in all cases.

AFM imaging of hydrophobin variant layers as Langmuir-Blodgett films

AFM images of Langmuir-Blodgett films (LB-films) of all HFBI-variants showed regular ordered raft-like structures (Figure 5A–D) of different variant-specific dimensions (e.g.: D30N/K32Q: ~150x150 nm, D40Q/D43N: ~120x120 nm, D40Q/D43N/R45Q/K50Q: ~350x350 nm and R45Q/K50Q: ~800x800 nm). It is possible that at the air-water interface all protein films consisted of larger crystalline domains, which broke down to smaller rafts during film transfer onto mica. The crystallinity of the protein films was confirmed by Fourier transformation of the phase contrast image data. The oblique structures of all HFBI-variants possessed unit cell vector dimensions of 5.4 ± 0.9 nm for a and b, and 120 ± 12° for Y. The deviation of the lattice constants from pure hexagonal packing (a=B and Y=120°) and differences between different samples can be explained by scanner hysteresis, creep and drift in the AFM. These phenomena become more dominant when imaging in relatively slow scan speeds.

EXAMPLE 2

Results with class II hydrophobin variants according to Lienemann *et al.* 2015 where that variant HFBI-R45Q/K50Q forms the strongest film, while with variant HFBI-D40Q/D43N/R45Q/K50Q (containing substitutions D40Q, D43N, R45Q, and K50Q) strong film and best production level were achieved. These variants were tested in 10 ml volume with 0.6% (w/v) fibre suspension comprising bleached never dried soft wood Kraft pulp (pine). 0.6 ml in total of 1% surfactant was added to 10 ml resulting in total volume 10.6 ml and surfactant concentration 0.57g/l = 0.057 %.

Total surfactant concentration was kept the same in all samples, but the mass ratio between SDS and HFBI variant varied. The ratios (w/w) tested were as follows: HFBI-variant to SDS : 0 (SDS only), 1:10, 1:3 and 1 (HFBI variant only).

Mechanical mixer (Dispermat) was used to mix hydrophobin, SDS and fibers. Mixer speed was adjustable, but generally about 3800 rpm used in the experiments. Foam volume was studied as a function of time. Foaming reached the maximum level within 1 min with this set-up. The change of foam volume was monitored by photography, where pictures were taken every 10 s. From the photographic images, the mixing times required for reaching 50% gas volume (t50) and 66% gas volume (t66) were determined. In addition, the maximal volume achieved (Φ_{max}) was determined. Collapse rate of foam was measured with 10 min recording, whereas pictures were taken every 1 min. From these data t½ for the foam can be estimated. The t½ value describes the time elapsed after cessation of mixing when liquid volume reaches half of its initial height (drainage volume).

Results

Figures 9 and 10 show results for foam forming ability and foam stability from six parallel experiments when SDS only is used. Figures 11 and 12 show corresponding results for two experiments obtained with mixture, where HFBI-R45Q/K50Q to SDS ratio is 1:10 (w/w). Similar results were obtained when HFBI-D40Q/D43N/R45Q/K50Q was used instead of HFBI-R45Q/K50Q.

Table 2 shows data for samples SDS only (0.057%), HFBI-R45Q/K50Q to SDS 1:10 or 1:3, HFBI-R45Q/K50Q only (0.057%), HFBI-D40Q/D43N/R45Q/K50Q to SDS 1:10 or 1:3 (w/w), and HFBI-D40Q/D43N/R45Q/K50Q only (0.057%). Liquid volume was 10.6 ml and total surfactant-protein concentration 0.057% (w/v) in all experiments.

Sample	t50 (s)	t66 (s)	Φmax (ml)	t½ (min)
SDS 0.057%	6.5±3.4	11.3±6.3	83±16.9	5.0±0.9
4550:SDS = 1/10	15±7.1	22.5±9.2	72.5±9.2	3.7±0.3
4550:SDS = 1/3	12.5±6.4	27.0±19.8	66.0±26.8	3.7±0.3
4550 0.057%*	~30	nr	24	< 1**
4x:SDS = 1/10	8.3±2.5	21.0±14.1	68.5±37.5	4.0±1.3
4x:SDS = 1/3	10.3±5.3	16.8±9.5	68.0±24.0	5.0±1.5
4x 0.057%*	~25	nr	25	<1**

4550 = HFBI-R45Q/K50Q, 4x = HFBI-D40Q/D43N/R45Q/K50Q, nr = not reached
* Single measurement, ** no clear interface between liquid and foam

After foamability and foam stability measurements, foams were pipetted to separate tubes, and the foam volume was monitored as a function of time. The time points show the time past after pipetting from the measuring glass. These results are presented in Figure 13.

Conclusions

The t50 and t66 values showed that SDS alone in fiber suspension was the fastest in foam formation compared to hydrophobins and hydrophobin-SDS -mixtures. The maximum fibre foam volumes (Φ_{Max}) were the highest with SDS, whereas with hydrophobin-SDS mixtures the maximum foam volumes were 80-85% of the corresponding values obtained for SDS alone. Foam volumes with bare hydrophobins were not high, being less than 30% of the maximum volume with SDS alone. Pipetting of fibre suspension seemed to affect greatly to foaming in studied experimental scale. The water drainage (shown by t½ values) was slightly faster in samples containing hydrophobins. In general, similarities in results with HFBI-R45Q/K50Q and HFBI-D40Q/D43N/R45Q/K50Q were seen. Based on these observations we can conclude that hydrophobins as such cannot replace SDS in the studied concentrations. However, the most interesting and promising results were obtained by monitoring the volume of the remaining fibre foam after foaming experiments. The fibre foams with SDS/hydrophobin mixtures lasted for longer time than with SDS indicating of higher strength. Where fiber foam with SDS fully collapsed within 50 minutes, the fiber foam volume with SDS-hydrophobin (HFBI-50R45Q/K50Q variant) was still approximately 85% of the original volume after four and a half hours. Even though the fiber foams with bare hydrophobins did not reach high volumes, the thick foams were still existing after one week.

EXAMPLE 3 A Larger-scale investigation

Similar experiments as described in example 2 were performed in larger scale with Class II hydrophobin HFBI-variant (HFBI-R45Q/K50Q) (Lienemann et al 2015). Hydrophobin and SDS with varying concentrations were tested in 400 ml liquid volume with 0.6 – 2.0% (w/v) fibre suspension comprising bleached never dried soft wood Kraft pulp (pine).

Foamability refers to the ease of foam generation and is a dynamic property of the foam-generating power of a liquid and the factors that help to attain immediate stabilisation of foam. On the other hand, foam stability refers to the time foam exists without significant changes in the bubble size distribution (BSD) of foam. In this study, we concentrate on wet foams, with large volumes of interstitial liquid between spherical bubbles. Therefore, in the beginning, the collapse of wet foam is dominated by drainage of liquid into the foam base. Foam stability is often measured using the time taken for foams to collapse. In this study, foaming dynamics (generation & collapse) were recorded with a CCD-camera. Images were acquired at 3 s interval. The volume of the foam-fibre mixture produced, and volume of drained liquid was monitored as a function of time. Foam stability was characterised by determining the time elapsed after cessation of mixing when the liquid volume reaches half of its initial height. Figure 14 shows the experimental set-up in the lab.

Fibre consistency was 0.6 – 2.0%. A transparent vessel (Ø 84 mm) containing the furnish was placed under the Netzsch Drill Pulp Agitator where it was clamped in (see Figure 1). The mixer speed was adjustable from 500 rpm to 6000 rpm. The mixing plate (Ø 53 mm) was placed just below the surface of the slurry before mixing so as to ensure the most effective mixing.

Figure 15 presents three images from the acquired sequence of images. The acquired image sequence (usually 300-700 images) was analysed afterwards using a semi-automatic method. It was assumed that the shape of the foam volume vs. time and water volume vs. time curves were linear between the measured data points. In the foamability curve, t=0 s corresponds the moment when the mixer was started, and in the foam stability curve, t=0 s corresponds to the moment when the mixer was stopped (Figure 15). The following quantitative measures of foamability and foam stability were calculated: $t(_{\phi 50\%})$: mixing time needed to reach 50% gas volume, $t(_{\phi 66\%})$: mixing time needed to reach 66% gas volume, ϕ_{Max}: maximum gas volume during the mixing period, ϕ_{End30s}: Air content 30 s after the cessation of mixing and $t_{1/2}$: half life time of foam.

The air content (Φ) and bubble size distribution (BSD) determine many important properties of the foam, such as viscosity, stability and drainage. In this study, BSD of the foam-fibre mixture was determined by collecting foam samples by dipping the cuvette (a Hele-Shaw cell, the internal diameter of 1.6 mm) into the foam. Foam samples were collected just before the cessation of mixing. Then, the cuvette was placed on a backlight panel, and images of the cuvette were taken with a camera. BSD curves were obtained by analysis of a large number of bubbles (400-800 bubbles) with an image analysis method. The Sauter mean radius r[3,2] was used for characterising the BSD of foam (Lappalainen and Lehmonen 2012).

The hydrophobin concentration in the drained liquids was determined with Ultra Performance Liquid Chromatography equipped with C4 column (Waters ACQUITY UPLC

1 Class CM-A System). Native HFBI was used as a standard. Retention of HFB (%) values were calculated by assuming that the rest of the original hydrophobin amounts remained in the foam.

Results

The foamability and foam stability results of HFBI-variant HFBI-R45Q/K50Q and SDS mixtures with pine Kraft pulp fibre suspension are listed in Table 3. T50 and T66 values show that changes in foam forming ability is not significantly changed when using hydrophobin-SDS mixtures with 2% fibre consistency. Also the maximum foam volumes (ϕ_{Max}) were at the same level. However, when comparing the t½ values, hydrophobins had a clear effect on the foam stability. The retention of hydrophobin in the foam was found to be critical for the foam stability (Figure 16). The more hydrophobin in the foam, the more stable was the foam. One very important factor for this is the amount of SDS. At 0.6 g/l concentration, the Gibbs elasticity reaches its maximum value and hence, the displacement process of small molecular weight SDS at the interface can be slow. At somewhat lower SDS concentrations the foam forming ability is not significantly hampered, but the effect on retention of hydrophobin is remarkable. When using 0.4 g/l of SDS and 0.2 g/l hydrophobin with 2% fibre consistency, the t½ value was 2.3 times (130%) higher than the corresponding value of the reference sample without hydrophobin.

Average bubble size (r[3,2]) of fiber-foam mixture made using hydrophobins is smaller (about 15%) than that without hydrophobin addition. Therefore, it can be assumed that hydrophobins stabilises the foam already during the foam generation and not just after the cessation of mixing.

Table 3. The foamability and foam stability of HFBI-variant HFBI-R45Q/K50Q and SDS mixtures with pine Kraft pulp fibre suspension.

Trial point	Furnish	Consistency	pH	Surfactant	Mixing speed and time	t50% (s)	t66% (s)	Ømax (%)	ØEnd30 (%)	t½ (s)	r[3,2] (µm)	Retention of HFB
REF without HFB	Softwood pulp	2.0%	6,6	SDS 0.4 g/l	4500 rpm, 3 min	10,6	73,7	69,3	68,4	630	58	0
Addition of HFB to pulp suspensio	Softwood pulp	2.0%	6,6	SDS 0.4 g/l + HFBIm 0.2 g/l	4500 rpm, 3 min	9,0	76,1	69,5	68,5	1475	48	86,5
Addition of HFB to foam-fibre mixture (at Φ=50%)	Softwood pulp	2.0%	6,6	SDS 0.4 g/l + HFBIm0.2 g/l	4500 rpm, 3 min	8,0	69,3	70,2	69,5	1502	49	91,4
Effect of pH	Softwood pulp	2.0%	5,0	SDS 0.4 g/l + HFBIm 0.2 g/l	4500 rpm, 3 min	11,5	64,0	69,9	68,8	1219	48	72,6
Effect of SDS/HFBI ratio	Softwood pulp	2.0%	6,6	SDS 0.6 g/l + HFBIm 0.4 g/l	4500 rpm, 3 min	8,1	63,5	70,0	69,4	1092	49	24,5
Effet of pulp consistency	Softwood pulp	0.6%	6,6	SDS 0.4 g/l + HFBIm 0.2 g/l	4500 rpm, 3 min	8,2	16,8	77,0	76,4	1103	58	84,3

HFBIm = HFBI-R45Q/K50Q variant (Lienemann et al 2015).

Conclusions

The use of hydrophobin as a mixture with SDS when foaming fibre suspension has a clear effect on the foam stability. The most important factor seems to be the amount of SDS: The amount should be high enough for foaming the fibre suspension, but low enough to allow hydrophobins to reach the air-water interface for stabilizing the foam.

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CLAIMS

1. A method for producing an open-cell foam, which comprises a step of introducing gas to an aqueous solution comprising a low-molecular-weight surfactant, hydrophobin or a variant thereof and fibers.
2. The method according to claim 1 further comprising a step of dewatering the obtained foam.
3. The method according to claim 1 or 2, wherein gas is introduced by mechanical mixing, blowing gas through a thin nozzle, blowing gas through a porous plug, or by the nucleation of gas bubbles in a liquid which is supersaturated.
4. The method according to any of claims 1 to 3, wherein hydrophobin is selected from class I and class II hydrophobins.
5. The method according to claim 4, wherein hydrophobin is class II hydrophobin.
6. The method according to claim 5, wherein class II hydrophobin is selected from the group consisting of wild-type HFBI, HFBII, SRHI and a variant thereof.
7. The method according to any of claims 1 to 6, wherein the variant comprises an amino acid sequence having a substitution of one or more charged amino acid residues against sterically related non-charged homologues in amino acid sequence of the mature hydrophobin HFBI of SEQ ID NO:2.
8. The method according to claim 7, wherein said substitution or substitutions is/are at positions 30, 32, 40, 43, 45 or 50 of SEQ ID NO:2.
9. The method according to claim 8, wherein hydrophobin is a variant that comprises an amino acid sequence set forth in SEQ ID NO:2 with one or more substitutions selected from the group consisting of D30N, K32Q, D40Q, D43N, R45Q, and K50Q, wherein the positions of the amino acid substitutions are numbered in reference to the positions in SEQ ID NO:2.
10. The method according to claim 9, wherein the variant comprises an amino acid sequence set forth in SEQ ID NO:10 (R45Q/K50Q) or SEQ ID NO:9 (D40Q/D43N/R45Q/K50Q).
11. The method according to any of claims 1 to 10, wherein the concentration of the hydrophobin in the aqueous solution is from 0.06 g/l to 2 g/l.

12. The method according to any of claims 1 to 11, wherein the low-molecular-weight surfactant is selected from the group consisting of sodium dodecyl sulphate (SDS), sodium laureth sulfate, sodium lauryl ether sulfate (SLES), ammonium lauryl sulfate (ALS) and sodium alkyl ether sulfonate (SAES).

13. The method according to claim 12, wherein the low-molecular-weight surfactant is SDS.

14. The method according to any of claims 1 to 13, wherein the hydrophobin to SDS ratio is in the range from 1:2 to 1:10 (w/w).

15. The method according to any of claims 1 to 14, wherein the hydrophobin to SDS ratio is 1:3.

16. The method according to any of claims1 to 15, wherein the fiber is selected from the group consisting of wood, soft wood, linen and cotton.

17. The method according to any of claims 1 to 16, wherein the mixture further comprises additives such as retention aid, filler, hydrophobizing agent, and wet and dry strength chemicals.

18. An open-cell foam comprising a low-molecular-weight surfactant, hydrophobin and fibers.

19. The open-cell foam according to claim 18, wherein the foam further comprises additives such as retention aid, filler, hydrophobizing agent, and wet and dry strength chemicals.

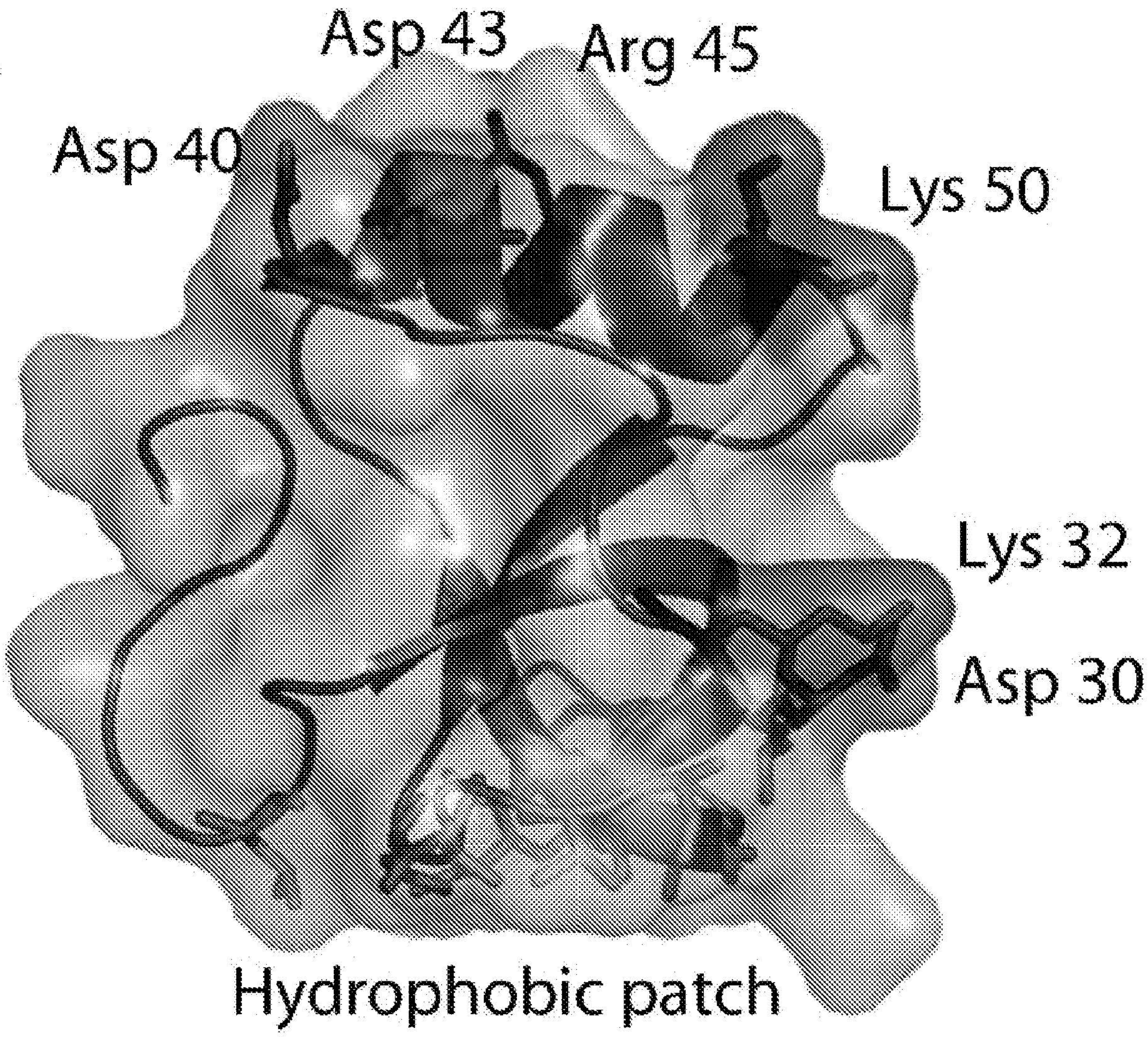
20. The open-cell foam according to claim 18 or 19, wherein the density of said foam is between 5 to 100 kg/m^3, and specific air flow resistance is from 3 to 500 kPas/m^2.

21. An insulating material which comprises the open-cell foam according to any of claims 18 to 20.

22. Noise and/or thermal insulation material which comprises the open-cell foam according to any of claims 18 to 20.

23. A package cushioning material which comprises the open-cell foam according to any of claims 18 to 20.

24. Use of the open-cell foam according to any of claims 18 to 20 in an insulating material, such as noise or thermal insulation material, or in package cushioning material.



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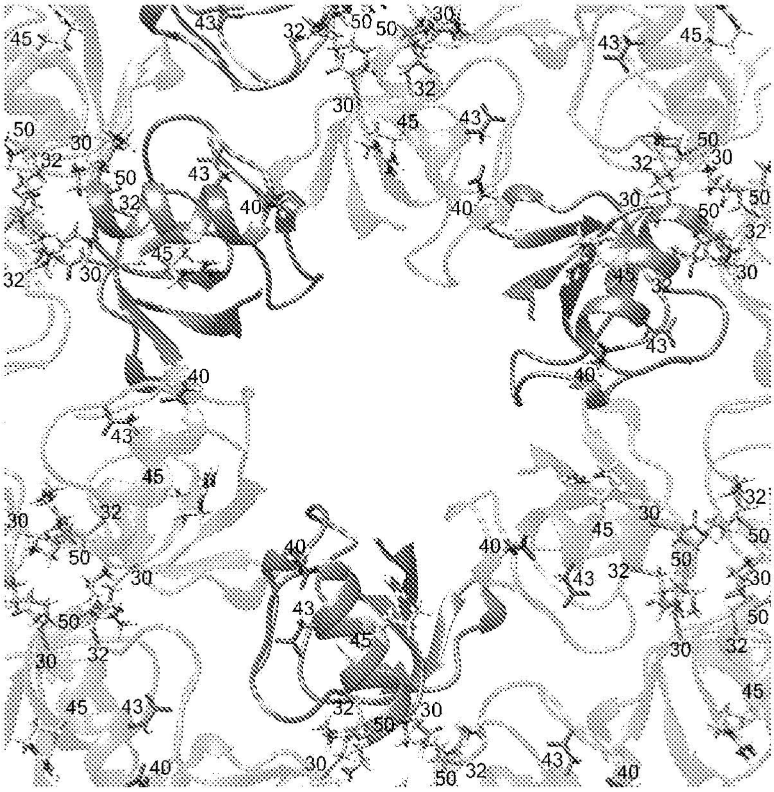
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50
30
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43
40

Figure 1B

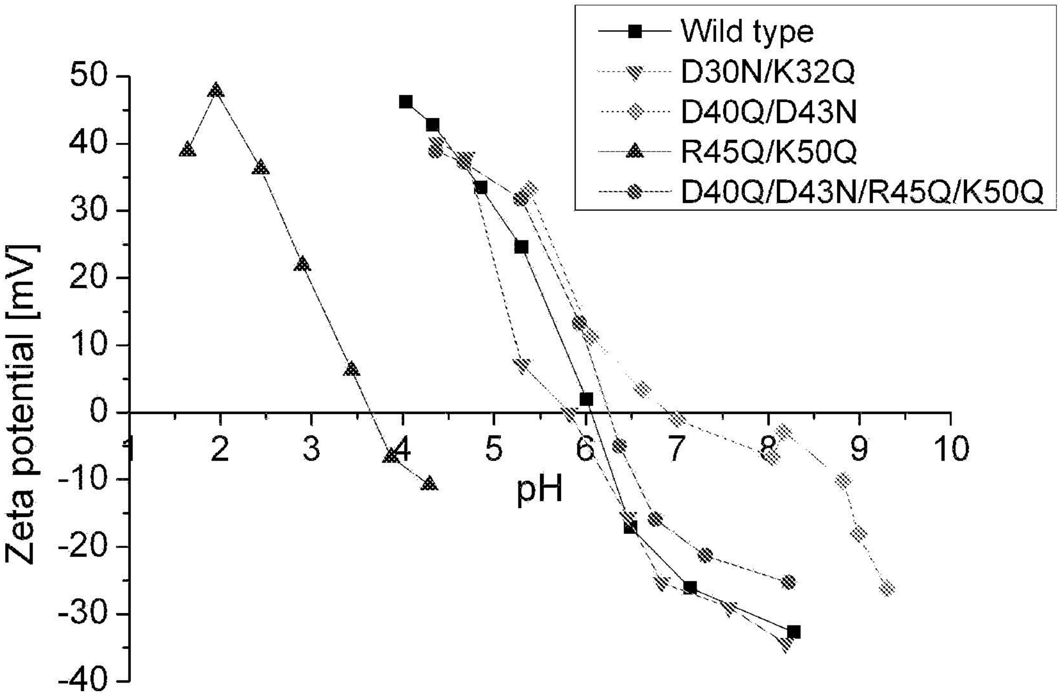
Wild type
D30N/K32Q
D40Q/D43N
R45Q/K50Q
D40Q/D43N/R45Q/K50Q
Zeta potential [mV]
50
40
30
20
10
0
-10
-20
-30
-40
pH
1
2
3
4
5
6
7
8
9
10

Figure 2

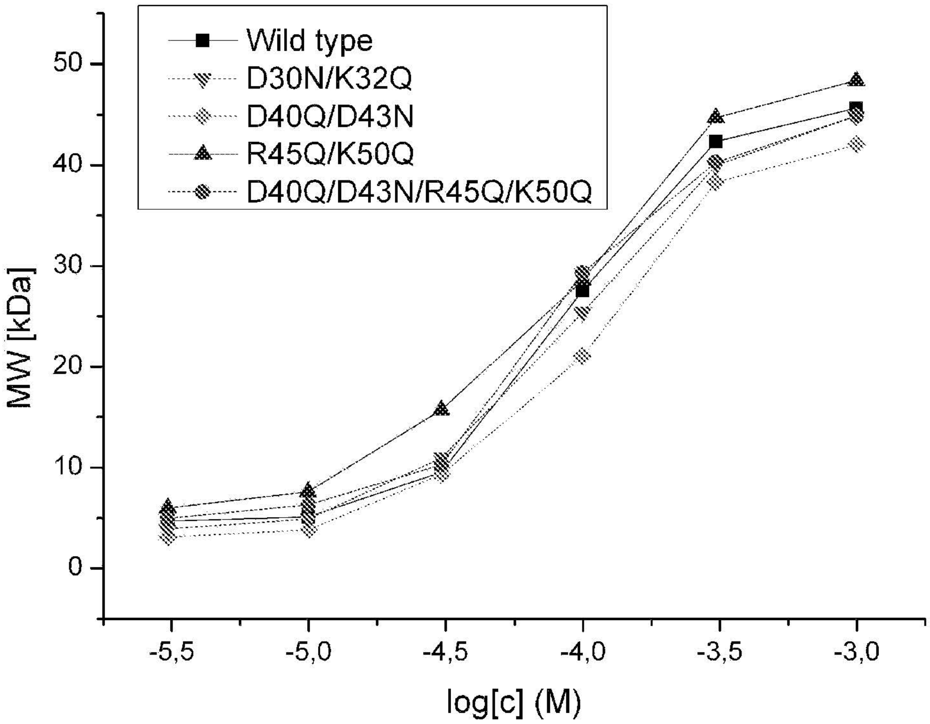
Wild type
D30N/K32Q
D40Q/D43N
R45Q/K50Q
D40Q/D43N/R45Q/K50Q
MW [kDa]
0
10
20
30
40
50
-5,5
-5,0
-4,5
-4,0
-3,5
-3,0
log[c] (M)

Figure 3

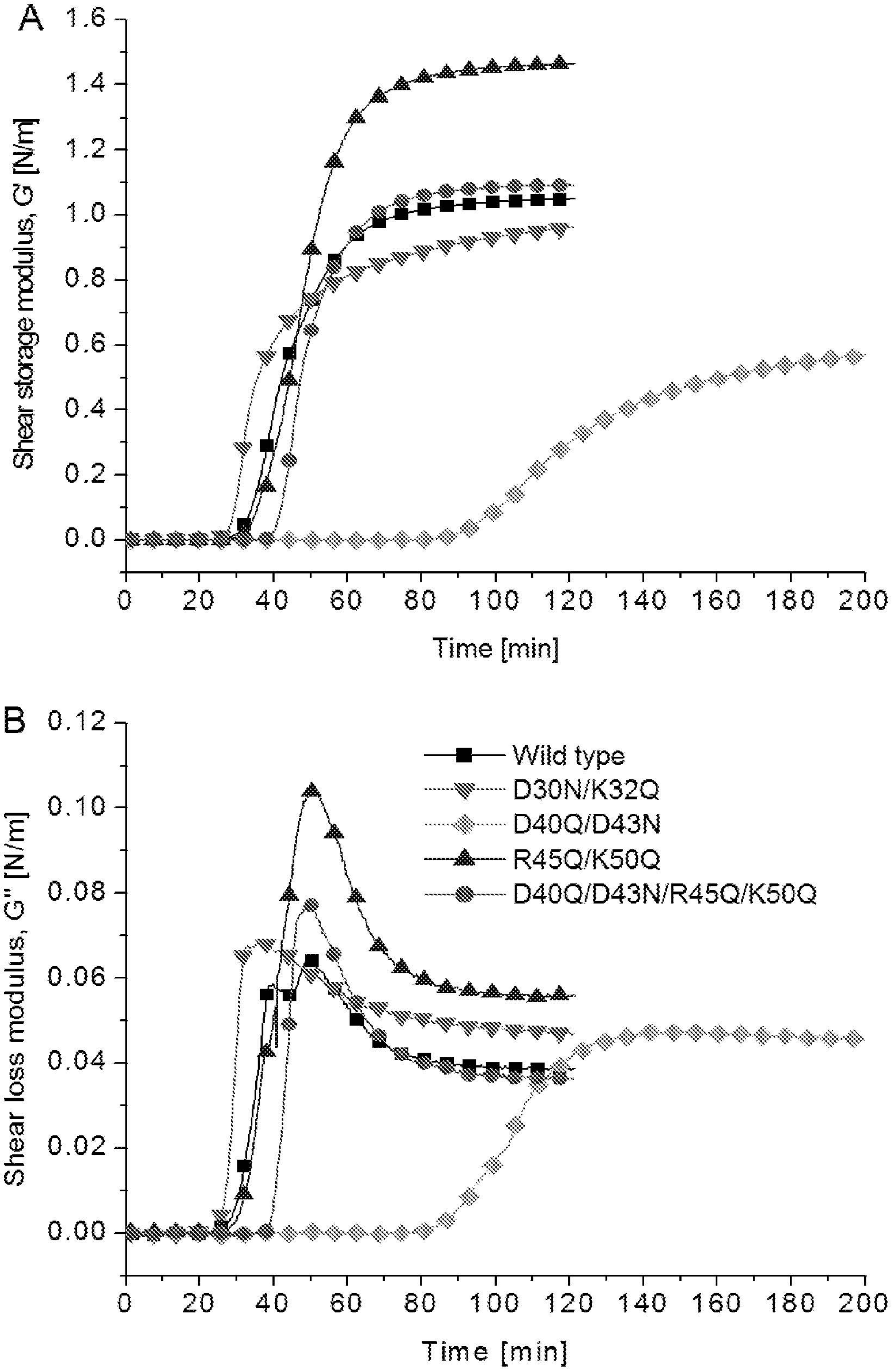
A
Shear storage modulus, G' [N/m]
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
20
40
60
80
100
120
140
160
180
200
Time [min]
B
Shear loss modulus, G'' [N/m]
0.12
0.10
0.08
0.06
0.04
0.02
0.00
Wild type
D30N/K32Q
D40Q/D43N
R45Q/K50Q
D40Q/D43N/R45Q/K50Q
Time [min]

Figure 4

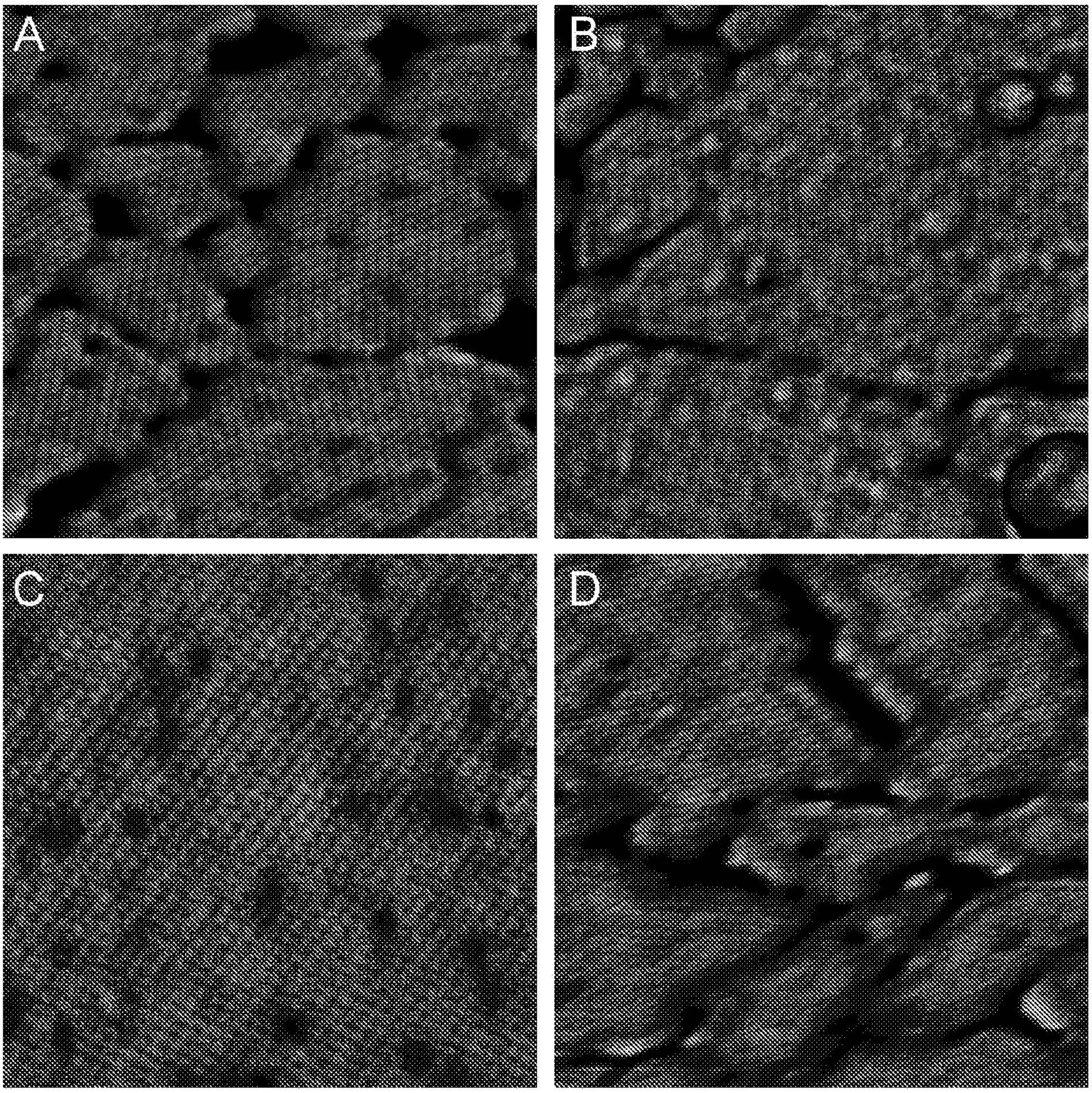

Figure 5

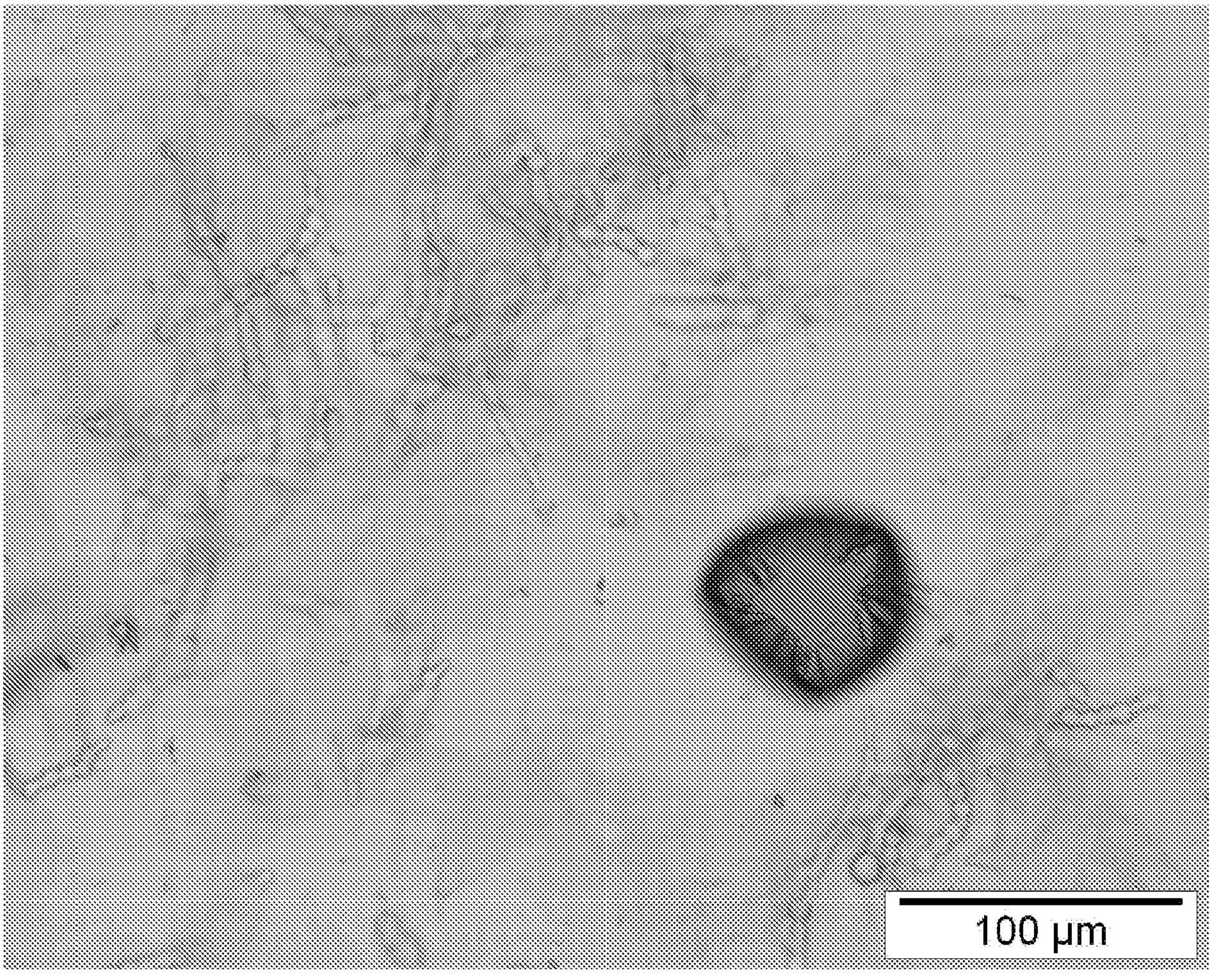

Figure 6

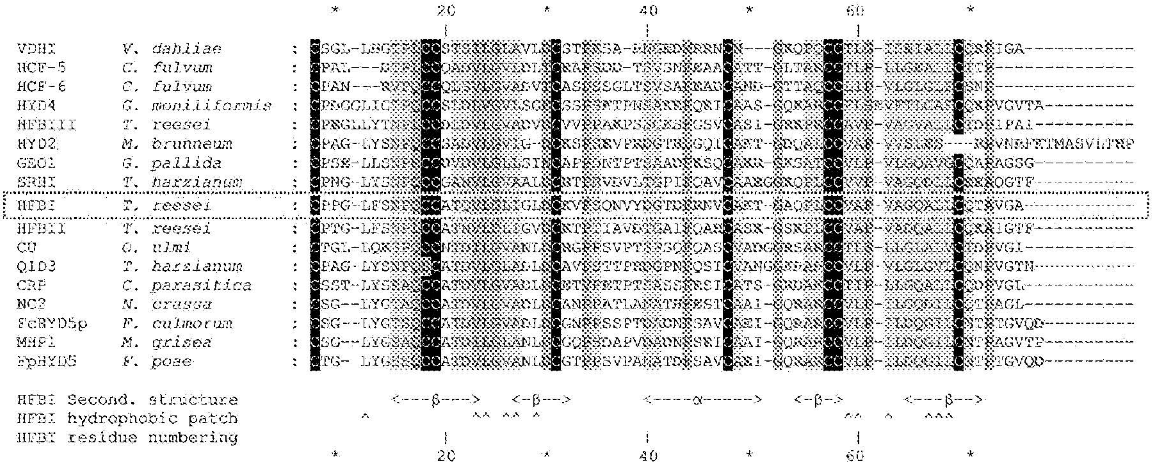
VDHI V. dahliae
HCF-5 C. fulvum
HCF-6 C. fulvum
HYD4 G. moniliformis
HFBIII T. reesei
HYD2 M. brunneum
GEO1 G. pallida
SRHI T. harzianum
HFBI T. reesei
HFBII T. reesei
CU O. ulmi
QID3 T. harzianum
CRP C. parasitica
NC2 N. crassa
FcHYD5p F. culmorum
MHP1 M. grisea
FpHYD5 F. poae
HFBI Second. structure
HFBI hydrophobic patch
HFBI residue numbering
20
40
60
β
α

Figure 7

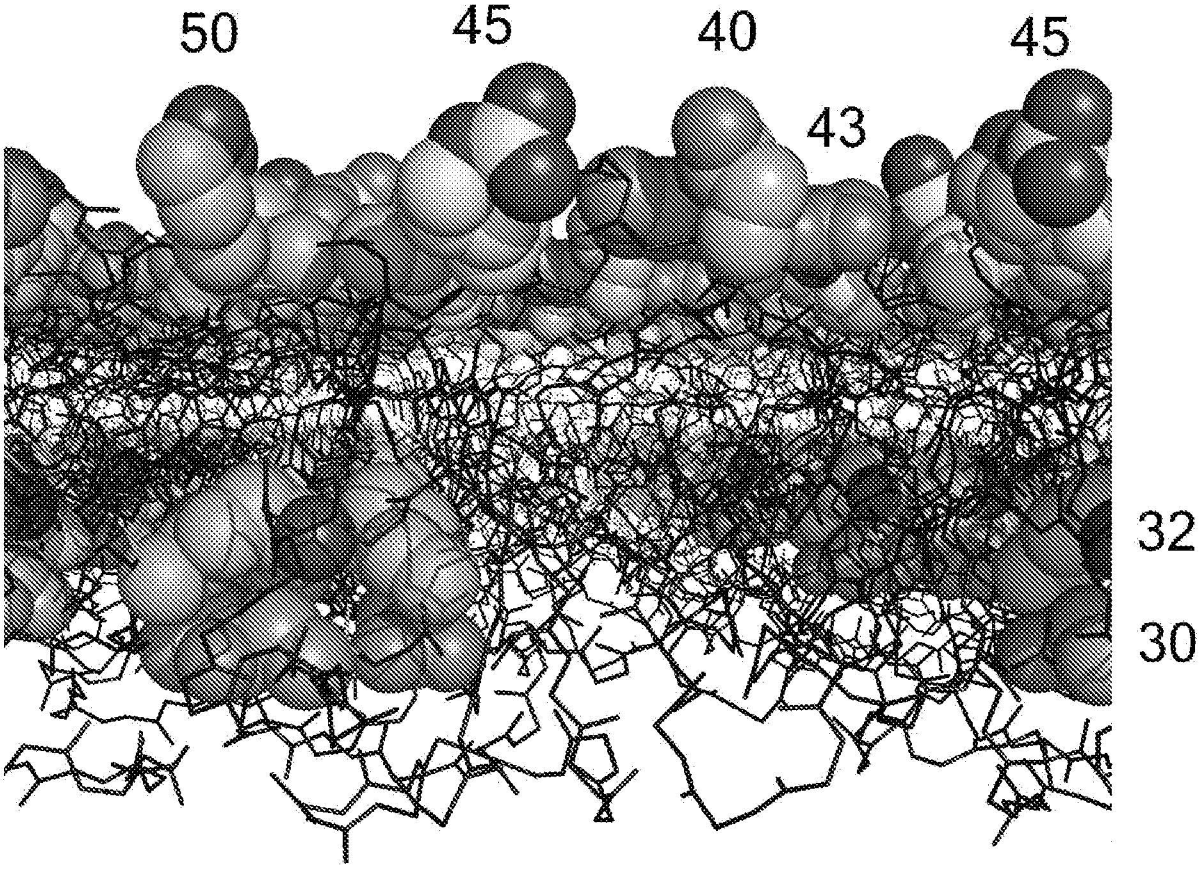

Figure 8

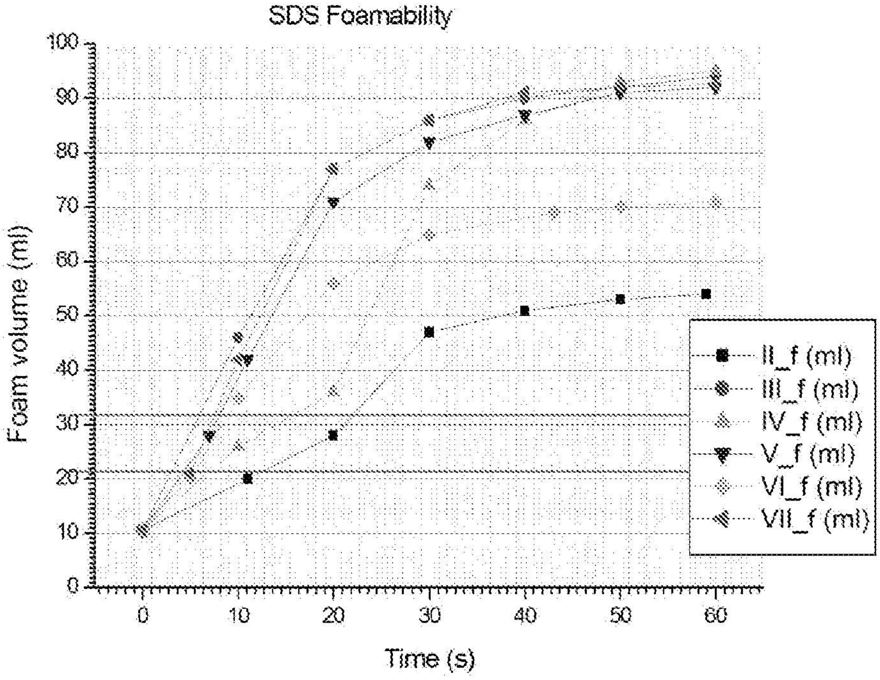

Figure 9

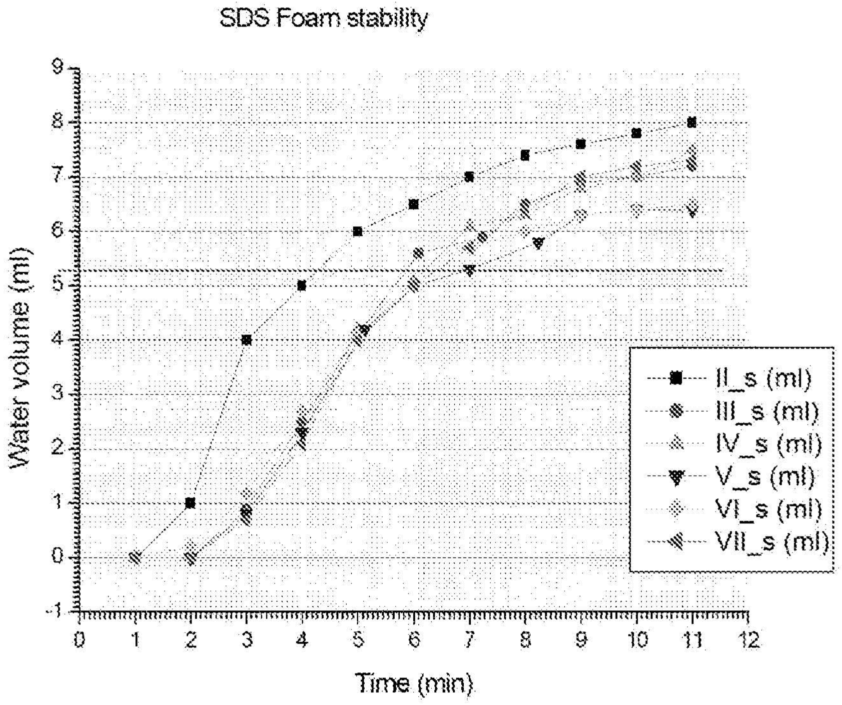

Figure 10

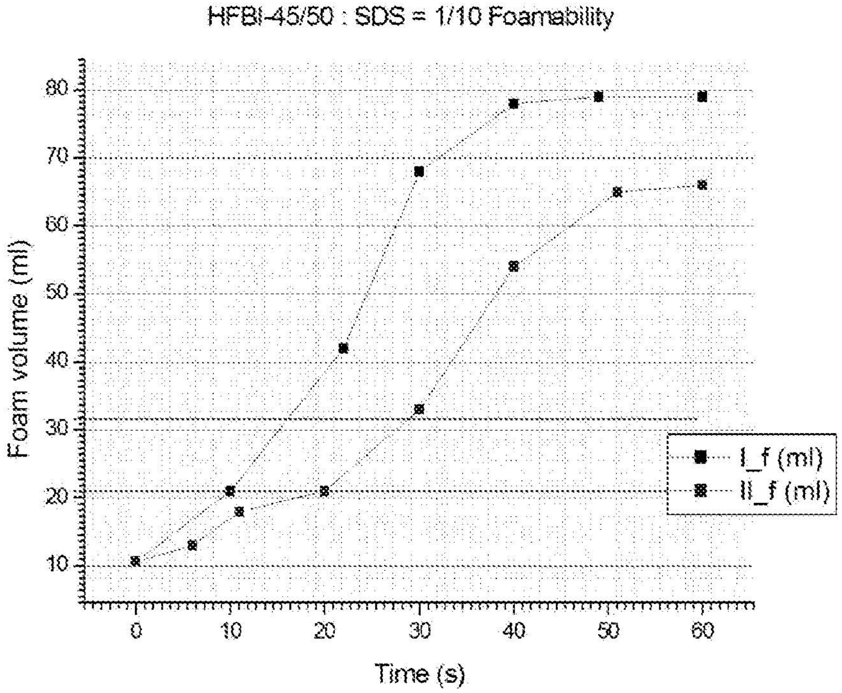

Figure 11

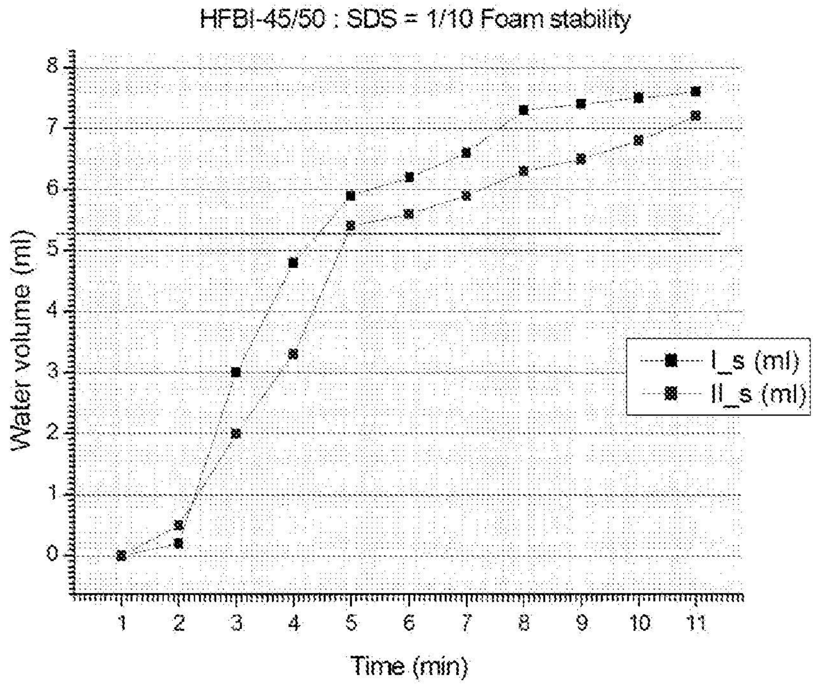

Figure 12

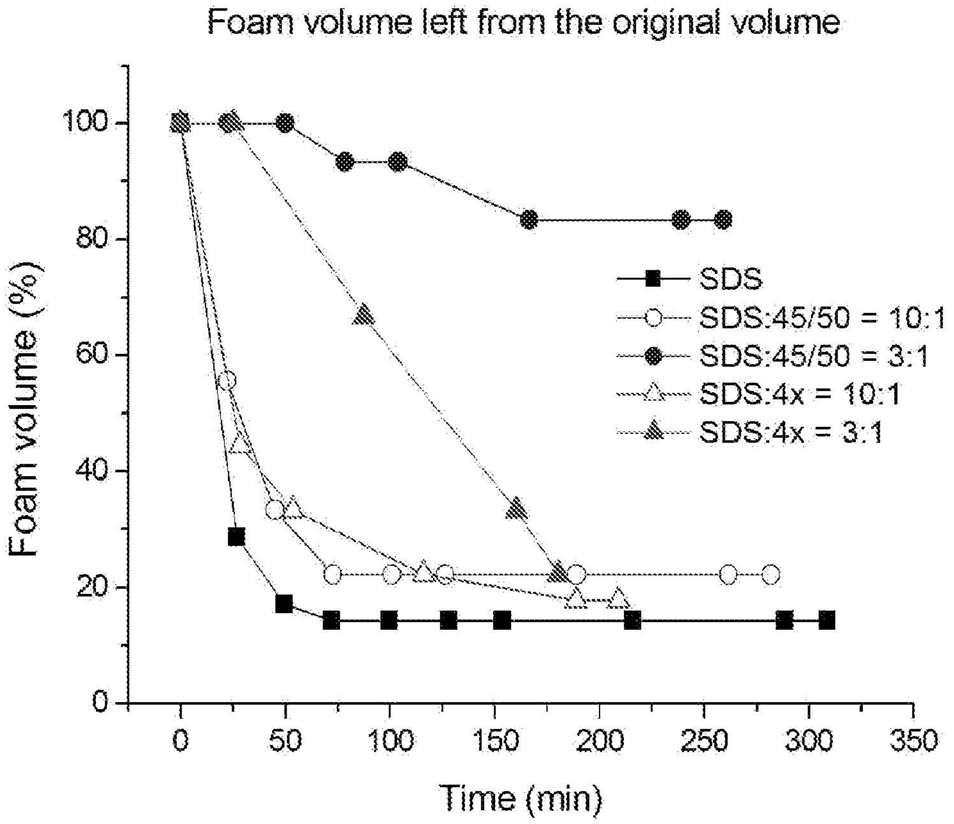

Figure 13

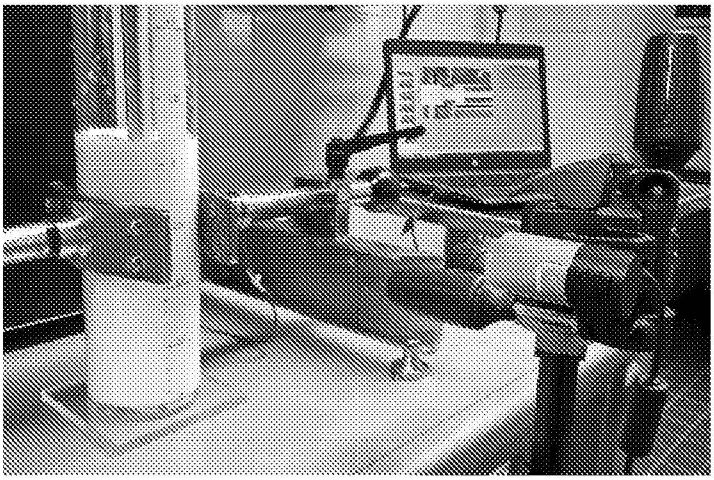

Figure 14

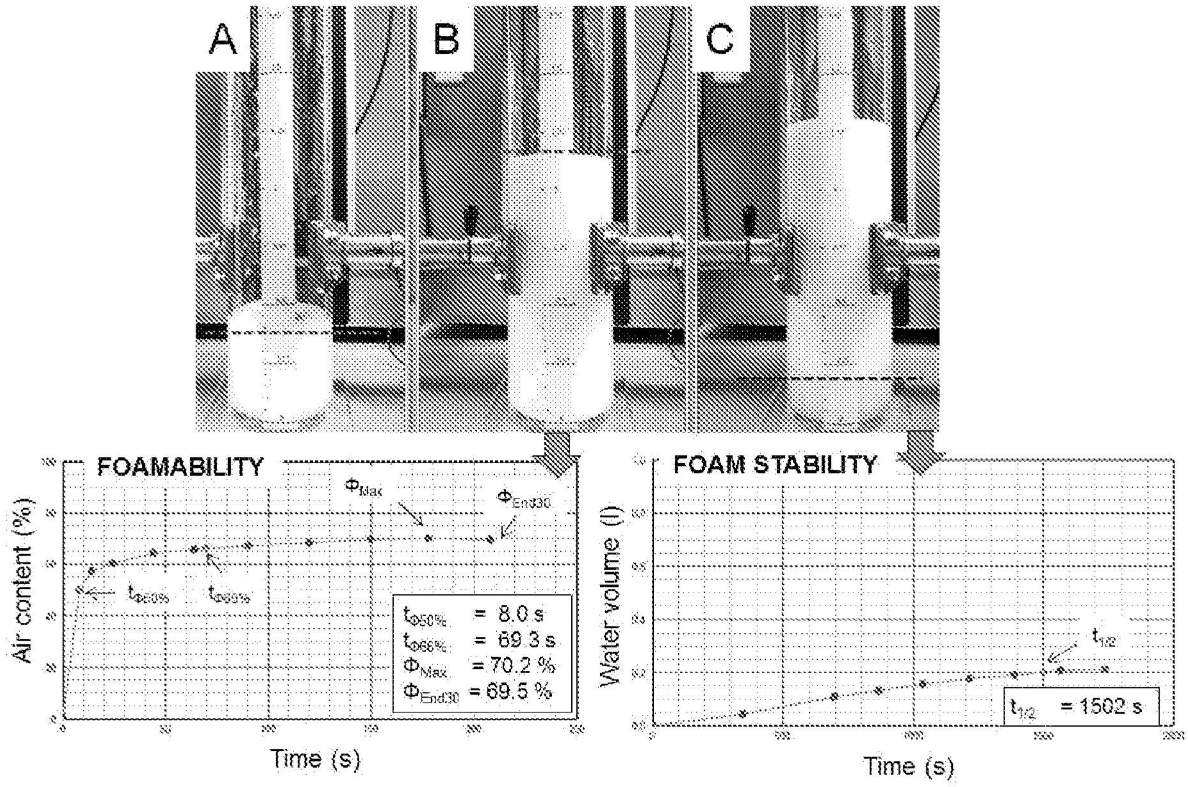

Figure 15

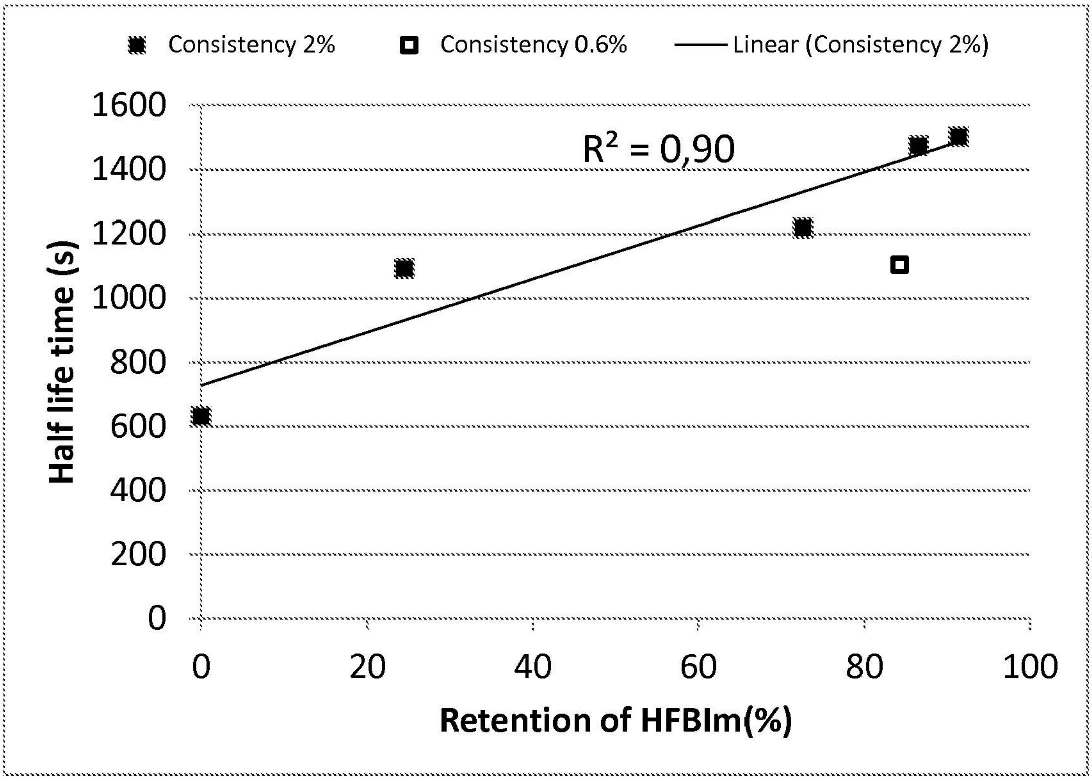

Figure 16

INTERNATIONAL SEARCH REPORT

International application No
PCT/FI2016/050389

A. CLASSIFICATION OF SUBJECT MATTER
INV. C08J9/00 C08J9/30 D21H21/56
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C08J D21H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2010/044308 A1 (BAUS ULF [DE] ET AL) 25 February 2010 (2010-02-25) claims 21,30,31 paragraph [0069] paragraph [0073] - paragraph [0074] paragraph [0110] - paragraph [0111] -----	1-24
A	WO 2015/066806 A1 (FPINNOVATIONS [CA]) 14 May 2015 (2015-05-14) claims 1,7,46 paragraph [0120] -----	1-24
A	US 2010/311629 A1 (BARG HEIKO [DE] ET AL) 9 December 2010 (2010-12-09) claims 1,7,11 paragraph [0052] paragraph [0078] paragraph [0144] ----- -/--	1-14

☒ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search
19 August 2016

Date of mailing of the international search report
30/08/2016

Name and mailing address of the ISA/
European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040,
Fax: (+31-70) 340-3016

Authorized officer
Costantini, Nicola

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INTERNATIONAL SEARCH REPORT

International application No
PCT/FI2016/050389

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	CN 103 030 924 B (UNIV SICHUAN) 3 September 2014 (2014-09-03) abstract claims 1-10 paragraph [0007] -----	1-24
Y	US 3 160 596 A (SPENCER FRANK R) 8 December 1964 (1964-12-08) claim 8 column 1, line 21 - line 30 column 1, line 54 - line 64 column 5, line 35 - line 45 column 6, line 18 - line 46 -----	1-24
A	WO 01/94436 A2 (BASF AG [DE]; BAUMGARTL HORST [DE]; GROSS BERND [DE]; GRAVE ISIDOR DE) 13 December 2001 (2001-12-13) claim 1 page 7, line 20 - line 37 -----	1-24

2

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/FI2016/050389

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2010044308 A1	25-02-2010	AT 471960 T	15-07-2010
		BR PI0807872 A2	17-06-2014
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		EP 2134775 A1	23-12-2009
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