

LOW PH AERATED PRODUCTS

Field of the invention

The present invention relates low pH aerated compositions, such as food
5 products, that include hydrophobins.

Background to the invention

A wide variety of food products contain introduced gas, such as air, nitrogen
and/or carbon dioxide. Typically, aerated food products tend to be of a relatively
10 neutral pH – approximately pH 6.0 to 7.5. Examples of such products include ice
cream, whipped toppings and whipped cream.

Most aerated food products comprise proteins and these are essential for both
the incorporation of air bubbles and their subsequent stability. However, dairy
15 proteins in particular tend to be pH sensitive. That is, their charge, interactions,
and their conformation, can alter as a function of pH. This adversely affects both
the foaming ability and the resulting stability of the foams made using protein,
particularly for aerated products of pH 5.4 and lower. This is because both the
surface activity and the solubility of many proteins are reduced as pH is lowered.
20 For example, sodium caseinate precipitates at about pH 4.6 to 4.8. This results in
both poor air incorporation and resulting instability of aerated products.

This fact has been described extensively in the literature and producing stable
foams at low pH is a known problem. However, although there are routes
25 described in the art that facilitate the formation of aerated food products at low
pH, they tend to have limitations. Such limitations are overcome by the current
invention. For example, gelatine is often added as a stabiliser which both
thickens the product and also aids in air stability. Most "mousse" products take
advantage of the use of gelatine. However, gelatine is an animal based stabiliser
30 which is not seen as a suitable ingredient by many consumers. Furthermore, use
of gelatine usually means that the product will be "set", i.e. will not flow. This

means one is limited to the type of products that can be made using this formulation technology.

Other methods take advantage of other chemical emulsifier systems such as sucrose esters and / or variants of mono-/di- glycerides of saturated fatty acids. Although these non-dairy based formulation technologies will form aeratable products at low pH, often significant quantities of emulsifier are required for long term stability (in excess of 3 weeks). This can have an undesirable influence on both taste and texture. Furthermore, significant quantities of chemical emulsifier in foods are not acceptable to the consumer.

Ideally, a suitable aerating agent would be pH insensitive (in terms of its foaming behaviour), be functional at low concentrations such that no undesirable impact on taste and texture was apparent, and could be used to form aerated foods where the foam was stable in excess of 3 weeks at chill or ambient temperature.

Summary of the invention

In our co-pending application, WO 06 / 010425, we have identified fungal proteins, termed hydrophobins, as being highly effective at stabilising aerated food products. We have now surprisingly found that low concentrations of hydrophobin protein (< 0.5 wt. %) can be mixed with acids to form solutions at low pH which are readily foamable and will form foams at chill and ambient temperatures that are stable in excess of 3 weeks. Since hydrophobin does not lead to gelation of the continuous phase or to undesired textures in the mouth, this means that stable foams can be prepared at low pH irrespective of continuous phase rheology. Therefore, the type of aerated product opportunities are wide, for example, aerated acidic smoothies and aerated tea beverages.

These opportunities are not limited to food products but can be applied to other aerated compositions which have a low pH.

Accordingly, the present invention provides an aerated composition having a pH of less than 5.5, which composition comprises hydrophobin.

In one embodiment, the hydrophobin is in a substantially isolated form.

In a preferred embodiment, the hydrophobin is present in an amount of at least
5 0.001 wt%, more preferably at least 0.01 wt%.

Preferably the hydrophobin is a class II hydrophobin.

The present invention further provides the use of a hydrophobin in a method of
10 inhibiting bubble coarsening in an aerated composition having a pH of less
than 5.5.

In a related aspect the present invention provides a method of inhibiting bubble
coarsening in an aerated composition having a pH of less than 5.5 which method
15 comprises adding hydrophobin to the composition prior to and/or during aeration
of the composition.

The present invention also provides the use of a hydrophobin in a method of
stabilising a foam in an aerated composition having a pH of less than 5.5.

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In a related aspect the present invention also provides a method of stabilising a
foam in an aerated composition having a pH of less than 5.5 which method
comprises adding hydrophobin to the composition prior to and/or during aeration
of the composition.

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Detailed description of the invention

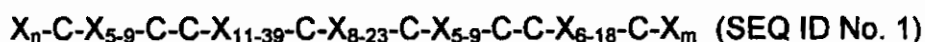
Unless defined otherwise, all technical and scientific terms used herein have the
same meaning as commonly understood by one of ordinary skill in the art (e.g. in
chilled confectionery/frozen confectionery manufacture, chemistry and
30 biotechnology). Definitions and descriptions of various terms and techniques used
in chilled/frozen confectionery manufacture are found in Ice Cream, 4th Edition,
Arbuckle (1986), Van Nostrand Reinhold Company, New York, NY. Standard
techniques used for molecular and biochemical methods can be found in

Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, 3rd ed. (2001) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. and Ausubel *et al.*, Short Protocols in Molecular Biology (1999) 4th Ed, John Wiley & Sons, Inc. - and the full version entitled Current Protocols in Molecular Biology).

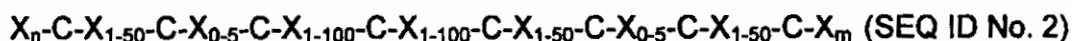
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Hydrophobins

Hydrophobins are a well-defined class of proteins (Wessels, 1997, Adv. Microb. Physio. 38: 1-45; Wosten, 2001, Annu Rev. Microbiol. 55: 625-646) capable of self-assembly at a hydrophobic/hydrophilic interface, and having a conserved
10 sequence:



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. In
15 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:



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, α -helix) (De Vocht *et al.*, 1998,
25 Biophys. J. 74: 2059-68).

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 (Wosten *et al.*, 1994, Embo. J. 13: 5848-54). The protein film can be visualised by any
30 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.

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Hydrophobins identified to date are generally classed as either class I or class II. Both types have been identified in fungi as secreted proteins that self-assemble at hydrophobic 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.

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Hydrophobin-like proteins have also been identified in filamentous bacteria, such as *Actinomycete* and *Streptomyces* 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 shown in SEQ ID Nos. 1 and 2, and are within the scope of the present invention.

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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. Particularly preferred hosts are food grade organisms, such as *Cryphonectria parasitica* which secretes a hydrophobin termed cryparin (MacCabe and Van Alfen, 1999, App. Environ. Microbiol 65: 5431-5435).

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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

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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 WO96/41882 which gives the sequence of hydrophobins identified in *Agaricus bisporus*; and Wosten, 2001, Annu Rev. Microbiol. 55: 625-646). Recombinant technology can also be used to modify hydrophobin sequences or synthesise 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. Preferred hosts are those that are considered food grade – 'generally regarded as safe' (GRAS).

Suitable fungal species, include yeasts such as (but not limited to) those of the genera *Saccharomyces*, *Kluyveromyces*, *Pichia*, *Hansenula*, *Candida*, *Schizosaccharomyces* 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".

- 5 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, Biochim Biophys
10 Acta. 1569: 139-50; Calonje et al., 2002, Can. J. Microbiol. 48: 1030-4; Askolin et al., 2001, Appl Microbiol Biotechnol. 57: 124-30; and De Vries et al., 1999, Eur J Biochem. 262: 377-85.

Aerated low pH compositions

- 15 By the term "low pH composition", we mean any composition where the pH of the aqueous phase is less than 5.5 for some or all of the product life-time. Preferably the pH is less than 5.4, 5.2 or 5.0. Typically, the pH is equal to or more than 1.0, preferably 3.0 or more, such as 4.0 or more. Typically, for an aerated food, a low pH product would exhibit a pH of from 3.0 to 5.4.

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- The term "aerated" means that gas has been intentionally incorporated into the product, such as by mechanical means. The gas can be any gas, but is preferably, particularly in the context of food products, a food-grade gas such as air, nitrogen or carbon dioxide. The extent of aeration is typically defined in terms
25 of "overrun". In the context of the present invention, %overrun is defined in volume terms as:

$$\frac{((\text{volume of the final aerated product} - \text{volume of the mix}) / \text{volume of the mix})}{\text{X } 100}$$

- The amount of overrun present in the product will vary depending on the desired
30 product characteristics. For example, the level of overrun in frozen yoghurt is typically from about 70 to 100%, and in confectionery such as mousses the overrun can be as high as 200 to 250 wt%. The level of overrun in some chilled

products, ambient products and hot products can be lower, but generally over 10%, e.g. the level of overrun in milkshakes is typically from 10 to 40 wt%.

The level of overrun in other products is preferably from 100 to 800%.

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It is not necessary for the foam to be homogeneous within the product. Nonetheless, in one embodiment, the foam is substantially homogeneous.

Aerated compositions of the invention include aerated food products. Other compositions include those where a foam is required within a continuous phase of low pH which can maintain its stability throughout the required time of product use.

Preferably an aerated composition of the invention will retain at least 50% of its original air phase volume, more preferably 75%, for a period of at least 3 weeks (typically measured after storage at chill temperatures (ca. 5°C)). The overrun does not have to be dispersed homogeneously throughout the product.

Preferably, the average bubble diameter in the composition will not change appreciably over a period of 3 weeks (typically measured after storage at chill temperatures (ca. 5°C)) from the average size when it was initially prepared at time $t = 0$. Preferably, the relative average bubble diameter (d_r) will change less than a factor 2.5 over a period of 3 weeks, and more preferably less than a factor of 2. The relative bubble diameter (d_r) at time $= t$ is as determined in the examples through the equation:

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$$d_r = \frac{d_t}{d_0}$$

Where d_0 is the average diameter immediately after preparation, i.e. $t = 0$, and d_t is the average bubble diameter at time $= t$.

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A suitable method for measuring changes in bubble size and foam volume is by using a light scattering technique. The Turbiscan TLab measurement system (Formulation, France) can conveniently be used, which analyses both the backscattered and transmitted light from the aerated sample of interest.

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The foam to be analysed is contained within a cylindrical sample cell (e.g. with a diameter of 25 mm, filled with 20 ml foam). A light source of wavelength $\lambda = 880\text{nm}$ is used to supply the incident light, and two optical sensors receive the light transmitted through the sample (180° from the incident light) and back
 10 scattered light (45° from the incident light) from the sample. In scanning mode, the optical sensors scan the height of the tube acquiring both transmitted and backscattered data as a function of sample height and time. Therefore, migration phenomena (such as creaming) and changes in particle size (such as bubble size) can be monitored over time. Relevant theory and examples of the use of
 15 the Turbiscan measurement system can be found in: Mengual et al., Colloids and Surfaces A, 1999, 152, 112-123; Rouimi et al., Food Hydrocolloids, 2005, 19, 467-478; Also, application notes and useful information can be obtained from the manufacturer's website: www.turbiscan.com

20 Experimentally, average bubble size changes are best observed by variations in the backscattered light through an area of the sample where other changes (such as foam collapse or bubble creaming) are not occurring. Here, we used central areas of the foam. The backscattering level (BS) is linked to the photon transport mean free path, λ^* , through the foam by the relation:

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$$BS = \frac{1}{(\lambda^*)^{\frac{1}{2}}}$$

λ^* is dependent upon the gas volume fraction, ϕ , and the bubble mean diameter d through:

$$\lambda^* = \frac{2d}{3\phi(1-g)Q}$$

Q and g are both optical parameters from Mie theory, where Q is the scattering
 30 efficiency factor and g is an asymmetry factor. For a foam of known volume

fraction of air, the change in the average bubble diameter can be monitored over time. This is calculated automatically through the Turbiscan software.

Exact measurement parameters that can be used are stated in the Examples.

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Foam stability (volume of foam as a function of time) and extent of creaming can also be determined by a visual method by observing these phenomena in foams sampled into measuring cylinders. Creaming (due to the buoyancy of the air bubbles) is a process which leads to vertical phase separation in the container
10 resulting in a large proportion of bubbles close to the upper surface and the depletion of bubbles at the bottom.

Aerated Food Products

Aerated food products of the invention typically fall into one of four groups – hot,
15 ambient (i.e. products stored and/or served at room temperature without the requirement for refrigeration/freezing), chilled or frozen. The term "food" includes beverages. Chilled aerated food products include smoothies and tea. Frozen aerated food products include frozen confections such as frozen yoghurt.

20 Suitable acids for use in low pH food products of the invention include, but are not limited to, ascorbic acid, citric acid, lactic acid, tartaric acid, carbonic acid, succinic acid, malic acid, gluconic acid, and mixtures thereof.

Food products may optionally contain other ingredients such as one or more of
25 the following: other proteins such as dairy proteins, either as pure ingredients or as liquid ingredients, e.g. milk or cream; oil or fat, notably in the form of an emulsified phase; sugars; salts; colours and flavours; chemical emulsifiers, such as monoglycerides, tea or coffee; fruit or vegetable purees / extracts / juice; stabilisers or thickeners, such as polysaccharides; preservatives; inclusions, such
30 as nuts, fruit, toffees. Preferably, food products of the invention do not contain gelatine.

Some particular examples of embodiments of the present invention are set out below:

5 In one embodiment, the product is an aerated beverage such as a milkshake, smoothie, carbonated drink, beer, or tea where a foam is required at low pH and which maintains stability throughout the product life-time. In this case, the overrun of the aerated product may be between 5 and 300%, and most preferably between 10 and 200%. The gas does not need to be dispersed homogeneously throughout the product.

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In a second embodiment, the product is an aerated set food such as a mousse, cheesecake, jam, whipped topping or cream, where a foam is required at low pH and which maintains stability throughout the product life-time. In this case, the preferred overrun is between 50 and 400%.

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In a third embodiment, the product is an aerated frozen dessert such as a sorbet, ice cream, or frozen yoghurt, where a foam is required at low pH and which maintains stability throughout the product life-time. The preferred overrun is between 50 and 300%.

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Preferably the aerated food product is an aerated confectionery product.

The amount of hydrophobin present in the product will generally vary depending on the product formulation and volume of the air phase. Typically, the product will
25 contain at least 0.001 wt%, hydrophobin, more preferably at least 0.005 or 0.01 wt%. Typically the product will contain less than 1 wt% hydrophobin. The hydrophobin can be from a single source or a plurality of sources e.g. the hydrophobin can be a mixture of two or more different hydrophobin polypeptides.

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Preferably the hydrophobin is a class II hydrophobin.

The present invention also encompasses compositions for producing an aerated food product of the invention, which composition comprises a hydrophobin. Such

compositions include liquid premixes, for example premixes used in the production of frozen confectionery products, and dry mixes, for example powders, to which an aqueous liquid, such as milk or water, is added prior to or during aeration.

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The compositions for producing an aerated food product of the invention, will comprise other ingredients, in addition to the hydrophobin, which are normally included in the food product, e.g. sugar, fat, emulsifiers, flavourings etc. The compositions may include all of the remaining ingredients required to make the food product such that the composition is ready to be processed, i.e. aerated, to form an aerated food product of the invention.

Dry compositions for producing an aerated food product of the invention will also comprise other ingredients, in addition to the hydrophobin, which are normally included in the food product, e.g. sugar, fat, emulsifiers, flavourings etc. The compositions may include all of the remaining non-liquid ingredients required to make the food product such that all that the user need only add an aqueous liquid, such as water or milk, and the composition is ready to be processed to form an aerated food product of the invention. These dry compositions, examples of which include powders and granules, can be designed for both industrial and retail use, and benefit from reduced bulk and longer shelf life.

Compositions for producing an aerated food product of the invention will typically have a pH of less than 5.5, or in the case of dry compositions, form a composition having a pH of less than 5.5 when water or milk is added to reconstitute the product to its usual final form.

The hydrophobin is added in a form and in an amount such that it is available to stabilise the air phase. By the term "added", we mean that the hydrophobin is deliberately introduced into the product for the purpose of taking advantage of its foam stabilising properties. Consequently, where ingredients are present or added that contain fungal contaminants, which may contain hydrophobin

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polypeptides, this does not constitute adding hydrophobin within the context of the present invention.

Typically, the hydrophobin is added to the product in a form such that it is capable
5 of self-assembly at an air-liquid surface.

Typically, the hydrophobin is added to the products or compositions of the invention in an isolated form, typically at least partially purified, such as at least 10% pure, based on weight of solids. By "added in isolated form", we mean that
10 the hydrophobin is not added as part of a naturally-occurring organism, such as a mushroom, which naturally expresses hydrophobins. Instead, the hydrophobin will typically either have been extracted from a naturally-occurring source or obtained by recombinant expression in a host organism.

15 In one embodiment, the hydrophobin is added to the product in monomeric, dimeric and/or oligomeric (i.e. consisting of 10 monomeric units or fewer) form. Preferably at least 50 wt% of the added hydrophobin is in at least one of these forms, more preferably at least 75, 80, 85 or 90 wt%. Once added, the hydrophobin will typically undergo assembly at the air/liquid interface and
20 therefore the amount of monomer, dimer and oligomer would be expected to decrease.

In one embodiment, the hydrophobin is added to the aerated compositions of the invention in an isolated form, typically at least partially purified.

25 The added hydrophobin can be used to stabilise the air phase in an aerated composition, generally by inhibiting bubble coarsening, i.e. hydrophobins have been found not only to stabilise foam volume but also the size of the bubbles within the foam.

30 The present invention will now be described further with reference to the following examples which are illustrative only and non-limiting.

Description of the figures

Figure 1: Average bubble diameter relative to that at time $t = 0$ as a function of time.

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Figure 2: Foams created using 0.1% HFBII and 0.5% xanthan at pH 3.5 and 5.2 (left and right, respectively) and stored at 5°C for 3 weeks. After this period of time, no loss of foam volume or bubble visible bubble growth has occurred.

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Figure 3: Foams created at pH 3.5 containing 0.5% xanthan and (left) 0.1% HFBII – 3 weeks storage, (centre) 1.5% LACTEM – 2 weeks storage, and (right) 0.5% hyfoama – 3 weeks storage. All stored at 5°C.

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Figure 4: Close up picture of foams created at pH 3.5 containing 0.5% xanthan and (left) 0.1% HFBII – 3 weeks storage, (right) 1.5% LACTEM – 2 weeks storage. Note that the example with HFBII shows a foam where bubbles are not visible whereas the foam with LACTEM shows visible bubbles that have grown during storage.

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Figure 5: Average bubble diameter relative to that at time $t = 0$ as a function of time for green tea containing foam.

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Figure 6: SEM images of the microstructure of sorbets created using 0.2% hygel and 0.1% HFBII, both fresh and after temperature abuse.

Figure 7: Higher magnification SEM images of the microstructure of sorbets created using 0.2% hygel and 0.1% HFBII, after temperature abuse.

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Figure 8: Photographs of sorbets created using 0.2% hygel and 0.1% HFBII after 2 weeks temperature abuse.

Figure 9: Photographs of an aerated fruit smoothie product containing 0.1% HFBII and 0.41% xanthan freshly made (left) and after 3 weeks storage at 5°C (right) showing little creaming or bubble growth.

- 5 Figure 10: Photographs of an aerated vinaigrette product containing 0.1% HFBII and 0.25% xanthan freshly made (left) and after 3 weeks storage at 5°C (right) showing little creaming or bubble growth.

Example 1: Aerated low pH products

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Aerated products were prepared comprising solutions containing an acid, xanthan gum, and one of three aerating agents (A-C) listed below.

A: Hyfoama DS

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B: Lactic acid ester of monoglyceride (Grinsted Lactem P 22, LACTEM)

C: Hydrophobin (HFBII) from *Trichoderma reesei* (HFBII was obtained from VTT Biotechnology, Finland, purified from *Trichoderma reesei* essentially as described in WO00/58342 and Linder et al., 2001, Biomacromolecules 2: 511-517).

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Details of the materials used are summarised in Table 1 and the formulations from which each of the foam samples was prepared are shown in Table 2 (Mixes A to C).

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Xanthan was added to each mix in order to prevent creaming of the foam. This allows full analysis of bubble size as a function of time without the complication of other destabilising factors such as creaming. In other words, we are measuring the stability of the foams to destabilising mechanisms such as disproportionation and coalescence.

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Mix preparation

For Mix A the protein and xanthan gum were blended and added slowly into agitated water at room temperature. The solutions were subsequently heated to

40°C to ensure that proteins were properly dissolved, with a total mixing time of 30 minutes. The mixes were cooled and stored at 5°C until further use.

For Mix B the Lactem and xanthan were dispersed into agitated water at room temperature. This dispersion was then heated to 60°C to ensure that the Lactem is properly dispersed, with a total mixing time of 30 minutes. The mixes were cooled and stored at 5°C until further use.

For Mix C the xanthan was added slowly to cold water with agitation and stirred for at least 30 minutes to ensure that the polymer was fully hydrated. Then, the required concentration of HFB II was added as an aliquot. The solution was then gently sonicated in a sonic bath for 30 seconds to fully dissolve the HFB II. The mix was cooled and stored at 5°C until further use.

Ingredient	Composition	Supplier
Hyfoama DS	65% protein	Quest
Grinsted Lactem P22 (Lactic acid ester)	100% fat (Lactic acid content 20-25%)	Danisco
Hydrophobin HFB II	Purified from <i>T. reesei</i>	VTT Biotechnology, Finland.
Xanthan Gum (Keltrol RD)	polysaccharide	CP Kelco

Table 1. Materials used

	Mix A	Mix B	Mix C
Ingredient	Concentration / wt%		
Hyfoama DS	0.5	--	--
Lactem	--	1.5	--
HFB II	--	--	0.1
Xanthan gum	0.5	0.5	0.5
Water	99.0	98.0	99.4

Table 2. Base Formulations used before addition of acid.

Aerating Process

Before aeration the sample solutions were acidified to the desired pH of either 5.4 or 3.5 using a 10 wt% solution of citric acid. These are summarised in Table 3.

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Mix / Aerated Product	pH
A1 (from Mix A)	5.4
A2 (from Mix A)	3.5
B1 (from Mix B)	5.4
B2 (from Mix B)	3.5
C1 (from Mix C)	5.2
C2 (from Mix C)	3.5

Table 3: pH of mixes after addition of citric acid.

80 mL of the acidified mix was sheared using a stirred pot apparatus for a timed duration that corresponded to obtaining 100% overrun. This equipment consists of a cylindrical, vertically mounted, jacketed stainless steel vessel with internal proportions of 105mm height and diameter 72mm.

The rotor used to shear the sample consists of a rectangular impeller of the correct proportions to scrape the inner surface of the vessel as it rotates (dimensions 72mm x 41.5mm). Also attached to the rotor are two semi-circular (60mm diameter) high-shear blades positioned at a 45° angle to the rectangular attachment. The rate was 1200 rpm and the steel vessel containing the rotor was cooled to 5°C during aeration.

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Post aeration, samples were stored at 5°C in Turbiscan vials or 100mL measuring cylinders before further analysis.

Measurement of Foam and Bubble Stability

The stability of the foam and the bubbles therein was measured using the Turbiscan TLab, the operating details of which are discussed above. This

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enables the determination of the following as a function of time: (1) The foam volume (i.e. measurement of overall air phase loss). (2) The average bubble size.

- 5 The produced foam was dosed into a Turbiscan glass sample tube up to a height of about 42 mm, corresponding to approximately 20 mL of foam. The equipment then scans and measures both the backscattered and transmitted light between the height of 2 and 55 mm. Measurements were taken over a period of several weeks, depending on the stability of the foam. Since data is collected over the
10 full sample height, from this, the mean values of the backscattering profiles between defined limits (lower and upper height) give specific information with respect to changes in the sample in that area, e.g. bubble size.

Bubble size: From the backscattered data measured between 20 and 30 mm, the
15 average bubble size was calculated automatically from the backscattered light. The refractive indices were taken as those of water and air. The air phase volume fraction of the foam was 0.5 (equating to 100% overrun). Although the air phase volume fraction of the foam can change over time, particularly if it is not stable and creaming takes place, we found that taking measurements between 20
20 and 30 mm height of the sample gave reliable size data unless the foam was very unstable. If the foam was highly unstable, then the size data needs to be treated with caution in a quantitative sense but can be compared with that of other foams.

Results and Discussion

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Stability as a function of time

The average bubble size (relative to that measured at $t = 0$ min) measured for each of the foamed mixes as a function of time is shown in Figure 1. At both pH 3.5 and 5.2, HFBII forms a foam where the bubbles remain stable far in excess of
30 those in the foams aerated using either Hyfoama or Lactem. The foam stabilised by HFBII remained stable for in excess of 3 weeks.

As well as significant bubble growth, the foams created using Hyfoama and Lactem collapsed (lost air phase volume) in the later stages of their short life-time. In the case of HFBII, no measured air phase volume was lost from the product.

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Figures 2 and 3 show images of foams created using HFBII, Lactem, or Hyfoama as the aerating agent. Figure 2 clearly demonstrates that the foam created using HFBII is highly stable at both pH 3.5 and 5.4. Even after 3 weeks storage, no foam collapse or visible bubble growth could be observed.

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Figures 3 and 4 again demonstrate the stability of the foam created using HFBII. The foam made using Lactem at pH 3.4 shows reasonable stability in terms of retaining the air phase volume, but it is clear that significant bubble growth has occurred, as can be seen from the close up picture in Figure 4 showing visible air bubbles. In the case of Hyfoama, significant bubble growth has occurred, but also air phase volume has been lost.

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Therefore, it is clear that hydrophobin can be used to form highly stable foams in the presence of acids. These foams retain their air phase volume over a period of 3 weeks at chill, and the bubble size does not change significantly during that time.

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Example 2: Aerated product containing tea extract

25 Preparation of Aerated Product

A mix containing tea extract of the following formulation was made. 0.1% HFBII, 0.5% xanthan, 0.16% green tea powder, and the remainder water. The mix was prepared as follows: The xanthan and green tea powder were slowly add to cold water with agitation and stirred for at least 30 minutes to ensure that the polymer was fully hydrated. Then, the required concentration of HFB II was added as an aliquot. The solution was then gently sonicated in a sonic bath for 30 seconds to fully dissolve the HFB II. The mix was cooled and stored at 5°C until further use.

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Before aeration the solution was acidified to the desired pH of 5.4 using a 10 wt% solution of citric acid. The mix was then aerated to 100% overrun using the stirred pot in the same manner as described for the mixes in Example 1. The aerated mix was then stored at 5°C and analysis of bubble size and foam volume was made as a function of time using the Turbiscan with the same experimental set up as described in Example 1.

Results and Discussion

The stability of the foam in terms of average bubble diameter is shown in Figure 5. Clearly, over a period of time there is minimal change in bubble size indicating that the bubbles are stable to significant change. Furthermore, the overall foam volume remained constant over this time period.

Example 3: Aerated and Frozen Fruit sorbet

Two sorbets were produced using the ingredients listed in Table 4. Product J was produced using the formulation for Mix J, described in Table 5. The air stabilising agent in this product was a commercially available aerating agent called Hygel, a hydrolysed milk protein. Product K was produced using the formulation shown for Mix K. The air stabilising protein in this product was hydrophobin, HFBII.

Ingredient	Details and Supplier
Sucrose	Tate and Lyle
LF9	63DE corn syrup, C*Trusweet 017Y4, Cerestar, UK
Xanthan gum (Keltrol RD)	CPKelco
Citric acid	Jungbunzlauer AG
Strawberry puree	SVZ International BV
Hydrophobin HFB II	Purified from <i>Trichoderma reesei</i> , purchased from VTT Biotechnology, Finland
Hygel 8293	A hydrolysed milk protein, minimum 80% protein, obtained from Kerry Bioscience, UK.

Table 4. Materials used

Ingredient	Mix J	Mix K
	Concentration / wt%	
HFB II	0	0.1
Hygel	0.2	0
Xanthan gum	0.2	0.2
Citric acid	0.2	0.2
Sucrose	10.5	10.5
LF9	17.3	17.3
Strawberry puree	20.0	20.0
Water	51.6	51.7

Table 5. Formulations

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Mix preparation

For Mix J all the ingredients were added to cold water, dispersed using a magnetic stirrer and heated to 80°C with continuous mixing. The solution was cooled rapidly to 5°C using a cooling bath set at -18°C. For Mix K, comprising hydrophobin, the same procedure was followed except that the HFB II was added to the cooled solution as an aliquot. The mixes were stored at 5°C before further processing. The pH of the unaerated mix was measured to be pH 4.

Aeration and freezing step

80 mL of cold mix was transferred into the stirred pot (described in Example 1) for aeration and freezing. Freezing was achieved by circulating coolant through the jacket surrounding the stirred pot. The mix was aerated and frozen to produce a sorbet product using the following shear and temperature regime: 100 rpm for 1 minute, switch on coolant circulation (at -18°C), then 1000rpm for 2 minutes, then 300 rpm until the torque reached 1 Nm (this occurred at a product temperature of -5°C). The sorbets were collected into suitable containers which had been cooled to below -20°C. The overrun of Product J was measured to be 113% and the overrun of Product K was 101%.

Storage and Temperature Abuse Regimes

The sorbet products were subsequently stored under two temperature regimes:

- 5 (a) "Fresh" samples were stored at -80°C for until they were analysed (about 1 week). At -80°C no structural changes occur, so that the microstructure is essentially the same as for a fresh sample.
 - (b) "Temperature abused" samples were stored at -10°C for 1 week. Some samples were also stored for a further week at -10°C .
- 10 After storage, the products were analysed by Scanning Electron Microscopy (SEM) as well as by visual inspection of the overall product quality.

Scanning Electron Microscopy

The microstructure of the products was visualised using low temperature
15 Scanning Electron Microscopy (SEM). To prepare specimens for microscopy, the sample was cooled to -80°C on dry ice and a section was cut. This section, approximately 6mm x 6mm x 10mm in size, was mounted on a sample holder using a Tissue Tek: OCTTM compound (PVA 11%, Carbowax 5% and 85% non-reactive components) on the point of freezing. The sample including the holder
20 was plunged into liquid nitrogen slush and transferred to a low temperature preparation chamber (Oxford Instrument CT1500HF) held under vacuum, approximately 10^{-4} mbar. The sample was fractured inside the chamber using a scalpel blade. The sample was then warmed up to -90°C for approximately 60 to 90 seconds so that ice slowly sublimed to reveal surface details. It was then
25 cooled to -110°C to end the sublimation. The sample was next coated with gold using argon plasma. This process also took place under vacuum with an applied pressure of 10^{-1} mbar and current of 6 milliamps for 45 seconds. The sample was then transferred to a conventional Scanning Electron Microscope (JSM 5600), fitted with an Oxford Instruments cold stage at a temperature of -150°C . The
30 sample was imaged and areas of interest were captured via digital image acquisition software.

Results

Figure 6 shows SEM images of the microstructures of: (left) product J, the comparative product; and (right) product K, according to the invention. The upper images show the fresh products and the lower ones show the temperature abused products.

5

The fresh products have similar microstructures with small, spherical air bubbles. However, after temperature abuse the comparative product J shows large air channels and very few discrete air cells, indicating that severe bubble coalescence has taken place. Product K according to the invention (i.e. containing hydrophobin) shows much less coalescence and channelling of the air phase and retains a large number of small discrete bubbles.

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Figure 7 shows the microstructure of the temperature abused samples at a higher magnification. Sorbet K shows the presence of small air bubbles (less than 100 μm in diameter) where as Sorbet J does not.

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Figure 8 shows a photograph of the comparative sorbet product (J) and the sorbet product comprising hydrophobin (K) after temperature abuse for 2 weeks. Sorbet J is darker than sorbet K due to the increase in bubble size in the sample. Furthermore, Sorbet J has decreased in volume due to loss of air, where as Sorbet K has not.

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From these images it is evident that a low pH sorbet containing hydrophobin (K) has a substantially more stable air phase than a comparative sorbet (J) containing a standard air stabilising milk protein.

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Example 4: Aerated fruit smoothie

An aerated fruit smoothie was prepared using a Vie Shots™ drink, produced by Unilever UK as a fruit puree base. The Vie Shot™ contained: banana puree (28%), orange juice concentrate (26%), carrot juice concentrate (23%), pumpkin juice concentrate (14%), orange pulp (4%), lemon juice concentrate, acerola cherry concentrate (1.5%), and apple pectin. The pH was measured (at room temperature) to be pH 4.17. Xanthan gum was slowly added to the fruit puree

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with stirring, to a concentration of 0.5 wt. %. This was then mixed for 20 minutes to allow the xanthan gum to hydrate fully. A known volume of 0.5 wt. % hydrophobin solution was aerated to 400% overrun using a hand held aerolatte™ device. This was added to the fruit puree to give an aerated fruit smoothie product with approximately 100% overrun, an overall hydrophobin concentration of 0.1 wt % and an overall xanthan concentration of about 0.41 wt %. The aerated fruit smoothie product was then stored at 5°C and its stability monitored over a period of three weeks. Figure 9 shows that after 3 weeks the aerated fruit smoothie product retained a stable air phase and that no significant bubble growth or creaming occurred.

Example 5: Vinaigrette dressing

An aerated dressing was prepared using Hellman's™ Light Vinaigrette as a base. The base contained: water, spirit vinegar, sugar, modified potato starch, garlic, salt, red pepper, preservative, parsley, black pepper, thyme and colour. The pH was measured (at room temperature) to be pH 3.58. Xanthan gum was slowly added to the vinaigrette with stirring, to a concentration of 0.3 wt. %. It was then mixed for 20 minutes to allow the xanthan gum to hydrate fully. A known volume of 0.5 wt. % hydrophobin solution was aerated to 400% overrun using a hand held aerolatte™ device. This was added to the vinaigrette dressing to give an aerated vinaigrette dressing product with approximately 100% overrun, an overall hydrophobin concentration of 0.1 wt % and an overall xanthan concentration of about 0.25 wt %. The product was then stored at 5°C and the stability monitored over a period of three weeks. Figure 10 shows that after 3 weeks there was no significant foam collapse or bubble growth. There was also no significant amount of creaming. Therefore, hydrophobin is able to adequately stabilise a foam in a pourable low pH dressing for at least 3 weeks.

The various features and embodiments of the present invention, referred to in individual sections above apply, as appropriate, to other sections, *mutatis mutandis*. Consequently features specified in one section may be combined with features specified in other sections, as appropriate.

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

CLAIMS

1. An aerated composition having a pH of less than 5.5, which composition comprises at least 0.001 wt% hydrophobin.
- 5 2. An aerated composition according to claim 1 comprising less than 1 wt% hydrophobin.
3. An aerated composition according to claim 1 or claim 2 wherein the
10 hydrophobin is in isolated form.
4. An aerated composition according to any one of claims 1 to 3 wherein the hydrophobin is a class II hydrophobin.
- 15 5. An aerated composition according to any one of the preceding claims which is an aerated food product.
6. An aerated food product according to claim 5 which is a chilled food product.
- 20 7. An aerated composition according to any one of the preceding claims having a pH of from 3.0 to 5.4.
8. An aerated composition according to any one of the preceding claims
25 having a pH of from 3.0 to 5.0.
9. Use of a hydrophobin in a method of inhibiting bubble coarsening in an aerated composition having a pH of less than 5.5.
- 30 10. Use of a hydrophobin in a method of stabilising a foam in an aerated composition having a pH of less than 5.5.