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(54) **METHOD FOR EXTRACTING HYDROPHOBIN FROM A SOLUTION**
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Process for extracting hydrophobin from a solution wherein carrageenan is added to the solution and the pH of the solution is brought below 3.5, and the ionic strength of the solution is below 0.5.

22 Claims, No Drawings

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METHOD FOR EXTRACTING HYDROPHOBIN FROM A SOLUTION

Matter enclosed in heavy brackets [] appears in the original patent but forms no part of this reissue specification; matter printed in italics indicates the additions made by reissue.

PRIORITY TO RELATED APPLICATIONS

This application is a reissue of U.S. Pat. No. 8,354,503 B2, which was filed Dec. 11, 2009, and was assigned U.S. application Ser. No. 12/636,157, which claims the benefit of European Application No. 08171868.6, filed Dec. 16, 2009. The entire contents of the above-identified applications are hereby incorporated by reference.

TECHNICAL FIELD OF THE INVENTION

The present invention relates to method for extracting hydrophobin from a solution. In particular it relates to a method for extracting hydrophobin in a fermentation process.

BACKGROUND TO THE INVENTION

Foaming is a common problem in aerobic, submerged fermentations. Foaming is caused by the sparging of gas into the fermentation medium for the purpose of providing oxygen for the growth of the aerobic organism being cultivated (e.g. bacteria, yeasts, fungi, algae, cell cultures). If the fermentation medium contains surface active components such as proteins, polysaccharides or fatty acids, then foam can be formed on the surface of the medium as the sparged gas bubbles disengage from the liquid. Foaming creates a number of problems including the undesirable stripping of product, nutrients, and cells into the foam, and can make process containment difficult. A known method for controlling foaming is to use antifoams, of which several types are commonly used: silicone-based (e.g. polydimethylsiloxanes), polyalkylene glycols (e.g. polypropylene glycol), fatty acids, polyesters and natural oils (e.g. linseed oil, soybean oil). Antifoams replace foam-forming components on bubble surfaces, resulting in destruction of the foam by bubble coalescence. Antifoams are added at the start of and/or during the fermentation.

When the fermentation product is intended for use in foods, personal products or medicine, it is highly desirable that the product is excreted by the producing organism into the fermentation medium (i.e. extra-cellular, rather than intra-cellular production). This avoids the need to disrupt the cells by physical or chemical means in order to release the product for recovery. By maintaining the cells intact, the cellular material can be easily separated from the product so that it is free of intracellular and genetic material which is usually regarded as an undesirable contaminant. This can be especially important when the producing organism has been genetically modified. However, extra-cellular production may intensify the degree of foaming in the fermenter, especially if the product facilitates foam formation or enhances foam stability, for example a biosurfactant or a hydrophobin. The use of antifoams presents a particular problem in the extra-cellular production of such foaming agents for two reasons: firstly the amount of antifoam required is increased because the foaming agent itself contributes to foaming in the fermenter. Secondly, it is not necessary to remove the antifoam from most fermentation products since it is present in low concentrations which do not affect the functionality of the product. However, when the

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fermentation product is a foaming agent, the antifoam must be substantially removed since the presence of antifoam in the product will impair its functionality.

Bailey et al, Appl. Microbiol. Biotechnol. 58 (2002) pp 721-727 disclose the production of hydrophobins HFB I and HFB II by the fermentation of transformants of *Trichoderma reesei*. An antifoam (Struktol J633) was used to prevent foaming and the hydrophobin was purified using aqueous two phase extraction. However separation methods such as aqueous two phase extraction or chromatographic processes are expensive and may require food-incompatible chemicals.

It has now been found that, rather than removing the antifoam from the solution it is possible to remove the hydrophobin.

TESTS AND DEFINITIONS

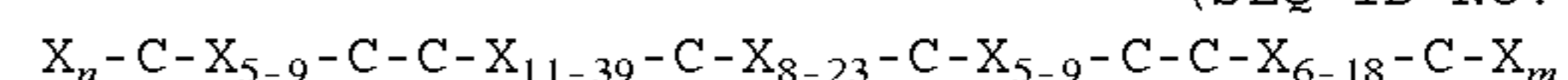
Hydrophobins

Hydrophobins can be obtained by culturing filamentous fungi such as 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). Similarly, surfactin can be obtained from *Bacillus subtilis* and glycolipids from e.g. *Pseudomonas aeruginosa*, *Rhodococcus erythropolis*, *Mycobacterium* species and *Torulopsis bombicola* (Desai and Banat, Microbiology and Molecular Biology Reviews, March, 1997, pp 47-64).

In EP 1 623 631 we have previously found that hydrophobins allow the production of aqueous foams with excellent stability to disproportionation and coalescence. Because hydrophobins are highly effective foaming agents, their presence in the fermentation medium presents a particular challenge for foam control.

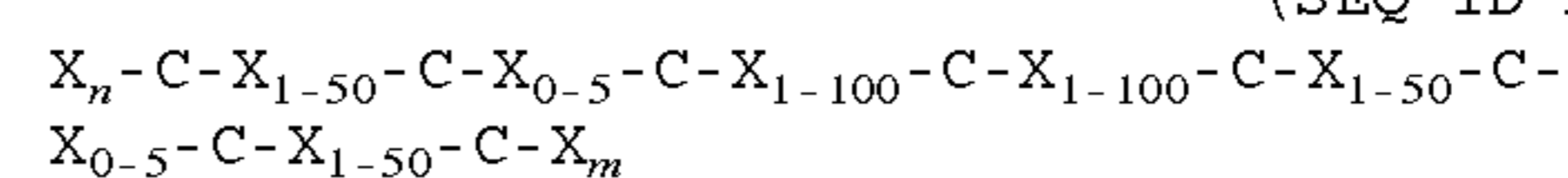
Hydrophobins are a well-defined class of proteins (Wessels, 1997, Adv. Microb. Physio. 38: 1-45; Wosten, 2001, Annu Rev. Microbiol. 55: 625-646) capable of self-assembly at a hydrophobic/hydrophilic interface, and having a conserved sequence:

(SEQ ID NO. 1)



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 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:

(SEQ ID NO. 2)



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, 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 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 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 generally relatively insoluble whereas those of class II hydrophobins readily dissolve in a variety of solvents. Preferably the hydrophobin is soluble in water, by which is meant that it is at least 0.1% soluble in water, preferably at least 0.5%. By at least 0.1% soluble is meant that no hydrophobin precipitates when 0.1 g of hydrophobin in 99.9 mL of water is subjected to 30,000 g centrifugation for 30 minutes at 20° C.

Hydrophobin-like proteins (e.g. "chaplins") have also been identified in filamentous bacteria, such as Actinomycete and Streptomyces sp. (WO01/74864; Talbot, 2003, *Curr. Biol.* 13: R696-R698). These bacterial proteins by contrast to fungal hydrophobins, may form only up to one disulphide bridge since they may 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.

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). For the purpose of the invention hydrophobins possessing at least 80% identity at the amino acid level to a hydrophobin that naturally occurs are also embraced within the term "hydrophobins".

Antifoams

The term "antifoam" includes both antifoams which are usually added before foaming occurs and also those which are usually added once the foam has formed (sometimes known as defoamers). A definition of antifoams used in the present invention is found in "Foam and its mitigation in fermentation systems"—Beth Junker—*Biotechnology Progress*, 2007, 23, 768-784.

Fermentation Process

The fermentation to produce hydrophobin is carried out by culturing the host cell in a liquid fermentation medium within a bioreactor (e.g. an industrial fermenter). The composition of the medium (e.g. nutrients, carbon source etc.), temperature and pH are chosen to provide appropriate conditions for growth of the culture and/or production of the foaming agent. Air or oxygen-enriched air is normally sparged into the medium to provide oxygen for respiration of the culture.

The antifoam may be included in the initial medium composition and/or added as required through the period of the fermentation. Common practice is to employ a foam detection method, such as a conductivity probe, which automatically triggers addition of the antifoam. In the present invention, the antifoam is preferably present at a final concentration of from 0.1 to 20 g/L, more preferably from 1 to 10 g/L.

The fermenter temperature during step i), i.e. during fermentation, may be above or below the cloud point of the

antifoam. Preferably the fermenter temperature is above the cloud point of the antifoam, since the antifoam is most effective at causing bubble coalescence and foam collapse above its cloud point. The fermenter temperature is generally chosen to achieve optimum conditions for growth of the host cells and/or production.

BRIEF DESCRIPTION OF THE INVENTION

It is the object of the invention to provide a process for extracting hydrophobin from a solution wherein carrageenan is added to the solution and the pH of the solution is brought below 3.5, preferably below 3.

In a first preferred embodiment of the invention, the solution is then filtered to produce a retentate and a filtrate, hydrophobin being recovered from the retentate. In a second preferred embodiment of the invention, the solution is submitted to a centrifugation step to produce a supernatant which is removed, leaving a remaining phase. Hydrophobin is then removed from the remaining phase.

Preferably, the process comprises the step of cultivating a host cell in a fermentation medium wherein the host cell extra-cellularly secretes hydrophobin; and the fermentation medium contains an antifoam. More preferably, the fermentation medium is aerated by sparging air or oxygen-enriched air into it.

Preferably the hydrophobin is HFBI or HFBII from *Trichoderma reesei*.

Preferably the host cell is a genetically-modified fungus, more preferably a yeast, most preferably *Saccharomyces cerevisiae*.

Preferably the ionic strength of the solution is below 0.5, preferably below 0.4, more preferably below 0.3, even more preferably below 0.2

Preferably, carrageenan is kappa or iota carrageenan, more preferably iota carrageenan.

Preferably also the carrageenan/hydrophobin ratio (w/w) is between 1:10 and 10:1, preferably above 1:5, more preferably above 1:1.

DETAILED DESCRIPTION OF THE INVENTION

The present invention will be further described in the following examples wherein the hydrophobin is always HFB II.

Example 1

Comparative

Starting concentration 145.4 $\mu\text{g}/\text{cm}^3$ of hydrophobin in 4.5 cm^3 of a 25 mM citric acid solution. The solution was filtered leading to a concentration in filtrate of 67.9 $\mu\text{g}/\text{cm}^3$ in 9 cm^3 .

There for 93% of the original hydrophobin filtered through.

Example 2

Comparative

Starting concentration 146.3 $\mu\text{g}/\text{cm}^3$ of hydrophobin in 4.5 cm^3 of a 25 mM sodium Citrate. The solution was filtered leading to a concentration in filtrate of 68.0 $\mu\text{g}/\text{cm}^3$ in 9 cm^3 .

Therefore again 93% of the original hydrophobin filtered through.

Example 3

Invention

Starting concentration 145.9 $\mu\text{g}/\text{cm}^3$ of hydrophobin in 4.5 cm^3 of a 25 mM citric acid solution+1% kappa carrageenan

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sheared gel. The solution was filtered leading to a concentration in filtrate of $3.8 \mu\text{g}/\text{cm}^3$ hydrophobin in 9 cm^3 .

Therefore, only 5% of the hydrophobin filtered through.

Then 9 cm^3 of 25 mM sodium citrate at pH 8 were passed through filter. The concentration in the filtrate was $40.9 \mu\text{g}/\text{ml}$, therefore 56% of the original hydrophobin was recovered that way.

Example 4

Comparative

Starting concentration $145.9 \mu\text{g}/\text{cm}^3$ of hydrophobin in 4.5 cm^3 of a 25 mM citric acid solution+1% kappa carrageenan sheared gel.

Then add 0.325 cm^3 NaOH (to go to pH 7.0) and filter
Concentration in filtrate= $75.6 \mu\text{g}/\text{cm}^3$ in 9 cm^3

Here, 100% of the hydrophobin ended in the filtrate despite the use of carrageenan, showing the importance of pH.

Example 5

Invention

Starting concentration $145.9 \mu\text{g}/\text{cm}^3$ of hydrophobin in 4.5 cm^3 of a 25 mM citric acid solution+1% kappa carrageenan sheared gel.

Then add solid NaCl to give a concentration of 0.5M NaCl and filter. The concentration in filtrate is $50.9 \mu\text{g}/\text{cm}^3$ in 9 cm^3 . So, about 70% of the original amount of hydrophobin filtered through despite the right pH and the use of 1% kappa carrageenan sheared gel.

Then 9 cm^3 of 25 mM sodium citrate at pH 8 passed through filter leading to a concentration in filtrate of $13.8 \mu\text{g}/\text{cm}^3$. Therefore, only 19% of the hydrophobin was recovered that way, showing the influence of the ionic strength on the whole process. The higher the ionic strength, the lower the recovery, everything else being equal.

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Example 6a

Invention

Starting concentration $145.9 \mu\text{g}/\text{cm}^3$ of hydrophobin in 4.5 cm^3 of a 25 mM citric acid solution+0.025% iota carrageenan.

The concentration in filtrate was $1.6 \mu\text{g}/\text{cm}^3$ in 9 cm^3 , only 2% of the original hydrophobin having passed through.

Then 9 cm^3 of 25 mM sodium citrate at pH 8 passed through filter, leading to a concentration in filtrate= $29.5 \mu\text{g}/\text{cm}^3$

Over 40% of the original hydrophobin was recovered.

Example 6b

Invention

Starting concentration $145.9 \mu\text{g}/\text{cm}^3$ of hydrophobin in 4.5 cm^3 of a 25 mM citric acid solution+0.025% kappa carrageenan.

The concentration in filtrate was $28.4 \mu\text{g}/\text{cm}^3$ in 9 cm^3 , 39% of the original hydrophobin having passed through.

This example shows that iota carrageenan performs better than kappa carrageenan when retaining hydrophobin

Example 7

Comparative

Starting concentration $145.9 \mu\text{g}/\text{cm}^3$ of hydrophobin in 4.5 cm^3 of a 25 mM citric acid solution+1% sheared pectin.

The concentration in filtrate was $57.3 \mu\text{g}/\text{cm}^3$ in 9 cm^3 , representing 79% of the original hydrophobin, showing that pectin does not work.

Example 8

Comparative

Starting concentration $145.9 \mu\text{g}/\text{cm}^3$ of hydrophobin in 4.5 cm^3 of a 25 mM citric acid and 1% N creamer 46.

The concentration in filtrate was $64.2 \mu\text{g}/\text{cm}^3$ in 9 cm^3 representing 88% of the original hydrophobin, showing that hydrophobic starch does not work.

SEQUENCE LISTING

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<211> LENGTH: 4106

<212> TYPE: PRT

<213> ORGANISM: Artificial

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<223> OTHER INFORMATION: Exemplary sequence used to illustrate invention.

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<223> OTHER INFORMATION: Xaa is any amino acid and can be shown 1 to 2000 times.

<220> FEATURE:

<221> NAME/KEY: REPEAT

<222> LOCATION: (2002)..(2010)

<223> OTHER INFORMATION: Xaa is any amino acid and can be shown 5 to 9 times.

<220> FEATURE:

<221> NAME/KEY: REPEAT

<222> LOCATION: (2013)..(2051)

<223> OTHER INFORMATION: Xaa is any amino acid and can be shown 11 to 39 times.

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 Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
 50 55 60
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 65 70 75 80
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<212> TYPE: PRT

<213> ORGANISM: Artificial

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<223> OTHER INFORMATION: Exemplary sequence used to illustrate

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<220> FEATURE:
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Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
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Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
 115 120 125

Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
 130 135 140

Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
 145 150 155 160

Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa

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165				170				175						
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		210				215				220				
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Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa	1060	1065	1070	
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Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa	1140	1145	1150	
Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa	1155	1160	1165	
Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa	1170	1175	1180	
Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa	1185	1190	1195	1200
Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa	1205	1210	1215	
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Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa				

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The invention claimed is:

1. Process for extracting hydrophobin from a solution comprising the steps of:

- a) adding carrageenan to the solution;
 - b) adjusting the pH of the solution to below 3.5;
 - c) filtering the solution to produce a retentate and a filtrate;
- and

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d) recovering the hydrophobin from the retentate;

[e) centrifuging the solution to produce a supernatant which is removed, leaving a remaining phase; and

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f) removing the hydrophobin from the remaining phase;]

wherein the resulting hydrophobin is suitable for use in foods, personal care products and medicine.

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2. Process according to claim 1 further comprising the step of cultivating a host cell in the solution including a fermentation medium wherein the host cell extra-cellularly secretes hydrophobin; and the fermentation medium contains an anti-foam.

3. Process according to claim 2 wherein the host cell is a genetically-modified fungus.

4. Process according to claim 3 wherein the host cell is a yeast.

5. Process according to claim 4 wherein the host cell is *Saccharomyces cerevisiae*.

6. Process according to claim 1 wherein the hydrophobin is HFBI or HFBII from *Trichoderma reesei*.

7. Process according to claim 1 wherein the solution has an ionic strength below 0.5.

8. Process according to claim 1 wherein the carrageenan is kappa or iota carrageenan.

9. Process according to claim 8 wherein the carrageenan is iota carrageenan.

10. Process according to claim 1 wherein the carrageenan/hydrophobin ratio (w/w) is between 1:10 and 10:1.

11. Process according to claim 1 wherein the carrageenan is a shear gel.

12. Process for extracting hydrophobin from a solution comprising the steps of:

- a) adding carrageenan to the solution;
- b) adjusting the pH of the solution to below 3.5;
- c) centrifuging the solution to produce a supernatant which is removed, leaving a remaining phase; and
- d) removing the hydrophobin from the remaining phase;

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wherein the resulting hydrophobin is suitable for use in foods, personal care products and medicine.

13. Process according to claim 12 further comprising the step of cultivating a host cell in the solution including a fermentation medium wherein the host cell extra-cellularly secretes hydrophobin; and the fermentation medium contains an antifoam.

14. Process according to claim 13 wherein the host cell is a genetically-modified fungus.

15. Process according to claim 14 wherein the host cell is a yeast.

16. Process according to claim 15 wherein the host cell is *Saccharomyces cerevisiae*.

17. Process according to claim 12 wherein the hydrophobin is HFBI or HFBII from *Trichoderma reesei*.

18. Process according to claim 12 wherein the solution has an ionic strength below 0.5.

19. Process according to claim 12 wherein the carrageenan is kappa or iota carrageenan.

20. Process according to claim 19 wherein the carrageenan is iota carrageenan.

21. Process according to claim 12 wherein the carrageenan/hydrophobin ratio (w/w) is between 1:10 and 10:1.

22. Process according to claim 12 wherein the carrageenan is a shear gel.

* * * * *