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[Continued on next page]

(54) **Title**: A POLYNUCLEOTIDE EXPRESSION CASSETTE

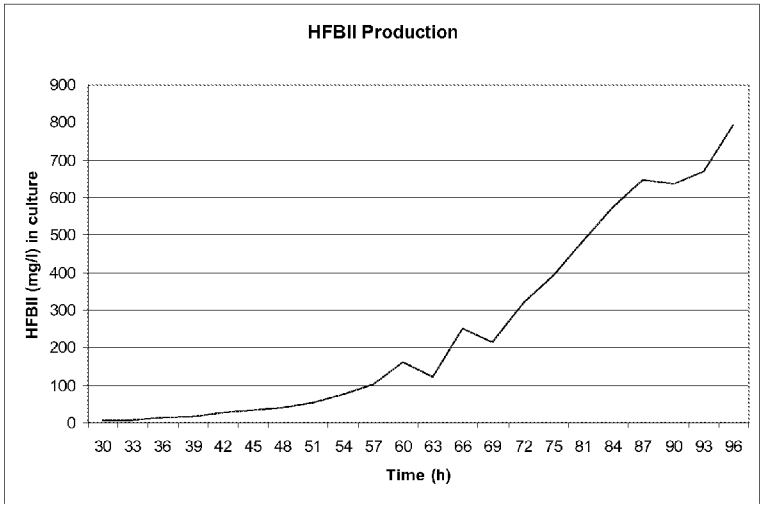

Figure 9

(57) **Abstract**: A polynucleotide expression cassette comprising an extracellular signal sequence operatively associated with an oligonucleotide sequence encoding the HFBII protein. The polynucleotide expression cassette is such that where a yeast host cell is transformed with the expression cassette, the cell is capable of synthesising at least 300 mg/l of the HFBII in a 100 hour period.

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A POLYNUCLEOTIDE EXPRESSION CASSETTE

Technical Field of the Invention

The present invention relates to a method of synthesising HFBII protein, a polynucleotide expression cassette that can be used to synthesise HFBII protein and a host cell comprising such a polynucleotide expression cassette. The present invention also relates to a method of synthesising heterologous proteins in general.

Background of the Invention

Hydrophobins are a family of surface-active proteins produced naturally by filamentous fungi such as *Trichoderma reesei*. They are small proteins (7-9 kDa) and are characterised by the presence of eight cysteine residues forming four disulphide linkages. Hydrophobins are remarkable for their high surface activity and the fact that they self-assemble at hydrophobic-hydrophilic surfaces or interfaces to form a robust, amphipathic layer. Two classes of hydrophobins have been identified and they are designated class I and class II. Class I hydrophobins (such as SC3 from *Schizophyllan commune*) form insoluble aggregates which can only be dissolved with strong acids. Class II hydrophobins (which include HFBI and HFBII from *Trichoderma reesei*) are more readily solubilised and can be dissolved in aqueous solutions to concentrations of at least 100mg/ml. It has been reported by Nakari-Setälä T. *et al* 1997 that the amino acid similarity of HFBII to HFBI is 69%.

However, the sequence similarity between class I hydrophobins and class II hydrophobins is low and it has been speculated that the two classes of hydrophobins evolved independently (Whiteford, J. R. *et al* 2002 Hydrophobins and the interactions between fungi and plants. Mol. Plant Pathol. 3, 391-400) meaning that similarities between the two classes may be limited.

The properties of hydrophobins outlined above have enabled them to be used in applications such as coatings, emulsion stabilisation and separation technologies. It has also been determined (see Cox A *et al*) that the class II hydrophobins HFBI and HFBII have surface elasticities far in excess of that known for any other protein and that, for HFBII, this property leads to the stabilisation of bubbles to the process of

disproportionation. In part, this appears to be due to the ability of HFBII to be absorbed at the air/water interface of bubbles which thereby stabilises the bubbles.

These properties of hydrophobins find particular application in the stabilisation of food products (e.g. frozen and chilled food products such as ice cream and mousses) which contain introduced gas in the form of bubbles of air, nitrogen and/or carbon dioxide. Hydrophobins are particularly suitable in this role because even relatively low levels of hydrophobins can provide excellent foam volume stability and inhibition of coarsening ("coarsening" being the increase in gas bubble size by processes such as disproportionation and coalescence).

While it is known that HFBII is a useful additive for foam stabilisation, it is difficult to produce HFBII in industrial quantities. Naturally, *Trichoderma reesei* produces HFBII only at low levels (Bailey *et al. infra*). It has been proposed, with regard to the related HFBI gene, to genetically modify *Trichoderma reesei* to increase the copy number of the HFBI gene (see Askolin *et al* and Bailey *et al*) but such techniques have tended to result in the majority of the expressed HFBI protein becoming bound to the cell wall, rather than being secreted into the medium in which the cells are cultured. This makes the HFBI protein difficult to extract.

WO96/41882 reports on the expression of two class I hydrophobins, HYPA and HYPB from *A. bisporus*, in yeast. However, it also acknowledges the difficulties in the secretion of recombinantly expressed hydrophobins from host cells due to the extraordinary properties of these proteins.

The present invention has arisen because the inventors have surprisingly found that recombinantly expressing the HFBII gene, together with a suitable extracellular signal peptide, in a host cell, results in a surprisingly high level of expression of the HFBII protein and secretion thereof from the host cell.

Summary of the Invention

According to one aspect of the present invention, there is provided a polynucleotide expression cassette comprising an extracellular signal sequence operatively associated with an oligonucleotide sequence encoding an HFBII protein.

Preferably, the polynucleotide expression cassette is such that a yeast host cell transformed with the polynucleotide expression cassette is capable of synthesising at least 300mg/1 of the HFBII protein in a 100 hour period.

Yeasts are a sub-division of the kingdom of fungi. They span a number of kingdom sub-divisions (phyla), but are distinguished by the fact that they are uni-cellular. By contrast, fungi which grow as multicellular organisms are known as moulds or filamentous fungi. Yeasts do not produce hydrophobins naturally, instead hydrophobins are naturally expressed exclusively by filamentous fungi such as *Trichoderma reesei*.

In preferred embodiments, the transformed yeast host cell is capable of synthesising at least 400mg/l, at least 500mg/l or at least 600mg/l of the HFBII protein in a 100 hour period.

The synthesis of at least 300mg/1, 400mg/l, 500mg/l or 600mg/l (the "threshold concentration") of the HFBII protein in a 100 hour period is achieved under optimal fermentation conditions. For example, in order to assess whether a polynucleotide expression cassette meets these criteria, a host cell is transformed with the expression cassette and then cultured at between 20°C and 32°C in rich medium containing yeast extracts. The culture is in a vessel of between 5 and 10l volume to permit oxygen transfer and the glucose feed is limited to the rate at which glucose is used by the yeast or is provided in a controlled feed. Suitable medium and protocols for determining HFBII synthesis levels are disclosed in the examples herein. For instance, in one embodiment, synthesis of HFBII by a particular yeast strain is measured using the fed batch fermentation protocol provided in Example 3. It is to be understood that the method may involve the synthesis of the threshold concentration of the HFBII protein (e.g. 300mg/l or more) in less than 100 hours as there is a tendency for the concentration of the protein to peak and then fall during culturing as foaming over takes place. However, at a time point during the 100 hour period, the concentration of HFBII reaches at least the threshold concentration.

It is to be noted that, in contrast to the present invention Bailey *et al* (infra) report on the synthesis of only 240 mg/1 of HFBII after 96 hours.

Typically, the extracellular signal sequence is located 5' upstream of the oligonucleotide sequence encoding the HFBII protein.

Conveniently, a yeast host cell transformed with the polynucleotide expression cassette and cultured in medium is capable of synthesising and secreting into the culture at least 300 mg/1 of the HFBII protein in a 100 hour period. The level of synthesis is measured in terms of the amount of protein present in the whole culture.

Preferably, the HFBII protein comprises a sequence at least 80% identical to SEQ. ID NO. 2, preferably at least 90%, 95% or 99% identical thereto.

Advantageously, a purified 1μM sample of the HFBII protein demonstrates a surface shear elasticity of at least 0.1Nm^{-1} at pH 7, 20°C after 10,000 seconds.

Conveniently, a purified 1μM sample of the HFBII protein demonstrates a surface shear elasticity of at least 0.5Nm^{-1} at pH 7, 20°C after 10,000 seconds.

Preferably, the extracellular signal sequence comprises a sequence at least 80% identical to SEQ. ID NO. 3, preferably at least 90%, 95% or 99% identical thereto. For example, the 5' ATG codon is optionally omitted.

Advantageously, the expression cassette further comprises a promoter for expression of a gene in a yeast cell. The promoter is located so as to control expression of the HFBII protein.

According to another aspect of the present invention, there is provided a vector comprising a polynucleotide expression cassette according to the present invention.

Conveniently, the vector is a plasmid or a YAC.

According to a further aspect of the present invention, there is provided a yeast host cell comprising a polynucleotide expression cassette according to the invention recombinantly incorporated therein.

Preferably, the cell is a food grade yeast strain, such as a strain complying with the USFDA GRAS designation.

Advantageously, the cell is of one of the following strains: *Saccharomyces cerevisiae, Kluyveromyces lactis, Schizosaccharomyces pombe, or Yarrowia lipolytica.*

Conveniently the cell is of strain CEN.PK102 or strain DXY1 rendered Hsp150 and pmt1 deficient, or a derivative thereof.

Preferably, the polynucleotide expression cassette is integrated into the genome of the yeast host cell.

Alternatively, the expression cassette is located in the cell as an extra-genomic vector.

According to another aspect of the present invention, there is provided a method of synthesising HFBII protein comprising expressing a polynucleotide sequence encoding the HFBII protein in a yeast host cell such that the HFBII protein is synthesised at a concentration of at least 300mg/1 in a 100 hour period.

Conveniently, the yeast host cell comprises a polynucleotide expression cassette according to the invention recombinantly incorporated therein.

Preferably, the method comprises maintaining the yeast host cells in a medium and raising the pH of the medium during the method.

Advantageously, the method comprises raising the pH of the medium by at least 1 on the pH scale.

Conveniently, the method comprises raising the pH of the medium from between pH 4.5 and pH 5.5 to between pH 6.0 and pH 7.0.

Preferably, the method comprises minimising the amount of foam in the layer above the culture such that after the 100 hour period the foam layer accounts for less than 5% of the volume of the culture, more preferably less than 1% of the volume.

According to another aspect of the present invention, there is provided a method for synthesising a heterologous protein in a yeast host cell that has been transformed with a

polynucleotide comprising a sequence encoding the heterologous protein comprising the steps of:

(i) maintaining the yeast host cell in a growth medium under growth conditions at a first pH value such that the yeast host cell multiplies; and

(ii) raising the pH of the medium to a second pH value, or transferring the multiplied yeast host cells to a growth medium at a second pH value, higher than the first pH value, and synthesising the heterologous protein.

It is preferred that the methods of the invention are performed using a yeast host cell of strain DXY1 rendered Hsp150 and pmt1 deficient.

Conveniently, the second pH value is at least 1 higher on the pH scale than the first pH value.

Preferably, the first pH value is between pH 4.5 and pH 5.5 and/or the second pH value is between pH 6.0 and pH 7.0.

Advantageously, the heterologous protein is a class II hydrophobin such as HFBII.

In the methods described herein the proteins (e.g. HFBII) are synthesised by yeast in a medium with a pH between 4 and 8, typically between 5 and 7.

In this specification, the percentage “identity” between two sequences is determined using the BLASTP algorithm version 2.2.2 (Altschul, Stephen F., Thomas L. Madden, Alejandro A. Schäffer, Jinghui Zhang, Zheng Zhang, Webb Miller, and David J. Lipman (1997), "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs", Nucleic Acids Res. 25:3389-3402) using default parameters. In particular, the BLAST algorithm can be accessed on the internet using the URL http://www.ncbi.nlm.nih.gov/blast/.

In this specification, the term “secretion” when used in relation to a protein translated in a cell means that the protein passes through the cell membrane and can be isolated from the other components of the cell without disrupting the cell membrane.

In this specification, “Strain A” refers to a yeast strain derived from *S. cerevisiae* DXY1 yeast strain (reported in Kerry-Williams S.M. *et al* Disruption of *Saccharomyces*

cerevisiae YAP3 gene reduces the proteolytic degradation of secreted recombinant human albumin, Yeast, 14, 161-169 (1998)) which has been rendered Hsp150 and pmt1 deficient using the techniques disclosed in WO95/033833 and US5,714,377, respectively.

In this specification, a "class II hydrophobin" is defined as an amphiphilic, surface active protein having one or more of the following features:

i) aggregates of the protein can be dissolved using aqueous dilutions of organic solvents;

ii) the sequence of the protein has at least 29% sequence identity to the sequence in SEQ ID NO. 2, preferably at least 50%, 60%, 70%, 80%, 90%, 95% or 99% sequence identity thereto;

iii) the protein comprises the consensus sequence of SEQ ID NO. 7; and

iv) the protein comprises a sequence with at least 80% identity with the consensus sequence of SEQ ID NO. 8.

Brief Description of the Figures

Figure 1 is a restriction map of the HFBII synthetic gene of SEQ. ID NO. 4.

Figure 2 is a schematic diagram of the *Saccharomyces cerevisiae* multi-copy integration vector pUR8569.

Figure 3 is a schematic diagram of the Hydrophobin expression plasmid pUR9011.

Figure 4 is a schematic diagram of plasmid pSAC35, which is a yeast expression vector described in WO2005/077042.

Figure 5 is a graph showing the results of HFBII production over time by CEN.PK102-3Δ*gal*Δ*pmt1*-pUR9110#2 yeast cells in accordance with one example of the present invention.

Figure 6 is a graph showing the results of HFBII production over time by Strain A-pDB3268 yeast cells in accordance with another example of the present invention.

Figure 7 is a graph showing the results of HFBII production over time by yeast cells under the same conditions as the example whose results are shown in Figure 6 except that increased stirring of the culture was carried out to reduce the foam layer on the culture.

Figures 8 to 10 are graphs showing the results, in triplicate, of HFBII production over time by Strain A-pDB3268 yeast cells, in which the pH of the culture was raised during the feed phase in accordance with another example of the present invention.

Brief Description of the Sequence Listing

SEQ. ID NO. 1 is the nucleotide sequence that encodes HFBII from *Trichoderma reesei*.

SEQ. ID NO. 2 is the protein sequence of HFBII as encoded by SEQ. ID NO. 1.

SEQ. ID NO. 3 is the nucleotide sequence of the SUC2 signal sequence from *S. cerevisiae.*

SEQ. ID NO. 4 is the nucleotide sequence encoding a fusion of the SUC2 signal sequence and the HFBII protein.

SEQ. ID NO. 5 is the protein sequence of HFBII as encoded by SEQ. ID NO. 4.

SEQ. ID NO. 6 is the nucleotide sequence of the plasmid pUR9011 whose construction is described in Example 1.

SEQ. ID NO. 7 is an amino acid consensue sequence for class II hydrophobins.

SEQ. ID NO. 8 is another amino acid consensue sequence for class II hydrophobins.

Sequence Listing Free Text

SEQ. ID NO. 4
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SEQ. ID NO. 5
<223> Synthetic Construct

SEQ. ID NO. 6
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Detailed Description

In one embodiment of the present invention a polynucleotide vector is provided which comprises an expression cassette. The expression cassette comprises an oligonucleotide sequence encoding the HFBII protein, as shown in SEQ. ID NO. 1. The oligonucleotide sequence is, at its 5' end, fused to the *SUC2* signal sequence, which is shown in SEQ. ID NO. 3. The expression cassette also comprises a strong yeast promoter such as GALF7.

In use, the polynucleotide is used to transform a host yeast cell such as a strain of *Saccharomyces cerevisiae*. Transformation is carried out by the lithium acetate/single-stranded carrier DNA/polyethylene glycol method (e.g. using the Sigma yeast transformation kit) for which further details may be found in Gietz RD, Woods RA Meth. Enzymol. 350: 87:96 Transformation of yeast by lithium acetate/single-stranded carrier DNA/polyethylene glycol method. Alternatively transformation may be carried out by electroporation. In some embodiments, the expression vector comprises rDNA sequences for the targeted integration of the expression vector into the host cell genome but in other embodiments, the oligonucleotide vector contains the necessary elements (such as an origin of replication and stability determining genes of the native 2 micron plasmid or centromere sequences) for stable extra-genomic reproduction.

After transformation of the yeast host cell, the transformed cell is cultured in medium such that expression of the HFBII protein takes place and the protein is secreted into the cell culture medium. The SUC2 signal sequence is cleaved off from the nascent protein during the expression and synthesis process. The HFBII protein is then extracted from the culture medium by filtration and purified. In the embodiments of the present invention, a concentration of at least 300 mg/l of the HFBII protein is secreted into the culture after 100 hours by the transformed cell culture. The level of secretion of the protein is measured in the whole culture, although the protein is predominantly present in the fermentation supernatant. This level of synthesis is achieved under optimal conditions including fermentation in a container of 10l volume, to permit a high oxygen transfer rate, and using separate batch and feed phases. In the batch phase, yeast growth is optimised using an excess of glucose. In the feed phase, yeast growth and protein synthesis are balanced and optimised by limiting the feed of glucose. This level of expression in a yeast host cell such as *S. cerevisiae* is surprising because hydrophobic proteins such as HFBII are not normally well expressed in yeast.

Because the protein is secreted into the cell culture medium, it is possible to recover the protein without disrupting the plasma membranes of the host cells. This avoids the need for an additional step to separate the protein from other cell components. In practice, around 10% of synthesised hydrophobin becomes attached to the external cell wall of the hosts cells. However, this hydrophobin is still regarded as being "secreted" because it can be recovered without disrupting the cell membranes, for example by washing the attached hydrophobin from the cell wall with ethanol.

In the above described embodiment, there is provided an oligonucleotide encoding the HFBII protein, in accordance with SEQ. ID NO. 1. However, it is to be appreciated that, in other embodiments, the oligonucleotide sequence is varied without significantly affecting the properties of the resulting HFBII protein. In particular, any oligonucleotide sequence which encodes the HFBII protein as set out in SEQ. ID NO. 2 may also be used in alternative embodiments. Furthermore, various additions, deletions and substitutions of amino acid residues of the HFBII protein sequence that is encoded may be made without significantly affecting the properties of the HFBII protein. Nevertheless, the HFBII protein must have at least 80% sequence identify to SEQ. ID NO. 2. Furthermore, such variant proteins demonstrate a surface shear elasticity of at least 0.1 Nm^{-1} at pH 7, 20°C after 10,000 seconds when at a concentration of at least 1μM, this being the principal functional characterisation of the HFBII protein. In preferred embodiments, the variant protein demonstrates a surface shear elasticity of at least 0.5 Nm^{-1} under the same conditions. These thresholds are derived from Cox A. *et al.* 2007 in which it is reported that the surface shear elasticity of HFBII protein is "far in excess" of proteins such as sodium caseinate, ß-casein and ß-lactoglobulin. Cox A. *et al* is hereby incorporated by reference, particularly as regards the methodology for determining surface shear elasticity of a protein. In one particular variant in accordance with certain embodiments amino acid residue number 216 of SEQ. ID NO: 1 (i.e. the C-terminal phenylalanine residue) is deleted.

Similarly, in alternative embodiments, a different extracellular signal sequence from *SUC2* may be used, such as a sequence with at least 80% sequence identity to SEQ. ID NO. 3. In particular, one of the 5' ATG codons of the sequence may be omitted. Alternatively, an entirely different extracellular signal sequence may be used, for example the pre-pro signal sequence of yeast mating factor alpha. What is important is that the signal

sequence results in the HFBII protein being secreted into the yeast cell growth medium rather than being retained within the cell.

In the embodiment described above, the polynucleotide vector is a plasmid. However, in alternative embodiments, the vector is of a different type such as a yeast artificial chromosome (YAC). In these embodiments, the YAC comprises the minimum elements required for independent reproduction, namely a telomere, centromere and replication of origin sequence elements.

In the above described embodiments, the yeast host cell is a strain of *Saccharomyces cerevisiae*. However, in alternative embodiments, other yeast strains are used, in particular, food grade yeast strains. One definition of food grade yeast strains are those complying with the GRAS (Generally Regarded As Safe) designation given by the USA Food and Drug Administration. While not limiting on the invention, preferred food grade yeast strains include: *Kluyveromyces lactis, Schizosaccharomyces pombe,* and *Yarrowia lypolytica*.

In order for the HFBII protein to be used in the applications described above, it is desirable, or even essential, for the protein to be non-glycosylated. Accordingly, it is advantageous for the yeast strain to have a reduced level of glycosylation of secreted proteins, for example by being deficient in expression of the *pmt1* gene. For this reason, particularly preferred strains of *Saccharomyces cerevisiae* are CEN.PK102-3Δ*gal1*Δ pmt1, Strain A, and derivatives thereof that, when transformed with an expression cassette of the invention, are capable of synthesising at least 300mg/L of the HFBII protein in a 100 hour period.

It has been observed that certain yeast strains (in particular Strain A which is disclosed herein) produce higher levels of heterologous proteins such as HFBII when maintained in a medium at a relatively high pH (i.e. around pH 6.5). However, in practice it is found that maintaining yeast strains at such pH levels permits contaminating bacteria (whose presence in batch fermentations is difficult to avoid) rapidly to outgrow the yeast strains and therefore limit effective yeast multiplication and protein production. Consequently, it has been found that maintaining yeast host cells in growth medium at a relatively low pH value (e.g. of around pH 5) during the early stages of a fermentation permits multiplication of yeast host cells while preventing bacterial growth. Once the yeast host cells have

multiplied to a significant level, the pH of the growth medium in which the cells are located is raised to around pH 6.5 (or, alternatively, the growth medium is replaced with a new growth medium with a pH of around 6.5). The higher pH level of the growth medium results in a greater level of production of the heterologous protein (e.g. HFBII) and the high quantity of the yeast biomass is, by this stage in the fermentation process, sufficient to ensure that it is not overgrown by any remaining bacterial contaminants.

It has also been observed that a higher yield of the HFBII protein is achieved if the layer of foam, which naturally forms at the top of the fermentation, is reduced or eliminated. It is believed that HFBII protein in solution otherwise migrates into the foam, thereby reducing the yield of the protein that can be recovered from the culture. Several means of reducing the presence of foam exist. In some embodiments, the fermentation is stirred at multiple (e.g. 3) levels within the fermentation vessel. In other embodiments, in which only a single stirrer is available, it has been found that by increasing the rate of stirring around 24 hours after initiation of the feed phase, foaming is reduced. In other embodiments, an anti-foaming agent such as Struktol J647 is provided. An anti-foaming agent may be provided in combination with one of the stirring techniques previously described.

Examples

Example 1 - Expression of HFBII in bakers yeast

An expression cassette for the *Trichoderma reesei* HFBII protein was constructed as follows. A synthetic gene (SEQ. ID NO. 4) was designed encoding the HFBII sequence (SEQ. ID NO. 1) fused to the *Saccharomyces cerevisiae SUC2* signal sequence (SEQ. ID NO. 3), based on the mature protein sequence of HFBII (Genbank accession number P79073, Nakari-Setala,T., Aro,N., Ilmen,M., Munoz,G., Kalkkinen,N. and Penttila,M (1997). Differential expression of the vegetative and spore-bound hydrophobins of *Trichoderma reesei*--cloning and characterization of the HFBII gene. Eur. J. Biochem. 248 415-423). The gene was constructed such that the 5' end contained a *Sac*I restriction enzyme recognition site and the 3' end a *Hin*dIII restriction enzyme recognition site (see SEQ. ID NO. 4 and the schematic diagram shown in Figure 1). The codons were optimised for expression in *S. cerevisiae* (using the codon usage database from Yasukazu Nakamura: http://www.kazusa.or.jp/codon) with the exception of sequences that would have generated unwanted *Sac*I or *Hind*III sites. The gene was synthesised commercially at Baseclear (Einsteinweg 5, P.O. Box 1336, 2333 CC Leiden, The Netherlands) and cloned in the cloning vector pGEMTteasy (Promega).

The resulting plasmid was digested with *Sac*I and *Hin*dIII and the 303bp fragment containing the expression cassette extracted and purified after agarose gel electrophoresis. This fragment was ligated with the 8914bp *Sac*I/*Hin*dIII fragment of the vector pUR8569 (a schematic diagram of which is shown in Figure 2), designed to integrate at the rDNA locus as described by Lopes et al (Lopes TS, Klootwijk J, Veenstra AE, van der Aar PC, van Heerikhuizen H, Raúe HA and Planta RJ. 1989 High-copy-number integration into the ribosomal DNA of *Saccharomyces cerevisiae*: a new vector for high-level expression Gene 15;79:199-206). The ligation mix was used to transform *Escherichia coli* TG1 (Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). Molecular Cloning: a Laboratory Manual, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory) by electroporation and ampicillin resistant transformants identified. Plasmids isolated from individual transformants were extracted and digested with *Sac*I and *Hin*dIII and those containing the 303 bp fragment identified. One such plasmid, pUR9011 (SEQ. ID NO: 6, a schematic diagram of which is shown in Figure 3), was chosen as the source of the expression cassette for expression in *S. cerevisiae* .

pUR9011 was digested with *Hpa*I and the 6559bp fragment isolated and purified after agarose gel electrophoresis. This fragment lacks the *E. coli* and ampicillin resistance gene DNA but contains the expression cassette for HFBII; rDNA sequences for the targeted integration of this fragment in the *S. cerevisiae* genome; the *leu2d* gene which acts as a selection marker for amplification of the cassette in l*eu2* auxotrophic mutants and a region of *S. cerevisiae* chromosome IX DNA that increases the stability of the integrated fragment, probably by ensuring that the integrated fragment is similar to that of a single rDNA repeat (Lopes TS, de Wijs IJ, Steenhauer SI, Verbakel J and Planta RJ., 1996, Factors affecting the mitotic stability of high-copy-number integration into the ribosomal DNA of *Saccharomyces cerevisiae* Yeast. 12(5):467-477).

This 6559bp fragment was used to transform the *S. cerevisiae* strain CEN.PK102-3Δ*gal*1 (*gal1:URA3, leu2, ura3*) to leucine prototrophy (Yeast Transformation by the LiAc/SS Carrier DNA/PEG Method. Methods in Molecular Biology:313:107-120 (2005)). The resulting transformants were screened for expression of HFBII by growth for 48 hours in YNB minimal medium (Difco Yeast Nitrogen Base, 2% glucose) followed by 48 hours growth in inducing medium (1% yeast extract, 2% peptone, 2%glucose, 0.5% galactose). The culture was extracted by addition of ethanol to 60% vol/vol and the cells removed by

filtration through a 2μm milipore filter. The presence of HFBII was assessed by HPLC analysis using the assay conditions shown at the end of this Example.

The results are shown in Table 1. The level of protein secretion is measured in terms of the amount in the whole culture.

Table 1

Hydrophobin production by CEN.PK102-3Δ*gal*1-pUR9110 transformants in shake flask culture.

Transformant	HFBII concentration in culture (mg/l)
CEN.PK102-3Δ*gal*1	0
CEN.PK102-3Δ*gal*1-pUR9011#12	8.5
CEN.PK102-3Δ*gal*1-pUR9011#13	8.3
CEN.PK102-3Δ*gal*1-pUR9011#14	4.3
CEN.PK102-3Δ*gal*1-pUR9011#16	3.4
CEN.PK102-3Δ*gal*1-pUR9011#18	4.3
CEN.PK102-3Δ*gal*1-pUR9011#19	6.6

It is clear that these transformants are capable of producing HFBII

The hydrophobin produced by these transformants was analysed by liquid chromatography mass spectroscopy (LCMS). The sample was diluted with 60 % aqueous ethanol to an approximate concentration of 200 μg/ml prior to analysis. HPLC separation was performed on a Vydac Protein C4 column (150 x 4.6 mm). Mobile phase A consisted of 0.025% TFA in water, mobile phase B was 0.025% TFA in acetonitrile. The following gradient programme was applied: T=0: 80% A, 20% B, T=30min: 30% A, 70% B, T=31min: 0% A, 100% B, T=34min: 0% A, 100% B; T=35min: 80% A, 20% B, followed by 10 min equilibration between runs. Flow rate was 1 ml/min, the column temperature was maintained at 30°C. The eluent from the HPLC column was split at a ratio of 1:4 introducing 200 μl/min into the electrospray ion source of the mass spectrometer. (Waters ZMD single quadrupole mass spectrometer (API))

Ionisation was achieved using the following parameters: Capillary Voltage: 3.0 kV, Cone: 30 V, Extractor: 4 V, RF Lense: 0.4 V, source temp: 100 °C, desolvation temp: 300°C, desolvation gas flow: 500 L/h, cone gas flow: 100 L/h. Full scan mass spectra were

recorded across the mass range m/z 500 – 2000 with a scan time of 2 secs. These results showed significant glycosylation of the hydrophobin.

Example 2 – Expression of HFBII in Yeast strain with impaired O-glycosylation

WO94/04687 describes the preparation of *S. cerevisiae* deficient in 0-mannosylation activity. To enable the expression of HFBII in a *S. cerevisiae* strain impaired in O-glycosylation, the 6559bp fragment of pUR9011 was used to transform the *S. cerevisiae* strain CEN.PK102-3Δ*gal*1Δpmt1 (*gal1:ura3, leu2, ura3, pmt1:URA3*) to leucine prototrophy . The resulting transformants were screened for HFBII production as described above.

The results are shown in Table 2.

Table 2

Hydrophobin production by CEN.PK102-3Δ*gal*Δ*pmt1*-pUR9110 transformants in shake flask culture.

Transformant	HFBII concentration in culture (mg/l)
CEN.PK102-3Δ*gal*1Δ*pmt1*	0
CEN.PK102-3Δ*gal*1-pUR9011#1	13.2
CEN.PK102-3Δ*gal*1-pUR9011#2	17.6
CEN.PK102-3Δ*gal*1-pUR9011#4	16.5
CEN.PK102-3Δ*gal*1-pUR9011#5	15.4
CEN.PK102-3Δ*gal*1-pUR9011#8	14.2
CEN.PK102-3Δ*gal*1-pUR9011#9	15.9

As is shown in Table 2, these transformants produced high levels of HFBII and analysis of the hydrophobin by LCMS showed no significant level of glycosylation.

Example 3 - Production of HFBII in small scale fermentation using strain CEN.PK102-3Δ*gal1*Δ*pmt1*-pUR9011#2.

Inoculum Preparation

A shake flask containing 50 ml YNB medium was inoculated with a 1.4 ml glycerol stock of the strain and incubated for 48 hours at 30 degrees C. at 120 rpm. Subsequently, the inoculum was transferred to a shake flask containing 500 ml medium consisting of 10 g/l Yeast extract (Difco), 20 g/l Bacto Peptone (Difco) and 20 g/l glucose and incubated for 24 hours, 30degrees C at 120 rpm.

Fed Batch Fermentations

The 5.5L batch medium consisted of 22 g/kg glucose, 10 g/kg yeast extract KatG (Ohly), 2.1 g/kg KH.$_2$PO.$_4$, 0.6 g/kg MgSO.$_4$.7H. $_2$O, 0.4 g/kg Struktol J647 (Schill & Seilacher), 10 g/kg Egli trace metals (a 100.times. solution of 5.5 g/l $CaCl_2$.2H.$_2$O, 3.73 g/l FeSO.$_4$.7H_2O, 1.4 g/l $MnSO_4$.1H_2O, 1.35 g/l $ZnSO_4$.7H_2O, 0.4 g/l $CuSO_4$.5H_2O, 0.45 g/l $CoCl_2$.6H_2O, 0.25 g/l $NaMoO_4$.2H_2O, 0.4 g/l H_3BO_3, 0.25 g/l KI, 30 g/l NaEDTA), 1 g/kg Egli vitamins (a 1000.times. solution of 5 g/l thiamine, 47 g/l meso-inositol, 1.2 g/l pyridoxin, 23 g/l pantothenic acid, 0.05 g/l biotin). The 4L feed medium contained 440 g/kg glucose, 3 g/l galactose (Duchefa), 25 g/kg yeast extract, 12 g/kg KH_2PO_4, 2.5 g/kg $MgSO_4$.7H_2O, 0.8 g/kg, Struktol J647, 20 g/kg Egli trace metals, 2 g/kg Egli vitamins.

The fed batch fermentations were performed in standard bioreactors with a working volume of 10 litres. Dissolved oxygen (DO_2) was measured with an Ingold DO_2 electrode (Mettler-Toledo) and controlled by automatic adjustment of the speed of the 6-bladed Rushton impeller to a maximum of 1000 rpm. The pH was measured with an Ingold Inpro 3100 gel electrode (Mettler-Toledo) and controlled using 3 M phosphoric acid (Baker) and 12.5% v/v ammonia (Merck). Temperature was measured by a PT100 electrode and controlled via a cooling jacket and cooling and heating fingers.

The batch phase was started by transferring 500 ml of full grown inoculum to the batch medium. The temperature was maintained at 30 degrees C and airflow at 2 l/min. DO_2 was controlled above 30% and the pH at 5.0. When the ethanol signal in the off-gas decreased below 100 ppm, the feed phase was started. In the feed phase the airflow was set to 6 l/min. The feed rate was applied according to an exponential profile required to maintain a growth rate of 0.06 l/h. The exponential feed continued until the DO_2 level in the fermenter decreased below 15% after which a linear feed rate was maintained.

Samples were taken for dry matter determination (by drying of approximately 1.6g of sample overnight in an oven at 150 degrees C) and analysis of hydrophobin levels. The hydrophobin was extracted from the culture by addition of ethanol to 60% v/v and quantified as described above. The results are shown in Table 3 and as a graph in Figure 5.

Table 3

Hydrophobin production by CEN.PK102-3Δ*gal*Δ*pmt1*-pUR9110#2 in a fed batch fermentation

Time (hours)	Dry Matter (g/kg)	HFBII level (mg/1) in culture
0	0.74	0.0
3	2.24	0.0
6	4.91	0.0
9	6.78	0.0
12	10.10	0.0
15	10.87	0.0
18	10.88	0.0
21	11.48	0.0
24	12.53	0.0
27	13.25	0.0
30	16.48	2.2
33	18.56	8.0
36	22.67	18.3
39	28.18	
42	34.48	61.5
45	40.44	73.2
48	45.64	103.3
51	53.29	146.4
54	58.85	186.4
57	61.76	239.3
60	66.81	272.5
63	70.21	294.2
66	69.86	314.2
69	74.15	339.5
72	76.21	345.6
75	79.63	382.6
78	83.08	372.4
81	85.12	398.0
84	86.24	388.2
87	89.09	401.1

As can be seen from Table 3, HFBII production was significant after 66 hours and very significant after 87 hours.

Example 4 - Expression of HFBII in a yeast with improved protein secretion properties

It is possible to increase the secretion capacity of yeast by selecting specifically for strains that have a higher capacity to secrete heterologous proteins (GB2239315 and Sleep D, Belfield GP, Ballance DJ, Steven J, Jones S, Evans LR, Moir PD and Goodey AR. (1991) *Saccharomyces cerevisiae* strains that over express heterologous proteins, Biotechnology N.Y. 9: 183-187). WO95/023857 shows how deletion of the protease

YAP3 and/or *KEX2* proteases also increases heterologous expression. Furthermore WO99/000504 describes how deletion of *UBC4* and/or *UBC5* genes increases plasmid copy number of the yeast 2μ plasmid and vectors based on the yeast 2μ plasmid. WO95/33833 describes how deletion of hsp150 can also improve heterologous protein production by removal of this specific contaminating yeast protein. Each of these references is hereby incorporated by reference, in particular as regards the production of increasing the secretion capacity of yeast.

The *S. cerevisiae* DXY1 yeast strain (reported in Kerry-Williams S.M. *et al* Disruption of *Saccharomyces cerevisiae YAP3* gene reduces the proteolytic degradation of secreted recombinant human albumin, Yeast, 14, 161-169 (1998)) was rendered Hsp150 and pmt1 deficient using the techniques disclosed in WO95/033833 and US5,714,377, respectively, in order to generate new Strain A. Thus Strain A has the following genotype: *leu2, pra1, ubc4, yap3, hsp150, pmt1*.

WO88/00802 describes a stable 2μ based yeast expression vector suitable for gene expression in such strains. A derivative of this vector, pSAC35 (a schematic diagram of which is shown in Figure 4) is described in WO2005/077042 and by Sleep *et al* (1991). An expression cassette for the synthetic HFBII gene was constructed. The expression cassette may be constructed in a similar manner to that described in WO2005/061718. The expression cassette contained the *S. cerevisiae PRB*1 promoter, the invertase signal sequence linked to the HFBII structural gene and the *S. cerevisiae ADH*1 terminator sequence. A *Not*I fragment carrying this expression cassette was ligated into pSAC35. The resulting plasmid, pDB3268 was introduced into *S. cerevisiae* strain Strain A using the LiAc technique. It is to be noted that the *SUC2* signal sequence encoded within this plasmid was modified and contains only a single N-terminal methionine. One of the resulting transformants was grown for 98h at 30°C in a shake flask culture containing YEPS (Yeast extract, 1 g/l, peptone 2 g/l, sucrose, 2 g/l) and was shown to produce 102 mg/l hydrophobin as determined by HPLC assay.

Strain A-pDB3268 was grown for 48 hours in BMMS (Difco yeast nitrogen base, without amino acids, without NH_4SO_4, 1.7 g/l, NH_4SO_4 5 g/l, citric acid monohydrate 6.09 g/l, $Na_2HPO_4.2H_2O$ 2.527 g/l, sucrose 20g/l) and 60 mg of cell dry weight used to inoculate a fermenter containing 6 l batch medium. The cell dry weight (CDW) was calculated using the following equation:

CDW g/L = [(OD600 * Dilution Factor) - 0.1904]/2.0827 OD600 must be in the 0.5 -1.0 range.

The temperature was maintained at 30 degrees C and airflow at 2 l/min. DO_2 was controlled above 30% and the pH at 6.5. When the ethanol signal in the off-gas decreased below 100 ppm, the feed phase was started. In the feed phase the airflow was set to 6 l/min. The feed rate was applied according to an exponential profile required to maintain a growth rate of 0.06 l/h. The exponential feed continued until the DO_2 level in the fermenter decreased below 15% after which a linear feed rate was maintained.

Samples were taken for dry matter determination (by drying of approximately 1.6g of sample overnight in an oven at 150 degrees C) and analysis of hydrophobin levels. The hydrophobin secreted by the yeast was extracted from the culture by addition of ethanol to 60% v/v and quantified as described above. The results are shown in Table 4 and as a graph in Figure 6.

Table 4

Hydrophobin production by Strain A-pDB3268 in a fed batch fermentation

Time (h)	Dry matter (g/kg)	HFBII ($mg.l^{-1}$) in culture
48	3	15
54	4	22
60	5	29
66	6	44
72	8	76
78	14	122
81	16	133
84	18	159
87	23	196
90	30	230
93	33	269
96	41	297
99	47	338
102	54	372
105	61	435
108	70	494
111	82	513

As can be seen from Table 4, the amount of HFBII secreted into the media was significant after around 99 hours and very significant after 105 hours, rising to an even higher level after 111 hours.

This experiment was repeated under the same conditions but with increased stirring starting 24 hours after start of the feed phase, in order to minimise the presence of foam on the culture. The results are shown in Table 5 and as a graph in Figure 7. Synthesis of the hydrophobin after 90 hours was higher with the increased stirring (491.59 mg/l compared with 230 mg/l). Synthesis of the protein after 90 hours was slightly reduced compared with synthesis after 87 hours, probably due to some loss of hydrophobin into the residual foam.

Table 5

Hydrophobin production by Strain A-pDB3268 in a fed batch fermentation with reduced foam

Time (hours)	Dry Matter (g/kg)	HFBII level (mg/1) in culture
51	15	104.66
54	17	130.81
57	21	157.59
60	24	195.97
63	28	232.47
66	33	282.05
69	38	332.76
72	44	399.74
75	50	447.41
78	57	531.56
81	65	583.75
84	75	564.72
87	85	584.70
90	88	491.59

Example 5 - Improving yield of the fermentation

As the fermentations at pH6.5 were shown to be prone to infection, and fermentation of Strain A-pDB3268 at pH5 gave a low biomass yield and less HFBII production, a fermentation protocol was devised in which the batch phase was carried out at pH5 and the feed phase was carried out at pH6.5.

The inoculum preparation and medium composition were the same as for the fermentation examples described above. The temperature was maintained at 30 degrees C and airflow at 2 l/min, the DO_2 was controlled above 30% and the pH at 5.0. When the ethanol signal in the off-gas decreased below 100 ppm, the feed phase was started. In the feed phase, the airflow was set to 6 l/min and the pH increased to pH6.5. The feed rate was applied according to an exponential profile required to maintain a growth rate of 0.06 l/h. The exponential feed continued until the DO_2 level in the fermenter decreased below 15% after which a linear feed rate was maintained.

Samples were taken for dry matter determination (by drying of approximately 1.6g of sample overnight in an oven at 150 degrees C) and analysis of hydrophobin levels. The hydrophobin produced by the yeast was extracted from the culture by addition of ethanol to 60% v/v and quantified as described above. The experiment was carried out in triplicate and the results are shown in Tables 6 to 8 and, graphically, in Figures 8 to 10, respectively.

Table 6

Hydrophobin production following raising of pH during fermentation

Time (h)	Dry matter (g/kg)	HFBII ($mg.l^{-1}$) in culture
48	7	38.36
51	7	50.90
54	10	64.61
57	12	80.80
60	14	105.61
63	18	134.82
66	21	173.42
69	25	212.40
72	29	270.10
75	33	348.61
78	39	384.33
81	44	464.01
84	50	565.82
87	58	622.73
90	68	616.48
93	74	705.89

Table 7

Hydrophobin production following raising of pH during fermentation

Time (h)	Dry matter (g/kg)	HFBII ($mg.l^{-1}$) in culture
30	4	6.44
33	3	7.11
36	4	12.37
39	4	18.06
42	5	25.70
45	7	32.41
48	8	39.49
51	10	52.49
54		77.46
57	11	103.02
60	17	162.68
63	23	122.25
66	23	249.23
69	36	215.22
72	38	319.05
75	43	391.93
81	49	484.24
84	55	572.37
87	64	647.03
90	73	637.61
93	82	668.21
96	94	793.00

Table 8

Hydrophobin production following raising of pH during fermentation

Time (h)	Dry matter (g/kg)	HFBII ($mg.l^{-1}$) in culture
36	4	7.26
39	4	
42	5	21.87
45	6	
48	7	43.15
51	9	
54	10	76.24
57	13	
60	16	114.59

63	19	151.26
66	23	177.78
69	27	222.37
72	32	265.64
75	38	361.92
78	44	394.49
81	50	453.26
84	57	518.72
87	64	592.11
90	72	685.12
93	83	677.62

Example 6 – Comparison of Secretion Levels

A study of prior art disclosures of hydrophobin expression in host cells was carried out. The results are shown in Table 9, in which are reported, *inter alia*: the hydrophobin expressed; the concentration of the hydrophobin expressed and incorporated into the cell wall; and the concentration of the hydrophobin secreted into the culture medium in which the cells were present.

It is to be noted that only Bailey *et al* report on the expression of HFBII and a comparatively low level is synthesised. Only US2007/0077619 and Tagu *et al* report on expression of hydrophobins in yeast host cells and in both cases expression of the hydrophobin was negligible. Accordingly, it is surprising that the present inventors have found that expression of HFBII in yeast host cells results in a high level of synthesis and secretion of HFBII.

Table 9

Comparison of prior art hydrophobin expression levels in fermentation

Reference	**Hydrophobin**	**Organism**	**Ferm scale**	**Expression level in fermentation (mg/L)**		
				Total expression	Secreted into medium	Bound to cell wall
Askolin et al. (2001) *Appl Micrbiol Biotechnol.* 57:124-130	HFBI (Class II)	Wild type *Trichoderma reesei* (QM9414)	15L	170	0	170
Askolin et al. (2001) *Appl Micrbiol*	HFBI	GM *T. reesei* with increased copy number of HFBI	15L	600	114	486

Biotechnol. 57:124-130		gene (VTT-D-98692)				
Bailey et al. (2002) *Appl Microbiol Biotechnol.* 58:721-727	HFBI	GM *T.reesei* with increased copy number (QM9414*hfb1*$^{+++}$)	10L	1400	100	1300
Bailey et al. (2002) *Appl Microbiol Biotechnol.* 58:721-727	HFBII	GM *T.reesei* with increased copy number (RutC-30 *hfb2*$^{+++}$)	10L	240	240	0
US 20070077619 BASF	DewA fusion protein (Class 1)	GM *Schizosaccharomyces pombe*		Barely detectable		
Scholtmeijer et al. (2001) *Appl Micobiol Biotechnol.* 56:1-8	SC3 (Class 1)	Wild type *Schizophyllum commune*	Unknown	60		
Hektor and Scholtmeijer (2005) *Curr Opinion Biotechnol.* 16:1-6	SC3	GM *T.reesei*	Unknown	60		
Scholtmeijer et al. (2001) *Appl Micobiol Biotechnol.* 56:1-8	Cerato ulmin (Class 2)	GM *E.coli*	Unknown	ca. 0.001		
Tagu et al, *Eur Journal of Histochemistry*, 46:23-29 (2002)	HYDPt-1	GM *Saccharomyces cerevisiae*		Not detected		

Example 7 – Culturing Parameters

In order to assess the effect of changes to the culturing parameters on the resulting concentration of synthesised HFBII protein, a series of culturing experiments were carried out under a range of conditions.

A reference batch was prepared as follows. Strain A-pDB3268 was grown for 48 hours in BMMS (see above) and 100 ml was used to inoculate the batch medium. 8 test batches were prepared as follows. Strain A-pDB3268 was grown for 48 hours in BMMS (see above) and 100 ml was used to inoculate 500ml YPD and grown for 24 hours to give a batch time of 30 hours. YPD was yeast-extract, pepton and dextrose broth medium comprising 2% yeast extract, 1% peptone and 2% glucose. To prepare YPD, all ingredients were dissolved in demi-water and sterilised at 121˚C for 15 minutes. The results and details of other culturing parameters are shown in Table 10.

It can be seen that all batches produced a concentration of at least 300mg/l of HFBII, or would have done had fermentation continued for 100 hours.

Footnotes to Table 10:

AF = Anti-foaming agent (concentration shown is relative to Reference Batch 2008003)
FB = Fed Batch
SS = Stirspeed

Remark 1: t= 71h, 4 L/min with 20% O2; t=76h, 6L/min, 20% O2; t=78 EtOH formed.
Remark 2: total airflow of 4L/min used, dO2=50%, cascade: SS (500~1000 rpm) and then O2 20%.
Remark 3: SS was not forced at min 800 rpm, so that the foam was out of the control.
Remark 4: after 76 h: airflow 4L/min with O2 20%. , SS max 1200 rpm
Remark 5: after 77 h: linear feed flow=244g/h.
Remark 6: 24h after Fed Batch, minimal SS was forced at 800 to control the foam, SS max was 1000, and it was cascaded with dO2.

Table 10

Batch Code	**Ino. Vol (ml)**	**AF**	**Airflow (L/min) and Stirspeed (rpm)**	**dO2 (%)**	**Feed (kg)**	**Growth Rate μ (h^{-1})**	**Total ferm time (h)**	**HFBII end hour (mg/l)**	**Max HFBII (mg/l)**
2008044	500	1X	6 L/min, 24 h after FB, SS_{min} forced 800	30	4	0.08	74	364	442
2008048	500	1X	SS_{max}=1200	50	4	0.08	66	363	394 (t=63h)
2008052	500	1X	6 L/min, 24 h after FB, SS_{min} forced 800	50	5.4	0.08	69	175	220
2008053	500	1X	6 L/min, 24 h after FB, SS_{min} forced 800	30	6.7	0.06	90	272	399 (72h)
2008056	500	1X	Remark 1	30	7.1	0.06	87	466	588 (72h)
2008057	500	1X	6 (after 52 h, 20% O2)	50	7.2	0.08	75	460	520 (60h)
2008063	500	1X	See Remark 2	50	7.2	0.06	90	303 (Remark 3)	681 (72h)
2008064	500	3X	Remark 4	30	6.7	Remark 5	90	687	757 (90h)
Reference (2008003)	100	1X	6 L/min, 24 h after FB, SS_{min} forced 800 (Remark 6)	30	4	0.06	93	685	693 (90h)

CLAIMS

1. A polynucleotide expression cassette comprising an extracellular signal sequence operatively associated with an oligonucleotide sequence encoding an HFBII protein, wherein a yeast host cell transformed with the polynucleotide expression cassette is capable of synthesising at least 300mg/l of the HFBII protein in a 100 hour period.

2. A polynucleotide expression cassette according to claim 1 wherein a yeast host cell transformed with the polynucleotide expression cassette and cultured in medium is capable of synthesising and secreting into the culture at least 300 mg/l of the HFBII protein in a 100 hour period.

3. A polynucleotide expression cassette according to claim 1 or 2 wherein the HFBII protein comprises a sequence at least 80% identical to SEQ. ID NO. 2, preferably at least 90%, 95% or 99% identical thereto.

4. A polynucleotide expression cassette according to any one of the preceding claims wherein a purified 1μM sample of the HFBII protein demonstrates a surface shear elasticity of at least $0.1Nm^{-1}$ at pH 7, 20°C after 10,000 seconds.

5. A polynucleotide expression cassette according to claim 4 wherein a purified 1μM sample of the HFBII protein demonstrates a surface shear elasticity of at least $0.5Nm^{-1}$ at pH 7, 20°C after 10,000 seconds.

6. A polynucleotide expression cassette according to any one of the preceding claims wherein the extracellular signal sequence comprises a sequence at least 80% identical to SEQ. ID NO. 3, preferably at least 90%, 95% or 99% identical thereto.

7. A polynucleotide expression cassette according to any one of the preceding claims further comprising a promoter for expression of a gene in a yeast cell.

8. A vector comprising a polynucleotide expression cassette according to any one of the preceding claims.

9. A vector according to claim 8 wherein the vector is a plasmid or a YAC.

10. A yeast host cell comprising a polynucleotide expression cassette according to any one of claims 1 to 7 recombinantly incorporated therein.

11. A yeast host cell according to claim 10 wherein the cell is a food grade yeast strain.

12. A yeast host cell according to claim 10 or 11 wherein the cell is of one of the following strains: *Saccharomyces cerevisiae, Kluyveromyces lactis, Schizosaccharomyces pombe, or Yarrowia lipolytica.*

13. A yeast host cell according to claim 10 wherein the cell is of strain CEN.PK102 or strain DXY1 rendered Hsp150 and pmt1 deficient.

14. A yeast host cell according to any one of claims 10 to 13 wherein the polynucleotide expression cassette is integrated into the genome of the yeast host cell.

15. A yeast host cell according to any one of claims 10 to 13 wherein the expression cassette is located in the cell as an extra-genomic vector.

16. A method of synthesising HFBII protein comprising expressing a polynucleotide sequence encoding the HFBII protein in a yeast host cell such that the HFBII protein is synthesised at a concentration of at least 300mg/l in a 100 hour period.

17. A method according to claim 16 wherein the yeast host cell comprises a polynucleotide expression cassette according to any one of claims 1 to 7 recombinantly incorporated therein.

18. A method according to claim 16 or 17 wherein the method comprises maintaining the yeast host cells in a medium and raising the pH of the medium during the method.

19. A method according to claim 18 comprising raising the pH of the medium by at least 1 on the pH scale.

20. A method according to claim 18 or 19 comprising raising the pH of the medium from between pH 4.5 and pH 5.5 to between pH 6.0 and pH 7.0

21. A method according to any one of claims 18 to 20 wherein the yeast host cell is of strain DXY1 rendered Hsp150 and pmt1 deficient.

22. A method for synthesising a heterologous protein in a yeast host cell that has been transformed with a polynucleotide comprising a sequence encoding the heterologous protein comprising the steps of:
(i) maintaining the yeast host cell in a growth medium under growth conditions at a first pH value such that the yeast host cell multiplies; and
(ii) raising the pH of the medium to a second pH value, or transferring the multiplied yeast host cells to a growth medium at a second pH value, higher than the first pH value, and synthesising the heterologous protein,
wherein the yeast host cell is of strain DXY1 rendered Hsp150 and pmt1 deficient.

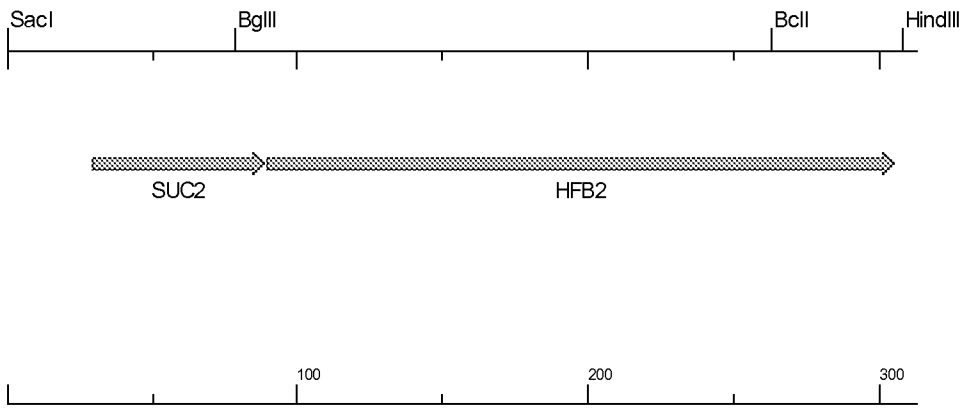
23. A method according to claim 22 wherein the heterologous protein is a class II hydrophobin.

24. A method according to claim 23 wherein the heterologous protein is an HFBII protein.

25. A method for synthesising a heterologous protein in a yeast host cell that has been transformed with a polynucleotide comprising a sequence encoding the heterologous protein comprising the steps of:
(i) maintaining the yeast host cell in a growth medium under growth conditions at a first pH value such that the yeast host cell multiplies; and
(ii) raising the pH of the medium to a second pH value, or transferring the multiplied yeast host cells to a growth medium at a second pH value, higher than the first pH value, and synthesising the heterologous protein,
wherein the heterologous protein is HFBII.

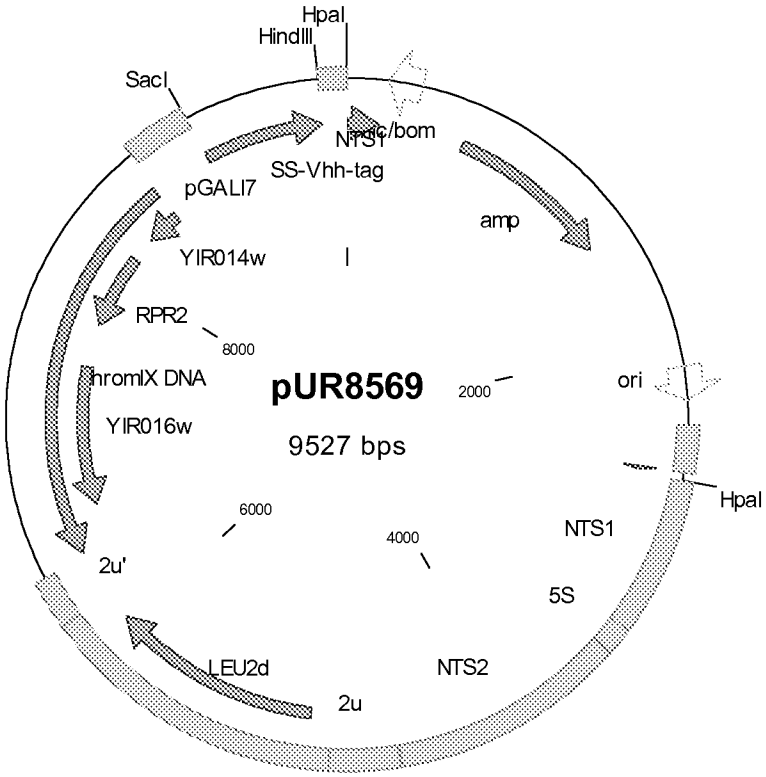
26. A method according to any one of claims 22 to 25 wherein the second pH value is at least 1 higher on the pH scale than the first pH value.

27. A method according to any one of claims 22 or 26 wherein the first pH value is between pH 4.5 and pH 5.5 and/or the second pH value is between pH 6.0 and pH 7.0.



Trichoderma reesei HFB2 (313 bps)

Figure 1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HindIII
SacI
NTS1
pGAL17
SS-Vhh-tag
amp
YIR014w
RPR2
8000
pUR8569
2000
ori
ChromIX DNA
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6000
4000
NTS1
2u'
5S
LEU2d
NTS2
2u

Figure 2

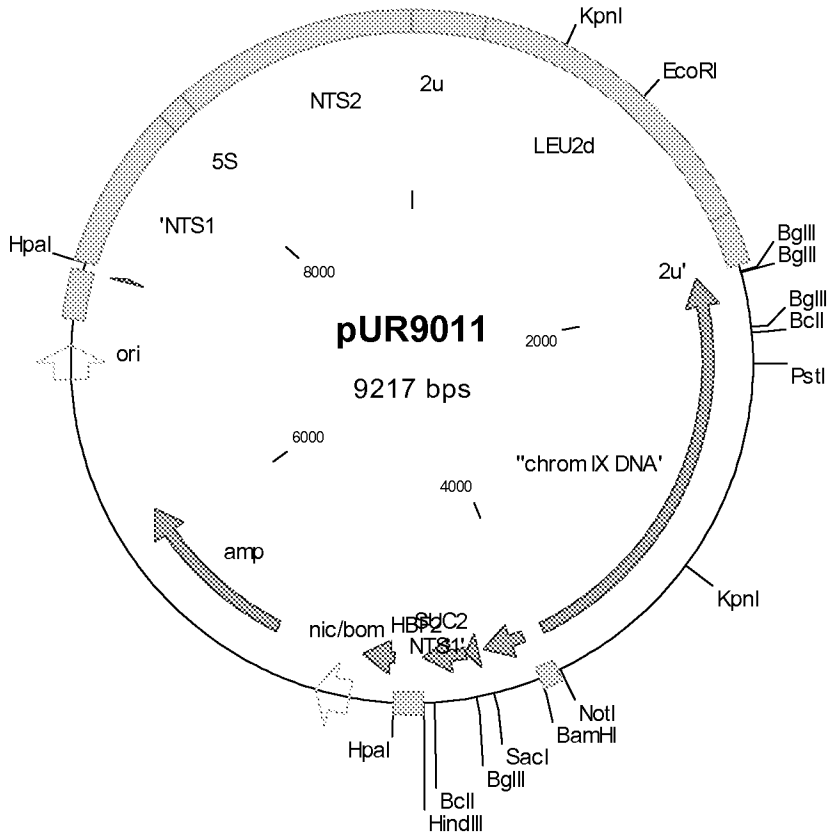
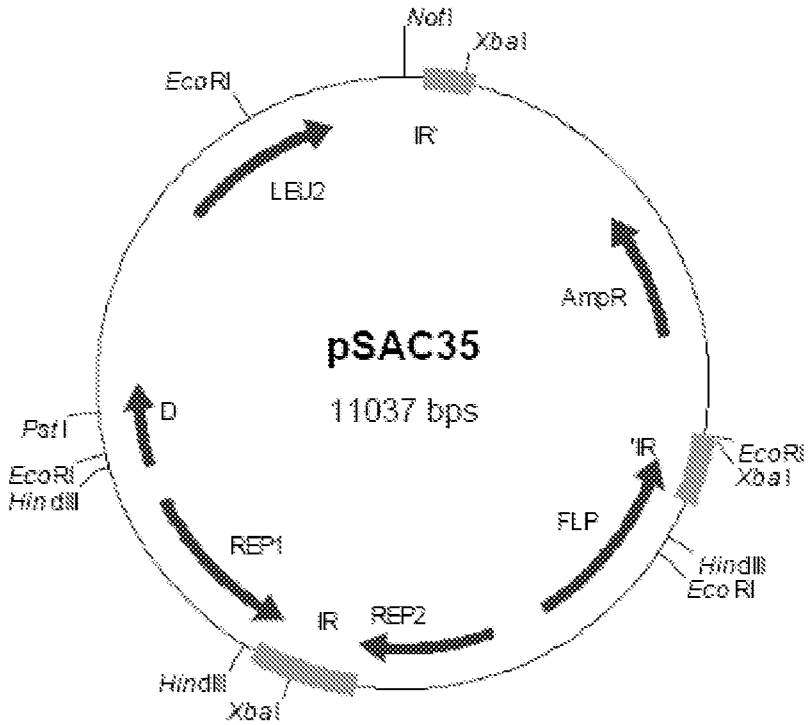

KpnI
EcoRI
2u
NTS2
LEU2d
5S
I
'NTS1
HpaI
8000
2u'
BglII
BglII
BglII
BclI
PstI
pUR9011
2000
ori
9217 bps
6000
"chrom IX DNA'
4000
amp
KpnI
nic/bom
HBP2
StdC2
NTS1'
NotI
BamHI
HpaI
SacI
BglII
BclI
HindIII

Figure 3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XbaI
EcoRI
IR'
LEU2
AmpR
pSAC35
11037 bps
D
PstI
EcoRI
HindIII
'IR
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FLP
REP1
HindIII
EcoRI
IR
REP2
HindIII
XbaI

Figure 4

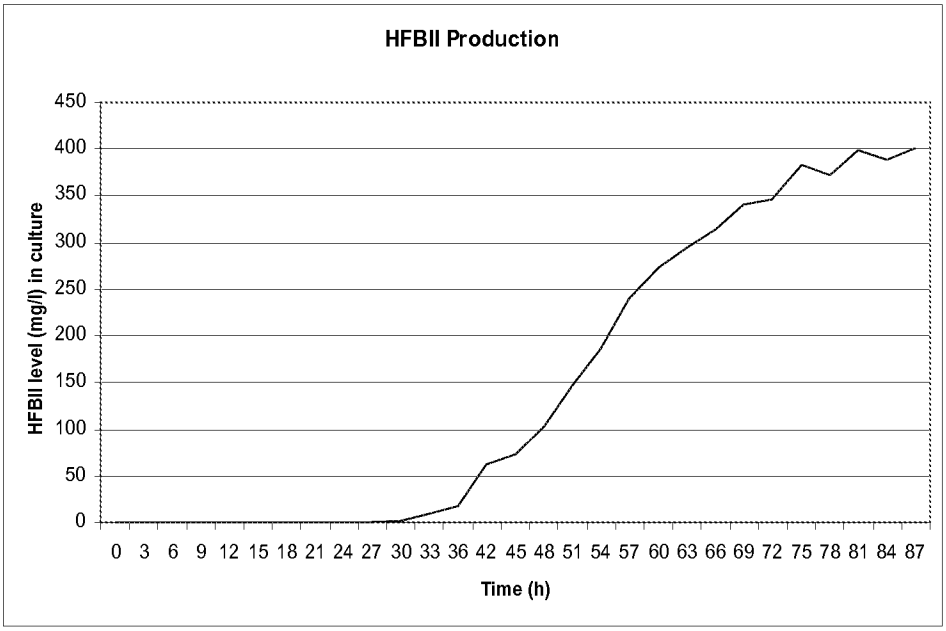

Figure 5

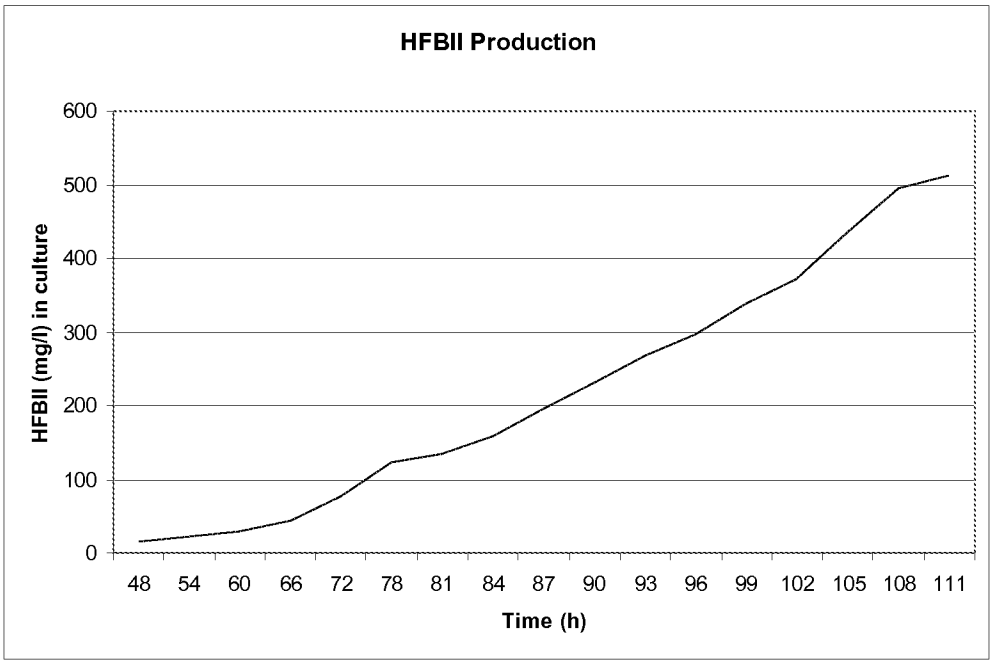

Figure 6

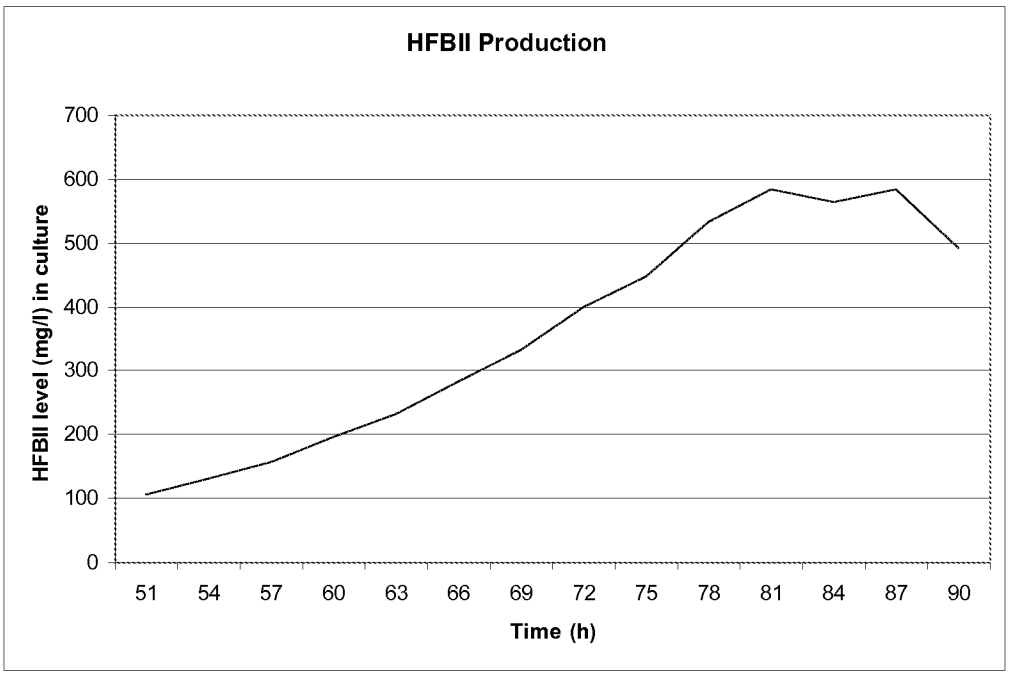

Figure 7

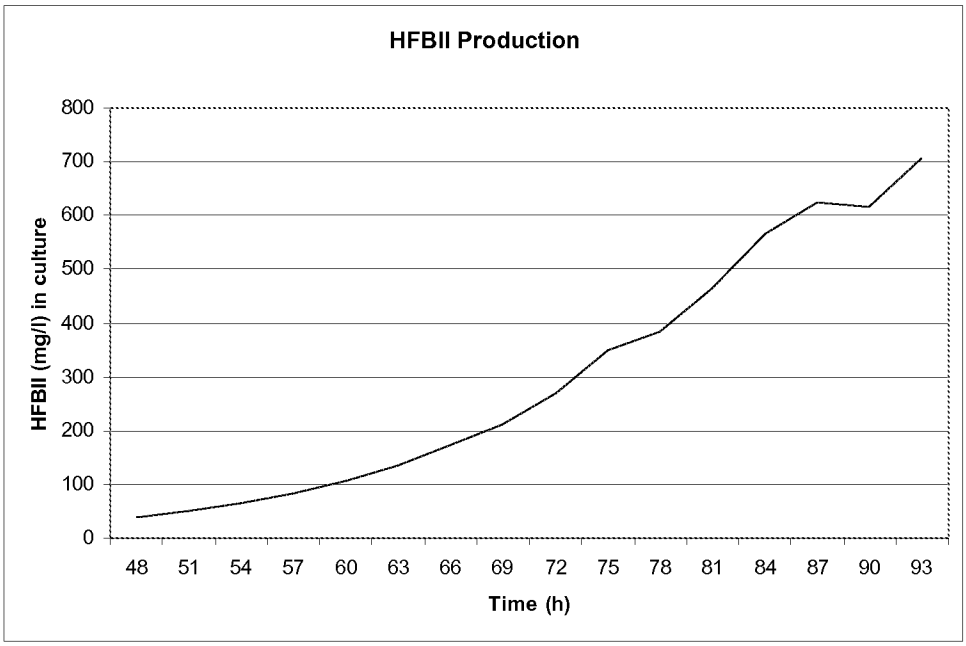

Figure 8

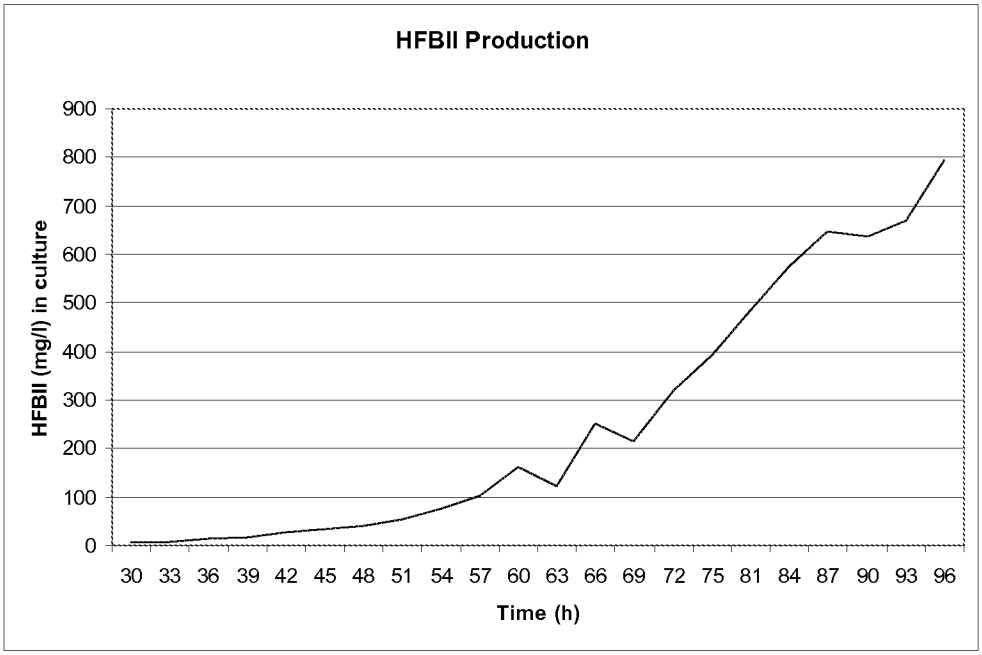

Figure 9

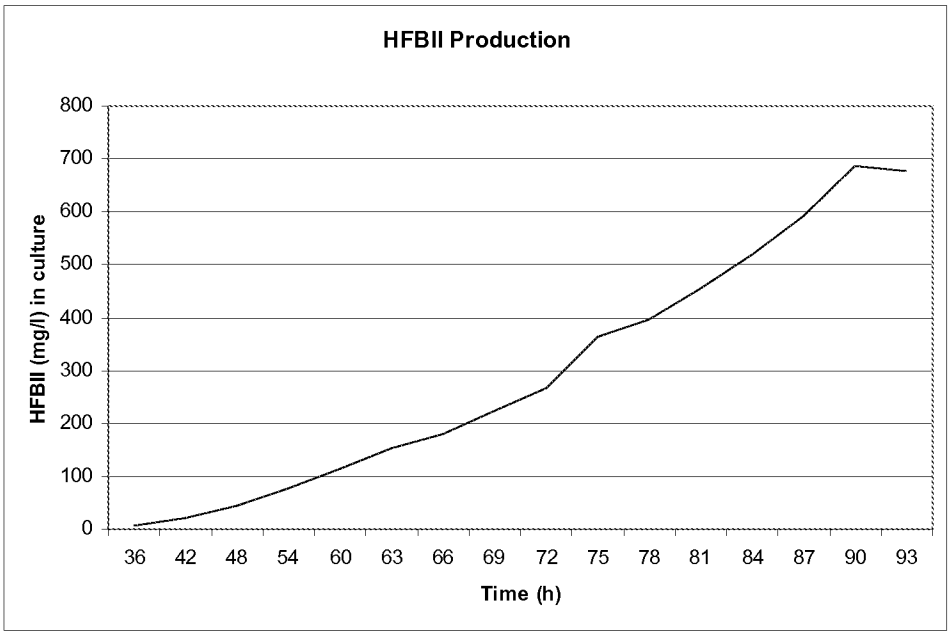

Figure 10

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2009/062254

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N15/62 C12N15/81 C12P21/02

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)
C12N C12R C12P

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 practical, search terms used)
EPO-Internal, EMBASE, BIOSIS, FSTA, Sequence Search, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BAILEY M J ET AL: "Process Technological effects of deletion and amplification of hydrophobins I and II in transformants of Trichoderma reesei" APPLIED MICROBIOLOGY AND BIOTECHNOLOGY, SPRINGER VERLAG, BERLIN, DE, vol. 58, 1 January 2002 (2002-01-01), pages 721-727, XP003015238 ISSN: 0175-7598	1-5
Y	page 722, column 1, line 34 - line 46 table 2	6-21

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[X] Further documents are listed in the continuation of Box C.

[X] 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 document 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	Date of mailing of the international search report
11 December 2009	30/12/2009

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

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2009/062254

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	ERIK BÖER ET AL: "Yeast expression platforms" APPLIED MICROBIOLOGY AND BIOTECHNOLOGY, SPRINGER, BERLIN, DE, vol. 77, no. 3, 9 October 2007 (2007-10-09), pages 513-523, XP019560739 ISSN: 1432-0614 page 513, column 1, line 16 - column 2, line 8	6-21
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Y	WO 96/41882 A (PROEFSTATION VOOR DE CHAMPIGNO [NL]; RIJKSLANDBOUWHOGESCHOOL [NL]; GRI) 27 December 1996 (1996-12-27) cited in the application page 17, line 3 - line 37 example 5 claims 1-37	6-21
Y	WO 2005/061718 A (DELTA BIOTECHNOLOGY LTD [GB]; SLEEP DARRELL [GB]; SHUTTLEWORTH GILLIAN) 7 July 2005 (2005-07-07) page 43, line 24 - page 62, line 29 example 1	13,21,22
Y	US 2003/143191 A1 (BELL ADAM [US] ET AL) 31 July 2003 (2003-07-31) paragraph [0360] - paragraph [0385] example 4	13,21,22
Y	US 2007/026013 A1 (ROSEN CRAIG A [US] ET AL) 1 February 2007 (2007-02-01) paragraph [0275] - paragraph [0288]	13,21,22
Y	KIM SE HOON ET AL: "Culture method to enhance the productivity of hepatitis B surface antigen (pre S2 + S-Ag) with recombinant Saccharomyces cerevisiae" BIOTECHNOLOGY TECHNIQUES, CHAPMAN & HALL, vol. 10, no. 4, 1 April 1996 (1996-04-01), pages 233-238, XP009112452 ISSN: 0951-208X page 233 - page 234; figure 1	18-20, 22,25-27

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INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2009/062254

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	CALADO CECILIA R C ET AL: "Optimisation of culture conditions and characterisation of cutinase produced by recombinant Saccharomyces cerevisiae" ENZYME AND MICROBIAL TECHNOLOGY, vol. 31, no. 1-2, 1 July 2002 (2002-07-01), pages 161-170, XP002515609 ISSN: 0141-0229 page 164, column 2, line 9 - page 165, column 2, line 4	18-20, 22,25-27
Y	BAE C S ET AL: "Improved process for production of recombinant yeast-derived monomeric human G-CSF" APPLIED MICROBIOLOGY AND BIOTECHNOLOGY, vol. 52, no. 3, September 1999 (1999-09), pages 338-344, XP002515608 ISSN: 0175-7598 page 340, column 2, line 32 - line 34 page 343, column 1, line 44 - column 2, line 1 figure 2	18-20, 22,25-27
A	NAKARI-SETALA T ET AL: "DIFFERENTIAL EXPRESSION OF THE VEGETATIVE AND SPORE-BOUND HYDROPHOBINS OF TRICHODERMA REESEI CLONING AND CHARACTERIZATION OF THE HFB2 GENE" EUROPEAN JOURNAL OF BIOCHEMISTRY, BERLIN, vol. 248, 1 January 1997 (1997-01-01), pages 415-423, XP002949768 ISSN: 0014-2956 page 418, column 1, line 1 - line 4	1-27
A	LINDER ET AL: "Hydrophobins: the protein-amphiphiles of filamentous fungi" FEMS MICROBIOLOGY REVIEWS, ELSEVIER, AMSTERDAM, NL, vol. 29, no. 5, 1 November 2005 (2005-11-01), pages 877-896, XP005107912 ISSN: 0168-6445 the whole document	1-27
A	WHITEFORD JAMES R ET AL: "Hydrophobins and the interactions between fungi and plants" MOLECULAR PLANT PATHOLOGY, WILEY-BLACKWELL PUBLISHING LTD, GB, vol. 3, no. 5, 1 September 2002 (2002-09-01), pages 391-400, XP009125460 ISSN: 1464-6722 [retrieved on 2002-08-30] table 1	1-27

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INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2009/062254

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	KUBICEK CHRISTIAN P ET AL: "Purifying selection and birth-and-death evolution in the class II hydrophobin gene families of the ascomycete Trichoderma/Hypocrea" BMC EVOLUTIONARY BIOLOGY, BIOMED CENTRAL LTD., LONDON, GB, vol. 8, no. 1, 10 January 2008 (2008-01-10), page 4, XP021032522 ISSN: 1471-2148 figure 2	1-27

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2009/062254

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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US 2007026013 A1	01-02-2007	NONE	