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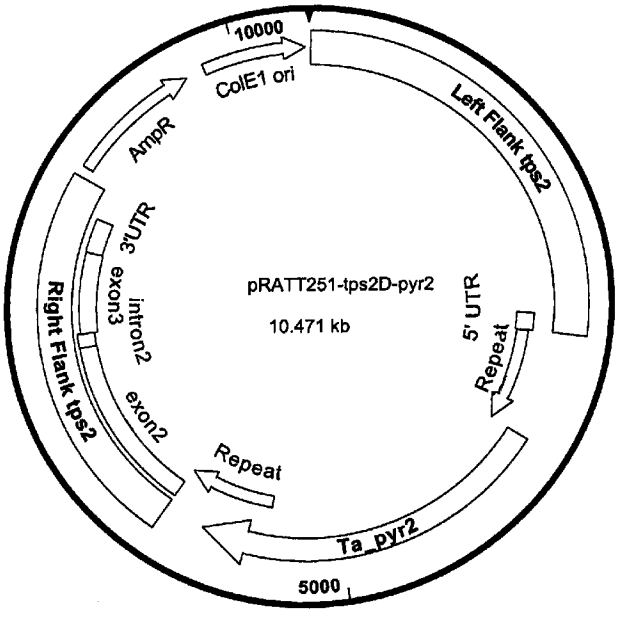

(57) Abrégé/Abstract:
Described are compositions and methods relating to variant filamentous fungi having altered growth characteristics. Such variants are well-suited for growth in submerged cultures, e.g., for the large-scale production of enzymes and other proteins for commercial applications.

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(54) **Title**: FILAMENTOUS FUNGI HAVING AN ALTERED VISCOSITY PHENOTYPE

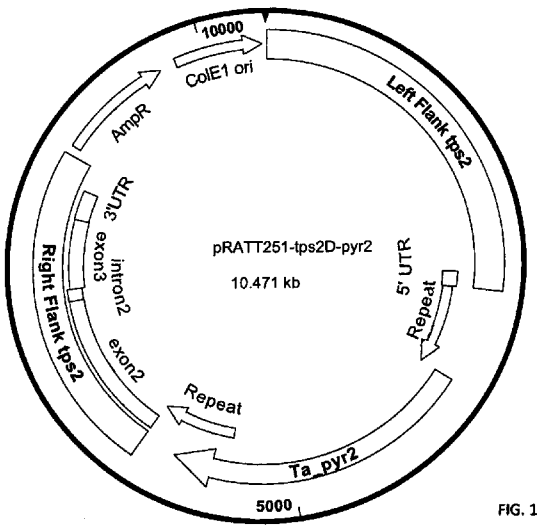

FIG. 1

(57) **Abstract**: Described are compositions and methods relating to variant filamentous fungi having altered growth characteristics. Such variants are well-suited for growth in submerged cultures, e.g., for the large-scale production of enzymes and other proteins for commercial applications.

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FILAMENTOUS FUNGI HAVING AN ALTERED VISCOSITY PHENOTYPE

[01]

TECHNICAL FIELD

[02] The present strains and methods relate to genetic mutations in filamentous fungi that give rise to strain variants having altered growth characteristics. Such variants are well-suited for growth in submerged cultures, *e.g.*, for the large-scale production of enzymes and other proteins or metabolites for commercial applications.

BACKGROUND

[03] Filamentous fungi are capable of expressing native and heterologous proteins to high levels, making them well-suited for the large-scale production of enzymes and other proteins for industrial, pharmaceutical, animal health and food and beverage applications. Filamentous fungi are typically grown in mycelial submerged cultures in bioreactors, which are adapted to introduce and distribute oxygen and nutrients into the culture medium (*i.e.*., broth). The morphological characteristics of the mycelium affect the rheological properties of the broth, thereby affecting bioreactor performance.

[04] Generally, the higher the viscosity of the broth, the less uniform the distribution of oxygen and nutrients, and the more energy required to agitate the culture. In some cases, the viscosity of the broth becomes sufficiently high to significantly interfere with the dissolution of oxygen and nutrients, thereby adversely affecting the growth of the fungi. Additionally, the power required to mix and aerate viscous broth can significantly increase the cost of production, and incur higher capital expenditures in terms of motors and power supplies.

SUMMARY

[05] Described are strains and methods relating to filamentous fungi having genetic alterations that give rise to altered viscosity phenotypes.

[06] In one aspect, a variant strain of filamentous fungus derived from a parental strain is provided, the variant strain comprising a genetic alteration that causes cells of the variant strain

to produce an altered amount of functional Tps2 protein compared to cells of the parental strain, wherein the cells of the variant strain are produced during aerobic fermentation in submerged culture cell broth that (i) requires an altered amount of agitation to maintain a preselected dissolved oxygen content compared to the cells of the parental strain, and/or (ii) maintains an altered dissolved oxygen content at a preselected amount of agitation, compared to the cells of the parental strain.

[07] In some embodiments, the altered amount of functional Tps2 protein is a reduced amount, and the variant strain produces during aerobic fermentation in submerged culture a cell broth that (i) requires reduced agitation to maintain a preselected dissolved oxygen content compared to the cells of the parental strain, and/or (ii) maintains an increased dissolved oxygen content at a preselected amount of agitation, compared to the cells of the parental strain.

[08] In some embodiments, the genetic alteration comprises a disruption of the *tps2* gene present in the parental strain. In some embodiments, disruption of the *tps2* gene is the result of deletion of all or part of the *tps2* gene. In some embodiments, disruption of the *tps2* gene is the result of deletion of a portion of genomic DNA comprising the *tps2* gene. In some embodiments, disruption of the *tps2* gene is the result of mutagenesis of the *tps2* gene.

[09] In some embodiments, disruption of the *tps2* gene is performed using site-specific recombination. In some embodiments, disruption of the *tps2* gene is performed in combination with introducing a selectable marker at the genetic locus of the *tps2* gene.

[10] In some embodiments, the variant strain does not produce functional Tps2 protein. In some embodiments, the variant strain does not produce Tps2 protein.

[11] In some embodiments, the variant strain further comprises a gene encoding a protein of interest. In some embodiments, the variant strain further comprises a disruption of the *sfb3* gene. In some embodiments, the variant strain further comprises a disruption of the *seb1*gene. In some embodiments, the variant strain further comprises a disruption of the *sfb3* and *seb1* genes. In some embodiments, the variant strain further comprises a disruption of at least one gene selected from the group consisting of the *sfb3* gene, the *seb1* gene, the *mpg1* gene, the *gas1* gene, and the *crz1* gene. In some embodiments, the variant strain produces substantially the same amount of, or more, protein per unit amount of biomass as the parental strain.

[12] In some embodiments, the filamentous fungus is a Pezizomycotina species. In some embodiments, the filamentous fungus is a *Trichoderma* spp., *Aspergillus* spp., *Fusarium* spp., *Scedosporium* spp., *Penicillium* spp., *Chrysosporium* spp., *Cephalosporium* spp., *Talaromyces* spp., *Geosmithia* spp., and *Neurospora* spp. In some embodiments, the filamentous fungus can include, but is not limited to, *Trichoderma reesei* (previously classified as *Trichoderma*

longibrachiatum and *Hypocrea jecorina*), *Aspergillus niger*, *Aspergillus fumigatus*, *Aspergillus itaconicus*, *Aspergillus oryzae*, *Aspergillus nidulans*, *Aspergillus terreus*, *Aspergillus sojae*, *Aspergillus japonicus*, *Scedosporium prolificans*, *Neurospora crassa*, *Penicillium funiculosum*, *Penicillium chrysogenum*, *Talaromyces (Geosmithia) emersonii*, *Fusarium venenatum*, and *Chrysosporium lucknowense*. In some embodiments, the filamentous fungus is *Trichoderma reesei*.

[13] In another aspect, a method for producing a variant strain of filamentous fungus cells is provided, comprising: introducing a genetic alteration into a parental strain of filamentous fungal cell, which genetic alteration alters the production of functional Tps2 protein compared to the cells of the parental strain, thereby producing a variant filamentous fungal cell that produces during aerobic fermentation in submerged culture a cell broth that (i) requires an altered amount of agitation to maintain a preselected dissolved oxygen content, compared to the cells of the parental strain, and/or (ii) maintains an altered dissolved oxygen content at a preselected amount of agitation, compared to the cells of the parental strain.

[14] In some embodiments, the genetic alteration reduces or prevents the production of functional Tps2 protein, thereby producing a variant filamentous fungal cell that produces during aerobic fermentation in submerged culture a cell broth that (i) requires reduced agitation to maintain a preselected dissolved oxygen content, compared to the cells of the parental strain, and/or (ii) maintains an increased dissolved oxygen content at a preselected amount of agitation, compared to the cells of the parental strain.

[15] In some embodiments, the genetic alteration comprises disrupting the *tps2* gene in a parental filamentous fungal cell using genetic manipulation. In some embodiments, the genetic alteration comprises deleting the *tps2* gene in a parental filamentous fungal cell using genetic manipulation. In some embodiments, the genetic alteration is performed using site-specific genetic recombination.

[16] In some embodiments, disruption of the *tps2* gene is performed in combination with introducing a selectable marker at the genetic locus of the *tps2* gene. In some embodiments, the variant strain produces substantially the same amount of, or more, protein per unit amount of biomass as the parental strain. In some embodiments, disruption of the *tps2* gene is performed in combination with disrupting the *sfb3* gene. In some embodiments, disruption of the *tps2* gene is performed in combination with disrupting at least one gene selected from the group consisting of the *sfb3* gene, the *seb1* gene, the *mpg1* gene, the *gas1* gene, and the *crz1* gene.

[17] In some embodiments, the variant strain produces substantially the same amount of, or more, protein per unit amount of biomass as the parental strain.

[18] In some embodiments, the filamentous fungus is a Pezizomycotina species. In some embodiments, the filamentous fungus is a *Trichoderma* spp., *Aspergillus* spp., *Fusarium* spp., *Scedosporium* spp., *Penicillium* spp., *Chrysosporium* spp., *Cephalosporium* spp., *Talaromyces* spp., *Geosmithia* spp., and *Neurospora* spp. In some embodiments, the filamentous fungus can include, but is not limited to, *Trichoderma reesei* (previously classified as *Trichoderma longibrachiatum* and *Hypocrea jecorina*), *Aspergillus niger*, *Aspergillus fumigatus*, *Aspergillus itaconicus*, *Aspergillus oryzae*, *Aspergillus nidulans*, *Aspergillus terreus*, *Aspergillus sojae*, *Aspergillus japonicus*, *Scedosporium prolificans*, *Neurospora crassa*, *Penicillium funiculosum*, *Penicillium chrysogenum*, *Talaromyces (Geosmithia) emersonii*, *Fusarium venenatum*, and *Chrysosporium lucknowense*. In some embodiments, the filamentous fungus is *Trichoderma reesei*.

[19] In some embodiments, the parental strain further comprises a gene encoding a protein of interest. In some embodiments, the gene encoding the protein of interest is present in the parental strain prior to introducing the genetic alteration that reduces or prevents the production of functional Tps2 protein. In some embodiments the protein of interest within the parental strain is encoded by an endogenous gene or a heterologous gene.

[20] In another aspect, a protein of interest produced by any of the aforementioned variant strains is provided.

[21] In yet another aspect, a filamentous fungus produced by any of the aforementioned methods and having any of the aforementioned properties is provided.

[22] In another aspect, a variant strain of filamentous fungus derived from a parental strain is provided, the variant strain comprising: (a) a genetic alteration that results in (i) a requirement for reduced agitation in submerged culture to maintain a preselected dissolved oxygen content, compared to the cells of the parental strain, and/or (ii) maintenance of an increased dissolved oxygen content in submerged culture at a preselected amount of agitation, compared to the cells of the parental strain, and (b) a gene encoding a protein of interest, wherein the gene encoding the protein of interest is present in the variant strain prior to the genetic alteration in (a).

[23] In some embodiments, the genetic alteration of the resulting variant strain comprises a disruption of the *tps2* gene present in the parental strain. In some embodiments, disruption of the *tps2* gene is performed in combination with introducing a selectable marker at the genetic locus of the *tps2* gene. In some embodiments, disruption of the *tps2* gene is performed in combination with disrupting the *sfb3* gene. In some embodiments, disruption of the *tps2* gene is performed in combination with disrupting the *seb1* gene. In some embodiments, disruption of

the *tps2* gene is performed in combination with disrupting at least one gene selected from the group consisting of the *sfb3* gene, the *seb1* gene, the *mpg1* gene, the *gas1* gene, and the *crz1* gene.

[24] These and other aspects and embodiments of present variant strains and methods will be apparent from the description, including the accompanying Figure.

BRIEF DESCRIPTION OF THE DRAWING

[25] Figure 1 is a map of the *tps2* disruption vector, pRATT251-tps2D-pyr2, as described in Example 1.

DETAILED DESCRIPTION

I. Overview

[26] The present strains and methods relate to variant strains of filamentous fungus cells having genetic modifications that affect their morphology and growth characteristics. When the variant cells are grown in submerged culture, they produce a cell broth that has different rheological properties compared to a cell broth comprising cells of the parental strain. Some of these variant strains are well-suited for the large-scale production of enzymes and other commercially important proteins.

II. Definitions

[27] Prior to describing the present strains and methods in detail, the following terms are defined for clarity. Terms not defined should be accorded their ordinary meanings as used in the relevant art.

[28] As used herein, "*Trichoderma reesei*" refers to a filamentous fungus of the phylum Ascomycota, subphylum Pezizomycotina. This organism was previously classified as *Trichoderma longibrachiatum*, or as *Hypocrea jecorina*.

[29] As used herein, the phrase "variant strain of filamentous fungus cells," or similar phrases, refer to strains of filamentous fungus cells that are derived (*i.e.*, obtained from or obtainable from) from a parental (or reference) strain belonging to the Pezizomycotina, *e.g.*, by genetic manipulation. In the present description, parental and variant strains can be described as having certain characteristics, such as genetic modifications, expression phenotypes, morphology, and the like; however, the skilled person will appreciate that it is technically the *cells of* the parental or variant strain that have such characteristics, and "the strains" are referred to for convenience.

[30] As used herein, the term "protein of interest" refers to a polypeptide that is desired to be expressed in a filamentous fungus. Such a protein can be an enzyme, a substrate-binding

protein, a surface-active protein, a structural protein, or the like, and can be expressed at high levels, and can be for the purpose of commercialization. The protein of interest can be encoded by an endogenous gene or a heterologous gene relative to the variant strain and/or the parental strain. The protein of interest can be expressed intracellularly or as a secreted protein.

[31] As used herein, the phrase "substantially free of an activity," or similar phrases, means that a specified activity is either undetectable in an admixture or present in an amount that would not interfere with the intended purpose of the admixture.

[32] As used herein, the terms "polypeptide" and "protein" (and/or their respective plural forms) are used interchangeably to refer to polymers of any length comprising amino acid residues linked by peptide bonds. The conventional one-letter or three-letter codes for amino acid residues are used herein. The polymer can be linear or branched, it can comprise modified amino acids, and it can be interrupted by non-amino acids. The terms also encompass an amino acid polymer that has been modified naturally or by intervention; for example, disulfide bond formation, glycosylation, lipidation, acetylation, phosphorylation, or any other manipulation or modification, such as conjugation with a labeling component. Also included within the definition are, for example, polypeptides containing one or more analogs of an amino acid (including, for example, unnatural amino acids, etc.), as well as other modifications known in the art.

[33] As used herein, functionally and/or structurally similar proteins are deemed "related proteins." Such proteins can be derived from organisms of different genera and/or species, or even different classes of organisms (*e.g.*, bacteria and fungi). Related proteins also encompass homologs determined by primary sequence analysis, determined by secondary or tertiary structure analysis, or determined by immunological cross-reactivity.

[34] As used herein, the term "derivative polypeptide/protein" refers to a protein, which is derived or derivable from a protein by addition of one or more amino acids to either or both the N- and C-terminal end(s), substitution of one or more amino acids at one or a number of different sites in the amino acid sequence, deletion of one or more amino acids at either or both ends of the protein or at one or more sites in the amino acid sequence, and/or insertion of one or more amino acids at one or more sites in the amino acid sequence. The preparation of a protein derivative can be achieved by modifying a DNA sequence, which encodes for the native protein, transformation of that DNA sequence into a suitable host, and expression of the modified DNA sequence to form the derivative protein.

[35] Related (and derivative) proteins include "variant proteins." Variant proteins differ from a reference/parental protein (*e.g.*, a wild-type protein) by substitutions, deletions, and/or

insertions at a small number of amino acid residues. The number of differing amino acid residues between the variant and parental protein can be one or more, for example, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 30, 40, 50, or more amino acid residues. Variant proteins can share at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or even at least about 99%, or more, amino acid sequence identity with a reference protein. A variant protein can also differ from a reference protein in selected motifs, domains, epitopes, conserved regions, and the like.

[36] As used herein, the term "analogous sequence" refers to a sequence within a protein that provides similar function, tertiary structure, and/or conserved residues as the protein of interest (*i.e.*, typically the original protein of interest). For example, in epitope regions that contain an α-helix or a β-sheet structure, the replacement amino acids in the analogous sequence preferably maintain the same specific structure. The term also refers to nucleotide sequences, as well as amino acid sequences. In some embodiments, analogous sequences are developed such that the replacement amino acids result in a variant enzyme showing a similar or improved function. In some embodiments, the tertiary structure and/or conserved residues of the amino acids in the protein of interest are located at or near the segment or fragment of interest. Thus, where the segment or fragment of interest contains, for example, an α-helix or a β-sheet structure, the replacement amino acids preferably maintain that specific structure.

[37] As used herein, the term "homologous protein" refers to a protein that has similar activity and/or structure to a reference protein. Homologs are not necessarily evolutionarily related. Thus, it is intended that the term encompasses the same, similar, or corresponding enzyme(s) (*e.g.*, in terms of structure and function) obtained from different organisms. In some embodiments, it is desirable to identify a homolog that has a quaternary, tertiary and/or primary structure similar to the reference protein. In some embodiments, homologous proteins induce similar immunological response(s) as a reference protein. In some embodiments, homologous proteins are engineered to produce enzymes with desired activity(ies).

[38] The degree of homology between sequences can be determined using any suitable method known in the art (see, *e.g.*, Smith and Waterman (1981) *Adv. Appl. Math.* 2:482; Needleman and Wunsch (1970) *J. Mol. Biol.*, 48:443; Pearson and Lipman (1988) *Proc. Natl. Acad. Sci. USA* 85:2444; programs such as GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package (Genetics Computer Group, Madison, WI); and Devereux *et al.* (1984) *Nucleic Acids Res.* 12:387-95).

[39] For example, PILEUP is a useful program to determine sequence homology levels. PILEUP creates a multiple sequence alignment from a group of related sequences using progressive, pair-wise alignments. It can also plot a tree showing the clustering relationships used to create the alignment. PILEUP uses a simplification of the progressive alignment method of Feng and Doolittle, (Feng and Doolittle (1987) *J. Mol. Evol.* 35:351-60). The method is similar to that described by Higgins and Sharp ((1989) *CABIOS* 5:151-53). Useful PILEUP parameters including a default gap weight of 3.00, a default gap length weight of 0.10, and weighted end gaps. Another example of a useful algorithm is the BLAST algorithm, described by Altschul *et al.* ((1990) *J. Mol. Biol.* 215:403-10) and Karlin *et al.* ((1993) *Proc. Natl. Acad. Sci. USA* 90:5873-87). One particularly useful BLAST program is the WU-BLAST-2 program (see, *e.g.*, Altschul *et al.* (1996) *Meth. Enzymol.* 266:460-80). Parameters "W," "T," and "X" determine the sensitivity and speed of the alignment. The BLAST program uses as defaults a word-length (W) of 11, the BLOSUM62 scoring matrix (see, *e.g.*, Henikoff and Henikoff (1989) *Proc. Natl. Acad. Sci. USA* 89:10915) alignments (B) of 50, expectation (E) of 10, M'5, N'-4, and a comparison of both strands.

[40] As used herein, the phrases "substantially similar" and "substantially identical," in the context of at least two nucleic acids or polypeptides, typically means that a polynucleotide or polypeptide comprises a sequence that has at least about 70% identity, at least about 75% identity, at least about 80% identity, at least about 85% identity, at least about 90% identity, at least about 91% identity, at least about 92% identity, at least about 93% identity, at least about 94% identity, at least about 95% identity, at least about 96% identity, at least about 97% identity, at least about 98% identity, or even at least about 99% identity, or more, compared to the reference (*e.g.*, wild-type) sequence. Sequence identity can be determined using known programs such as BLAST, ALIGN, and CLUSTAL using standard parameters. (See, *e.g.*, Altschul, *et al.* (1990) *J. Mol. Biol.* 215:403-410; Henikoff *et al.* (1989) *Proc. Natl. Acad. Sci. USA* 89:10915; Karin *et al.* (1993) *Proc. Natl. Acad. Sci USA* 90:5873; and Higgins *et al.* (1988) *Gene* 73:237-244). Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information. Also, databases can be searched using FASTA (Pearson *et al.* (1988) *Proc. Natl. Acad. Sci. USA* 85:2444-48). One indication that two polypeptides are substantially identical is that the first polypeptide is immunologically cross-reactive with the second polypeptide. Typically, polypeptides that differ by conservative amino acid substitutions are immunologically cross-reactive. Thus, a polypeptide is substantially identical to a second polypeptide, for example, where the two peptides differ only by a conservative substitution. Another indication that two nucleic acid sequences are substantially

identical is that the two molecules hybridize to each other under stringent conditions (*e.g.*, within a range of medium to high stringency).

[41] As used herein, the term "gene" is synonymous with the term "allele" in referring to a nucleic acid that encodes and directs the expression of a protein or RNA. Vegetative forms of filamentous fungi are generally haploid, therefore a single copy of a specified gene (*i.e.*, a single allele) is sufficient to confer a specified phenotype.

[42] As used herein, the terms "wild-type" and "native" are used interchangeably and refer to genes, proteins, or strains found in nature.

[43] As used herein, "deletion of a gene," refers to its removal from the genome of a host cell. Where a gene includes control elements (*e.g.*, enhancer elements) that are not located immediately adjacent to the coding sequence of a gene, deletion of a gene refers to the deletion of the coding sequence, and optionally adjacent enhancer elements, including but not limited to, for example, promoter and/or terminator sequences.

[44] As used herein, "disruption of a gene" refers broadly to any genetic or chemical manipulation, *i.e.,* mutation, that substantially prevents a cell from producing a function gene product, *e.g.*, a protein, in a host cell. Examples of methods of disruption include complete or partial deletion of any portion of a gene, including a polypeptide-coding sequence, a promoter, an enhancer, or another regulatory element, or mutagenesis of the same, where mutagenesis encompasses substitutions, insertions, deletions, inversions, and combinations and variations, thereof, any of which mutations substantially prevent the production of a function gene product. A gene can also be disrupted using RNAi, antisense, or any other method that abolishes gene expression.

[45] As used herein, the terms "genetic manipulation" and "genetic alteration" are used interchangeably and refer to the alteration/change of a nucleic acid sequence. The alteration can included but is not limited to a substitution, deletion, insertion or chemical modification of at least one nucleic acid in the nucleic acid sequence.

[46] As used herein, "aerobic fermentation" refers to growth in the presence of oxygen.

[47] As used herein, the term "cell broth" refers collectively to medium and cells in a liquid/submerged culture.

[48] As used herein, the term "cell mass" refers to the cell component (including intact and lysed cells) present in a liquid/submerged culture. Cell mass can be expressed in dry or wet weight.

[49] As used herein, the term "rheology" refers to a branch of physics dealing with the deformation and flow of matter.

[50] As used herein, "viscosity" is a measure of the resistance of a fluid to deformation by mechanical stress, such as shear stress or tensile stress. In the present context, viscosity can also refer to the resistance of a cell broth comprising filamentous fungus cells to mechanical stress, *e.g.*, as provided by a rotor/impeller. Because the viscosity of a cell broth can be difficult to measure directly, indirect measurements of viscosity can be used, such as the dissolved oxygen content of the culture broth at a preselected amount of agitation, the amount of agitation required to maintain a preselected dissolved oxygen content, the amount of power required to agitate a cell broth to maintain a preselected dissolved oxygen content, or even colony morphology on solid medium.

[51] As used herein, an "altered-viscosity" variant strain of filamentous fungus cells refers to a variant strain that produces a cell broth that has a reduced or increased viscosity (*i.e.*, reduced or increased resistance to shear or tensile stress) compared to an equivalent cell broth produced by a parental strain. Generally, comparable cell broths or equivalent cell broths have comparable cell masses. Preferably, the difference between a variant, altered viscosity strain and a parental strain, with respect to any direct or indirect measure of viscosity, is at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, or even at least 50%, or more. Methods for comparing the viscosity of filamentous fungus cells broth are described herein.

[52] As used herein, a "reduced-viscosity" variant strain of filamentous fungus cells refers to a variant strain that produces a cell broth that has reduced viscosity (*i.e.*, reduced resistance to shear or tensile stress) compared to an equivalent cell broth produced by a parental strain. Preferably, the difference between a variant, altered viscosity strain and a parental strain, with respect to any direct or indirect measure of viscosity, is at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, or even at least 50%, or more.

[53] As used herein, "dissolved oxygen" (DO) refers to the amount of oxygen (O_2) present in a liquid medium as measured in vol/vol units. The dissolved oxygen level can be maintained at a high level, *e.g.,* between 170-100% and 20%, between 100-80% and 20%, between 70% and 20%, between 65% and 20%, between 60% and 20%, between 55% and 20%, between 50% and 20%, between 45% and 20%, between 44% and 20%, between 43% and 20%, between 42% and 20%, between 41% and 20%, between 40% and 20%, between 35% and 20%, between 30% and 20%, and between 25% and 20% throughout the fermentation. In particular, the dissolved oxygen can be high at the beginning of the fermentation and to be permitted to fall as the fermentation progresses. The dissolved oxygen level can be controlled by the rate at which the

fermentation is agitated, e.g. stirred, and/or by the rate of addition of air or oxygen. The culture can be agitated, *e.g.,* stirred at between 400-700 rpm and the dissolved oxygen level is maintained above 20%, above 25%, above 30%, above 35%, above 40%, above 45%, above 50% and above 55% or more by altering the air or oxygen flow rate and impeller speed.

[54] As used herein, a "primarily genetic determinant" refers to a gene, or genetic manipulation thereof, that is necessary and sufficient to confer a specified phenotype in the absence of other genes, or genetic manipulations, thereof. However, that a particular gene is necessary and sufficient to confer a specified phenotype does not exclude the possibility that additional effects to the phenotype can be achieved by further genetic manipulations.

[55] As used herein, a "functional polypeptide/protein" is a protein that possesses an activity, such as an enzymatic activity, a binding activity, a surface-active property, or the like, and which has not been mutagenized, truncated, or otherwise modified to abolish or reduce that activity. Functional polypeptides can be thermostable or thermolabile, as specified.

[56] As used herein, "a functional gene" is a gene capable of being used by cellular components to produce an active gene product, typically a protein. Functional genes are the antithesis of disrupted genes, which are modified such that they cannot be used by cellular components to produce an active gene product, or have a reduced ability to be used by cellular components to produce an active gene product.

[57] As used herein, variant cells "maintain or retain a high level of protein expression and/or secretion" compared to a parental strain if the difference in protein expression between the variant strain and a parental strain is less than about 20%, less than about 15%, less than about 10%, less than about 7%, less than about 5%, or even less than about 3%.

[58] As used herein, host cells have been "modified to prevent the production of a specified protein" if they have been genetically or chemically altered to prevent the production of a functional protein/polypeptide that exhibits an activity characteristic of the wild-type protein, particularly an activity that promotes elongation of hyphae or otherwise increases the viscosity of a filamentous fungus in liquid culture. Such modifications include, but are not limited to, deletion or disruption of the gene encoding the protein, modification of the gene such that the encoded polypeptide lacks the aforementioned activity, modification of the gene to affect post-translational processing or stability, and combinations, thereof.

[59] As used herein, a "protein of interest" is a protein that is desired to be produced in a submerged culture of filamentous fungus cells. Generally, proteins of interest are commercially important for industrial, pharmaceutical, animal health, and food and beverage use, making them desirable to produce in large quantities. Proteins of interest are to be distinguished from the myriad

other proteins expressed by the filamentous fungus cells, which are generally not of interest as products and are mainly considered background protein contaminants.

[60] As used herein, a variant strain produces "substantially the same amount" of protein per unit amount of biomass as a parental strain if the amount of protein produced by the variant strain is no more than 20% reduced, no more than 15% reduced, no more than 10% reduced, an even no more than 5% reduced compared to the amount of protein produced by the parental strain, wherein the amount of protein is normalized to the total amount of biomass of cells from which protein production is measured, wherein biomass can be expressed in terms of either wet (*e.g.*, of cell pellet) or dry weight.

[61] As used herein, a variant strain produces "substantially more protein per unit amount of biomass" than a parental strain if the amount of protein produced by the variant strain is at least 5% increased, at least 10% increased, at least 15% increased, or more, compared to the parental strain, wherein the amount of protein is normalized to the total amount of biomass of cells from which protein production is measured, wherein biomass can be expressed in terms of either wet (*e.g.*, of cell pellet) or dry weight.

[62] As used herein, "fluorochromes" are fluorescent dyes. Preferred fluorochromes bind to cellulose and/or chitin in the cell walls of fungi.

[63] As used herein, the singular articles "a," "an," and "the" encompass the plural referents unless the context clearly dictates otherwise. The following abbreviations/acronyms have the following meanings unless otherwise specified:

CFU	colony forming units
EC	enzyme commission
kDa	kiloDalton
kb	kilobase
MW	molecular weight
w/v	weight/volume
w/w	weight/weight
v/v	volume/volume
wt%	weight percent
°C	degrees Centigrade
H_2O	water
H_2O_2	hydrogen peroxide
dH_2O or DI	deionized water
dIH_2O	deionized water, Milli-Q filtration
DO	dissolved oxygen
g or gm	gram
µg	microgram
mg	milligram
kg	kilogram
lb	pound

µL and µl	microliter
mL and ml	milliliter
mm	millimeter
µm	micrometer
mol	mole
mmol	millimole
M	molar
mM	millimolar
µM	micromolar
nm	nanometer
U	unit
ppm	parts per million
sec and "	second
min and '	minute
hr and h	hour
EtOH	ethanol
eq.	equivalent
N	normal
PCR	polymerase chain reaction
DNA	deoxyribonucleic acid
FOA	fluoroorotic acid
UV	ultraviolet
A_{540}	absorbance measured at a wavelength of 540 nm
CMC	carboxymethyl cellulose
rpm	revolutions per minute
Δ	relating to a deletion
CER	CO_2 evolution rate
bp	base pairs

III. Filamentous fungal strain with altered Tps2 protein production

[64] In one aspect, a variant strain of filamentous fungus derived from a parental strain is provided, the variant strain comprising a genetic alteration that causes cells of the variant strain to produce an altered amount of functional Tps2 protein compared to cells of the parental strain. The cells of the variant strain subsequently produce, during aerobic fermentation in submerged culture, a cell broth that requires an altered amount of agitation to maintain a preselected dissolved oxygen content, or a cell mass that maintains an altered dissolved oxygen content at a preselected amount of agitation, compared to the cells of the parental strain.

[65] In some cases, the genetic alteration causes cells of the variant strain to produce a reduced amount of functional Tps2 protein compared to cells of the parental strain, and the resulting cell broth requires reduced agitation to maintain a preselected dissolved oxygen content, or maintains a higher dissolved oxygen content at a preselected amount of agitation, compared to the cells of the parental strain. In such cases, it is believed that the cell mass of the variant strain exhibits reduced viscosity compared to the cell mass of the parental strain, which accounts for the observations relating to dissolved oxygen content and agitation as described in the Examples.

[66] The reduction in the amount of functional Tps2 protein can result from disruption of the *tps2* gene present in the parental strain. Because disruption of the *tps2* gene is a primary genetic determinant for conferring a reduced viscosity phenotype to the variant strain, such variant strains need only comprise a disrupted *tps2* gene, while all other genes can remain intact. In some cases, the variant strains can optionally include additional genetic alterations compared to the parental stain from which they are derived. Such additional genetic alterations are not necessary to confer a reduction in viscosity but can further reduce viscosity or confer other advantages for the variant strain.

[67] Disruption of the *tps2* gene can be performed using any suitable methods that substantially prevent expression of a function *tps2* gene product, *i.e.*, the Tps2 protein. Exemplary methods of disruption as are known to one of skill in the art include but are not limited to: Complete or partial deletion of the *tps2* gene, including complete or partial deletion of, *e.g.*, the Tps2-coding sequence, the promoter, the terminator, an enhancer, or another regulatory element. Disruption of the *tps2* gene can also be performed by the complete or partial deletion of a portion of the chromosome that includes any portion of the *tps2* gene. Particular methods of disrupting the *tps2* gene include making nucleotide substitutions or insertions in any portion of the *tps2* gene, *e.g.*, the Tps2-coding sequence, the promoter, the terminator, an enhancer, or another regulatory element. Preferably, deletions, insertions, and/or substitutions (collectively referred to as mutations) are made by genetic manipulation using sequence-specific molecular biology techniques, as opposed to by chemical mutagenesis, which is generally not targeted to specific nucleic acid sequences. Nonetheless, chemical mutagenesis can be used to disrupt the *tps2* gene.

[68] Mutations in the *tps2* gene can reduce the efficiency of the *tps2* promoter, reduce the efficiency of a *tps2* enhancer, interfere with the splicing or editing of the *tps2* mRNA, interfere with the translation of the *tps2* mRNA, introduce a stop codon into the Tps2-coding sequence to prevent the translation of full-length Tps2 protein, change the coding sequence of the Tps2 protein to produce a less active or inactive protein or reduce Tps2 interaction with other nuclear protein components, change the coding sequence of the Tps2 protein to produce a less stable protein or target the protein for destruction, cause the Tps2 protein to misfold or be incorrectly modified (*e.g.*, by glycosylation), or interfere with cellular trafficking of the Tps2 protein.

[69] In one embodiment, these and other genetic manipulations is to reduce or prevent the expression of a functional Tps2 protein, or reduce or prevent the normal biological activity of the Tps2 protein, thereby producing a morphology change that results in a reduced viscosity phenotype.

[70] In other cases, the genetic alteration increases or restores the expression of a functional Tps2 protein, or increases the normal biological activity of the Tps2 protein, thereby producing a morphology change that results in an increased or restored viscosity phenotype. Exemplary genetic alterations that increase or restore Tps2 function are those that introduce addition copies of the *tps2* gene into a cell, increase the efficiency of the *tps2* promoter, enhancer, or other control element, increase the translation of the mRNA encoding the Tps2 protein, increase the stability of mRNA encoding the Tps2 protein, introduce changes in the *tps2* gene that increase the activity or stability of the Tps2 protein, introduce changes in the *tps2* gene that modulate the interaction with other proteins or nucleic acids and the like. Other genetic alterations that increase or restore Tps2 function are those that reverse the effect of genetic alterations, which reduce or prevent the expression of a functional Tps2 protein.

[71] Filamentous fungus cells for manipulation and use as described are generally from the phylum Ascomycota, subphylum Pezizomycotina, particularly fungi that have a vegetative hyphae state and include a homolog of the *tps2* gene. Such organisms include filamentous fungus cells used for the production of commercially important industrial and pharmaceutical proteins, including, but are not limited to *Trichoderma* spp., *Aspergillus* spp., *Fusarium* spp., *Scedosporium* spp., *Penicillium* spp., *Chrysosporium* spp., *Cephalosporium* spp., *Talaromyces* spp., *Geosmithia* spp., and *Neurospora* spp. Particular organisms include, but are not limited to, *Trichoderma reesei* (previously classified as *Trichoderma longibrachiatum* or *Hypocrea jecorina*), *Aspergillus niger*, *Aspergillus fumigatus*, *Aspergillus itaconicus*, *Aspergillus oryzae*, *Aspergillus nidulans*, *Aspergillus terreus*, *Aspergillus sojae*, *Aspergillus japonicus*, *Scedosporium prolificans*, *Neurospora crassa*, *Penicillium funiculosum, Penicillium chrysogenum, Talaromyces (Geosmithia) emersonii, Fusarium venenatum*, and *Chrysosporium lucknowense*.

[72] The gene *tps2* encodes a trehalose-phosphate phosphatase involved in the synthesis of the disaccharide trehalose. Trehalose is a stress induced sugar that buffers the refolding of denatured proteins in the cytoplasm and ER (Singer *et al.* 1998, Simola, *et al.* 2000). This disaccharide is produced in large quantities by diverse organisms in response to a variety of stresses. In yeast, trehalose stabilizes proteins at high temperatures and assists in refolding heat damaged proteins (Simola, M *et al.* 2000).

[73] A disruption in the *tps2* homologue *orl*A in *A. nidulans* results in reduced chitin and increased sensitivity to high temperature (Borgia, P. *et al.* 1996). Disruption of *orl*A in *A. fumigatus* results in increased sensitivity to calcoflour white and Congo red. Based on these results, and not wishing to be bound to a theory, it is believed that the alteration of *tps2* expression

and/or activity in filamentous fungi can alter the cell wall, thereby producing a more compact cellular morphology characterized by shorter hyphae and/or a more yeast-like appearance. The present disclosure provides experimental evidence of the association of Tps1 with altered morphology.

[74] Using BLAST to search publicly available genome sequences of filamentous fungi and yeast using the *T. reesei* Tps2 amino acid sequence as query, homologs were found, although the function of these proteins was heretofore unknown. The amino acid sequences of the *T. reesei* (SEQ ID NO:1), *B. bassiana* (SEQ ID NO:2), *S. cerevisiae* S288c (SEQ ID NO:3), *A. flavus* NRRL 3357 (SEQ ID NO:4), *A. oryzae* (SEQ ID NO:5), *A. clavatus* (SEQ ID NO:6), and *T. stipitatus* ATCC 10500 (SEQ ID NO:7) Tps2 proteins are shown below.

[75] The predicted amino acid sequence of the *Trichoderma reesei* Tps2 protein is shown below as SEQ ID NO: 1:

MARRESLSEIRAANPELFLTGNIISATFNIPHAVTYHKGGAWDLKPRRGQSALIDSFAYLSSDATP
WNHTVVAWTGEIANPDNDPLSPPDTPSAAATTIGAANSLSAPVPIDATTRLPTPPPVDGLWIPKA
DQTRLEHQLSHSTTIRTVPVWLADQSEATDDGIMLKDQARWRRYAEHDLYTLFHYKQHEPTDG
RKERAQWADYYRMNQKFANKIIEIYKPGDVVIVHDYYLMLLPSMLRQRAPKMYISFFLHSPFPS
SEFLRCLPRRKEVLEGVLGANLVGFQSYSYSRHFLSCCTRILGFPSDTLGIDAYGSRVQVGVFPIG
IDAAKVETAAWADTVNEKHAAVLKMYEGKKIIVGRDRLDSVRGVAQKLQAFERFLELYPHWR
EKVVLIQVTSPTSIEAEKGDPENKNASRVNELITKINGEYGSLGFSPVQHYPQYLSQAEYFALLRA
ADIGLITSVRDGMNTTSLEYVVCQKDSNGPLILSEFSGTAGSLRDAIIHINPWDLTGVAEKINAALE
MSEEERVKMQTSLYTHVTTQNVQSWITKFIRKFHAALSETNSVTSTPLLDRALLLSRYRAAKKR
LFMFDYDGTLTPIVREPSAAVPSERIIRYLQSLASDPRNAVWIISGRDQEFLQQHLGHIPRIGFSAE
HGSFMRDPGSDEWVNLAEKFDMGWQAEVMEVFQRYTDKVPGSFIERKRCALTWHYRLAEPEQ
GLHMSRECHRELETGIAQRWEVEVMPGKANIEVRPTFINKGEIAKRLVATYHNPGAAPTDKDPY
PGKIEFALCSGDDFTDEDMFRSLNGACGTILEDQHVFTVTVGASTKVTLAKWHLLEPEDVIECV
GLLAGAGDPASLERVGEVNLAALSQVEGHIPAEEL

[76] The amino acid sequence of the *B. bassiana* Tps2 protein is shown below as SEQ ID NO: 2:

MARRESLSEILAANPELSLSGSIISAAFNIPHALTYRKGGDWGLKPRGGQSALFDSFAYLSSSANP
FKHTVVSWTGEIDSPQGPLEPEPQRPRSTTVGVSSLNPLSAPIPVDGIVQLPTPPSSDGLWLPKAD
QERLEHQLSNDKTIRTVPVWLADEDEITPDGIMLRDQGRWRGYAHRDLYSLFHYKQHEPSDGR
KEKIEWADYYRMNQKFAAKILEIYKPGDIVIIHDYFLMLLPSMLRQAVPNMYISFYLHCPFPSSEF
LRCLPRRREVLEGILGSNLVGFQSYSYSRHFLSCCTRILGFPSDTLGVDAYGSRVQVGVFPIGIDA
AKVEKLAWASSVDEKYDALKKMYAGKKIIVGRDRLDSVRGVVQKLQAFDRFLEMYSEWREK
VVLIQVTSPTNKVADKEDGEHKTSTRVNELVMQINGKYGSLGFSPVQHYPQYINQDEYFALLRA

ADIGLITSVRDGMNTTSLEYVVCQKDGHGPLILSEFSGTAASLSDAIHINPWDLTDVAGKINGAL
TMPDDARSKMQSRLYEHVTTQTVQSWITKFIRRIHSVLGDKSIQHSTPLLDRALLLSQYRAASKR
IFMFDYDGTLTPIVREPSAAVPSEKLLESLKILAAEPRNSVWIISGRDQEFLTQHLGHIPELGFSAE
HGSFMRDPGSQEWINLADKFDMGWQNEVIDVFQKYTDKVTGSFIERKRCAITWHYRLADPEQG
LHMSRVAHKEVEETVAKKWDVEVMAGKANIEVRPTFINKGEIVKRLISRYHNPGLVADEGDRN
AGRIEFALCSGDDFTDEDMFRSLNGVSGSVLDADHVFTVTVGPSTKVTLARWHLLEPADVVDC
VTLLSEQKGHLALERMGEVNLAALSSVEGHIPTA

[77] The amino acid sequence of the *S. cerevisiae* Tps2 protein is shown below as SEQ ID NO: 3:

MTTTAQDNSPKKRQRIINCVTQLPYKIQLGESNDDWKISATTGNSALFSSLEYLQFDSTEYEQHV
VGWTGEITRTERNLFTREAKEKPQDLDDDPLYLTKEQINGLTTTLQDHMKSDKEAKTDTTQTAP
VTNNVHPVWLLRKNQSRWRNYAEKVIWPTFHYILNPSNEGEQEKNWWYDYVKFNEAYAQKIG
EVYRKGDIIWIHDYYLLLLPQLLRMKFNDESIIIGYFHHAPWPSNEYFRCLPRRKQILDGLVGANR
ICFQNESFSRHFVSSCKRLLDATAKKSKNSSNSDQYQVSVYGGDVLVDSLPIGVNTTQILKDAFT
KDIDSKVLSIKQAYQNKKIIIGRDRLDSVRGVVQKLRAFETFLAMYPEWRDQVVLIQVSSPTANR
NSPQTIRLEQQVNELVNSINSEYGNLNFSPVQHYYMRIPKDVYLSLLRVADLCLITSVRDGMNTT
ALEYVTVKSHMSNFLCYGNPLILSEFSGSSNVLKDAIVVNPWDSVAVAKSINMALKLDKEEKSN
LESKLWKEVPTIQDWTNKFLSSLKEQASSNDDMERKMTPALNRPVLLENYKQAKRRLFLFDYD
GTLTPIVKDPAAAIPSARLYTILQKLCADPHNQIWIISGRDQKFLNKWLGGKLPQLGLSAEHGCF
MKDVSCQDWVNLTEKVDMSWQVRVNEVMEEFTTRTPGSFIERKKVALTWHYRRTVPELGEFH
AKELKEKLLSFTDDFDLEVMDGKANIEVRPRFVNKGEIVKRLVWHQHGKPQDMLKGISEKLPK
DEMPDFVLCLGDDFTDEDMFRQLNTIETCWKEKYPDQKNQWGNYGFYPVTVGSASKKTVAKA
HLTDPQQVLETLGLLVGDVSLFQSAGTVDLDSRGHVKNSESSLKSKLASKAYVMKRSASYTGA
KV

[78] The amino acid sequence of the *A. flavus* Tps2 protein is shown below as SEQ ID NO:4:

MSSEQRTTPAKIPSDQPDPVLVGPGVKVLGEEAYTKASTATPIPGGEKKQSFTTDAPSYFSKTPGE
KMSSESSNATPTTPAQAAKDARSRIELLRRLSLRETPKVLEADLRQQHPGLRLSGRIISAAFCIPY
KVYYRRESSWELKPRPGTSALFDSLAYLGSEETNWSHTLVGWTGEVEPVPEDTVPLQQIPINTSA
KLPAATNGTAKPLNKAAAPVPVDANQRPPSHPLLDGFTVSQDDRSRLDAQLSSGRYGKIAPVW
LSAETEIPEDTIFLEDQGRWRRYAERELYPLLHYKQHGPTDGRSERNWWADYVRMNRLFADRIL
KEYQEGDIVWIHDYHLFLLPSMLRQRIPNIYIGFFLHAPFPSSEFMRCLAKRKEVLTGVLGANMIG
FQTFSYSRHFSSCCTRVLGFDSNSAGVDAYGAHVAVDVFPIGIDAKAIQNIAFGASEIENAVTGIR
KLYAGKKIIVGRDRLDSVRGVAQKLQSFEVFLERYPEWRDKVVLIQVTSPTSVEEEKEENKIASQ
ISNLVSTINGRFGSLSFSPVKYYPQYLSQHEYFALLRVADVGLITTVRDGMNTTSLEYIICQQQSH

GPLILSEFSGTAGTLSSAIHINPWDTAGVAGAINQALTMSPESKKASHQKLYKHVTTNTVSAWST
QYLSRLLTNLSSFDQSVATPALDRAKLLKQYRKARKRLFMFDYDGTLTPIVKDPQAAIPSDRVL
RTIKTLAADSRNAVWIISGRDQAFLDEWMGHIPELGLSAEHGCFIRKPRSDDWENLAERSNMGW
QKEVMEIFQHYTERTQGSFIERKRVALTWHYRRADPEYGAFQARECRKHLEETVGKRWDVEV
MAGKANLEVRPTFVNKGFIASRLVNEYGTGPGQAPEFIFCSGDDFTDEDMFRALQKFDLPQDHV
YSVTVGASSKQTSASWHLLEPADVIETVTMLNSSSTQDY

[79] The amino acid sequence of the *A. oryzae* Tps2 protein is shown below as SEQ ID NO:5:

MSSEQRTTPAKIPSDQPDPVLVGPGVKVLGEEAYTKASTATPIPGGEKKQSFTTDAPSYFSKTPGE
KMSSESSNATPTTPAQAAKDARSRIELLRRLSLRETPKVLEADLRQQHPGLRLSGRIISAAFCIPY
KVYYRRESSWELKPRPGTSALFDSLAYLGSEETNWSHTLVGWTGEVEPVPEDTVPLQQIPINTSA
KLPAATNGTAKPLNKAAAPVPVDANQRPPSHPLLDGFTVSQDDRSRLDAQLSSGRYGKIAPVW
LSAETEIPEDTIFLEDQGRWRRYAERELYPLLHYKQHGPTDGRSERNWWADYVRMNRLFADRIL
KEYQEGDIVWIHDYHLFLLPSMLRQRIPNIYIGFFLHAPFPSSEFMRCLAKRKEVLTGVLGANMIG
FQTFSYSRHFSSCCTRVLGFDSNSAGVDAYGAHVAVDVFPIGIDAKAIQNIAFGASEIENAVTGIR
KLYAGKKIIVGRDRLDSVRGVAQKLQSFEVFLERYPEWRDKVVLIQVTSPTSVEEEKEENKIASQ
ISNLVSTINGRFGSLSFSPVKYYPQYLSQHEYFALLRVADVGLITTVRDGMNTTSLEYIICQQQSH
GPLILSEFSGTAGTLSSAIHINPWDTAGVAGAINQALTMSPESKKASHQKLYKHVTTNTVSAWST
QYLSRLLTNLSSFDQSVATPALDRAKLLKQYRKARKRLFMFDYDGTLTPIVKDPQAAIPSDRVL
RTIKTLAADSRNAVWIISGRDQAFLDEWMGHIPELGLSAEHGCFIRKPRSDDWENLAERSNMGW
QKEVMEIFQHYTERTQGSFIERKRVALTWHYRRADPEYGAFQARECRKHLEETVGKRWDVEV
MAGKANLEVRPTFVNKGFIASRLVNEYGTGPGQAPEFIFCSGDDFTDEDMFRALQKFDLPQDHV
YSVTVGASSKQTSASWHLLEPADVIETVTMLNSSSTQDY

[80] The amino acid sequence of the *A. clavatus* Tps2 protein is shown below as SEQ ID NO:6:

MSASQDSPSAKVLDGQPNPVIVGPGMKSLGEDAYTQAANVTPSLDTDKKHPVDSDAPSYFANIP
DTQPSADVNSPATPADAAKSAKSPIELLHRLSLNRTPLVPDFDPREQYPGLNLTGRFISAAFCIPY
KVYYRPGSDWELKPRPGTSALFDSFAYLGSEETKWSHTLVGWTGEVEPIQETPASLQQIPVNAG
AKLPPALNGVAVPLSKAAAPVPVDSSQRPPSHPLLEGFTVPQEDRARLDGQLGSGRYGKIAPVW
LSDESEEPEESSTIFLEDQGKWRRYAEKELYPLLHYKQHGPTDGRSERKWWGDYVRMNRLFAD
RILEEYKEGDIVWIHDYHLFLLPSLLRQRIPNIYIGFFLHAPFPSSEFMRCLAKRKEVLTGVLGSNM
IGFQTFSYSRHFSSCCTRVLGFESNSAGVDAYGAHVAVDVFPIGIDVKAIQKAAFGPANIENAVV
ALRNLYAGKKIIVGRDRLDSVRGVAQKLQAFEAFLERYPEWRDKVVLIQVTSPTSVEEEKEDPE
NKIASQISNLVSTINGRFGSISFSPVKYYPQYLSQHEYFALLRVADVGLITTVRDGMNTTSLEYILC
QQNTHSPLILSEFSGTAGPLSSAIHINPWDTIGVAEAINEALTMSPEEKRLQHVHLYKHVTTNTVL

TWSNQFVTRLLTNLSSFDQSVATPALDRATVLKQYRKARKRLFMFDYDGTLTPIVKDPQAAIPS
DRVLRNIKTLAADPRNAVWIISGRDQAFLDEWMGHIPELGLSAEHGCFIRKPRSDDWENLAESS
DMGWQKEVVEVFQHFTERTQGSFIERKRVALTWHYRRADPEYGAFQARECRKQLEETVAKRW
DVEVMAGKANLEVRPTFVNKGFIASRLVDEYGTGPGQAPEFVLCLGDDFTDEDMFRALKKANL
PADHVYSVTVGASSKQTEASWHLLEPADVIGTISVLNNSSSAQEY

[81] The amino acid sequence of the *T. stipitatus* Tps2 protein is shown below as SEQ ID NO:7:

MASEQGAPDKIPPNQPNPVIVGPGLSALGEEAYVDASTATPAVVPATTTTANADGAADSYFSQV
PGTATAIKDAYAKSPMSPADAASGVTSGPELLRRLSLMGGAHLTPATPVTDPRADHPGLQLTGR
IISASLCIPYKVAHQPGADWELSPRSGTSALFDSFAHLASDRSPWNHTLVGWTGEVEEIVSKRAP
LQPVSANGVPTAPLPVNKASAPVPVDLSQQVQSPVDGVLVSAADRERLERQLKSSKYGRILPVW
AIPESDEPQDDILLQDQSRWRRYAERELYPLLHYKQNGPSDGRSERKWWTDYMRLNRLFADRI
AGTYQAGDIVWIHDYHLFLLPNLLRQRIPNIFIGFFLHSPFPSSEYMRCLAKRKEVLTGVLGANMI
GFQTYSYSRHFSSCCTRVLGFESNSAGVDAYGAHVAVDVFATGIDAQNVQRAAFGSAETEQVV
ANIKKLYAGKKIIVGRDRLDSVRGVAQKLQAFEAFLEKYPHWHDKVVLIQVTSPTSMEEQKEDP
ENKIGSQVSSLVSTINGRFGSLSFTPVQYHPQYISPQEYFSLLRVADVGLITSVRDGMNTTSLEYV
LCQQGNHGPLILSEFSGTAAMLTSAIHINPWDTSGVAAAIDQALSMSEKEKVERHQVAYRHVTS
NTVSMWSQHYLNRLLTNLSSFDQSIATPALDRAQVLKQYRKAKKRLFMFDYDGTLTPIVKDPQ
AAIPSDRVLRNIKSLAADPRNSVWIISGRDQAFLDEWMGHIPELGLSAEHGCFIRKPRSDDWENL
AAQSDMSWQKDVMDIFQHYTERTQGSFIERKRVALTWHYRRADPEYGAFQAKECRKHLENTV
MKKYDVEVMAGKANLEVRPTFVNKGFIVTRLLNEYAKGEAPEFMFCSGDDFTDEDMFRALRH
SNLPQEHIFSVTVGASSKQTLASWHLLEPADVIATIGMLNGTSMGAEYS

[82] In some embodiments of the present compositions and methods, the amino acid sequence of the Tps2 protein that is altered in production levels has a specified degree of overall amino acid sequence identity to the amino acid sequence of SEQ ID NOs: 1, 2, 3, 4, 5, 6, or 7, *e.g.*, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or even at least about 99% identity, to SEQ ID NOs: 1, 2, 3, 4, 5, 6, or 7. The nucleotide sequences encoding each amino acid sequence can be identified from a BLAST search for each corresponding protein as is know to one skilled in the art.

[83] In some embodiments of the present compositions and methods, the *tps2* gene that is disrupted encodes a Tps2 protein that has a specified degree of overall amino acid sequence identity to the amino acid sequence of SEQ ID NOs: 1, 2, 3, 4, 5, 6, or 7, *e.g.*, at least about

70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or even at least about 99% identity, to SEQ ID NOs: 1, 2, 3, 4, 5, 6, or 7.

[84] The amino acid sequence information provided herein readily allows the skilled person to identify a Tps2 protein, and the nucleic acid sequence encoding a Tps2 protein, in any filamentous fungi, and to make appropriate disruptions in the *tps2* gene to affect the production of the Tps2 protein. The polynucleotide sequences encoding SEQ ID NOs: 1, 2, 3, 4, 5, 6, or 7 can be found in the GenBank or JGI databases, as are known to one of skill in the art.

[85] In another aspect, a method for altering the morphology of filamentous fungus cells is provided. The variant filamentous fungus cells exhibit altered growth morphology on solid medium and produce cell broth having different viscosities when grown in submerged culture compared to parental cell growth and cell broth viscosities.

[86] In some cases, the method comprises disrupting the *tps2* gene in a parental strain using suitable genetic methods, wherein during aerobic fermentation the disrupted *tps2* variant strain produces during aerobic fermentation in submerged culture a cell broth that requires reduced agitation to maintain a preselected dissolved oxygen content, or maintains an increased dissolved oxygen content at a preselected amount of agitation, compared to the cells of the parental strain. Such methods can be used to disrupt the *tps2* gene in any manner described above and elsewhere as are known to one of skill in the art. Preferably, disruption of the *tps2* gene is performed by genetic manipulation using sequence-specific molecular biology techniques, as opposed to chemical mutagenesis, which is generally not targeted to specific nucleic acid sequences. However, chemical mutagenesis can be used to achieve satisfactory results.

[87] In some embodiments, the parental strain into which the reduced viscosity phenotype is introduced creating a reduced viscosity strain already comprises a gene of interest intended to be expressed at high levels. In this manner, the present methods obviate the need to introduce a gene of interest into a pre-existing reduced viscosity strain for production. Thus, the present methods can be used to produce a reduced viscosity variant strain of filamentous fungus cells from a parental strain already comprising a gene of interest.

VI. Utility

[88] The use of reduced viscosity strains of filamentous fungi is known to improve the distribution of oxygen and nutrients in a submerged culture, reduce the amount of energy required to agitate a submerged culture, and increase the cell mass present in the culture, leading to increased

protein production. Moreover, the present variant strains of filamentous fungus offer significant advantages over previously-described reduced viscosity strains.

[89] First, the present variant strains can have a fully defined genome, making them well-suited for subsequent genetic manipulation, complementation, mating, and the like Second, the present strains are still capable of high levels of protein production, for example, by the manipulation(s) that resulted in the attendant viscosity alteration. Third, reduced viscosity strains can be produced from essentially any parental strain, including parental strains that already produce a protein intended for high level expression (*i.e.*, a protein of interest), already encoding a selectable marker, or already including other features that are desirable in a production host. Thus, the present strain and methods eliminate the need to transfer a gene encoding a protein of interest into a preexisting reduced viscosity production strain.

[90] The present strains and methods find use in the production of commercially important protein in submerged cultures of filamentous fungi. Commercially important proteins include, for example, cellulases, xylanases, pectinases, lyases, proteases, kinases, amylases, pullulanases, lipases, esterases, perhydrolases, transferases, laccases, catalases, oxidases, reductases, chlorophyllases, hydrophobin, chymosin, carbonic anhydrase, hymidylate synthase, dihydrofolate reductase, tyrosine kinases, multi-drug resistance proteins (e.g., ABC P-gp proteins), CAD (carbamyl-P synthase, aspartate transcarbamylase, dihydroorotase), topoisomerases, ribonucleotide reductase, and antibodies and other enzymes and non-enzyme proteins capable of being expressed in filamentous fungi. Such proteins can be suitable for industrial, pharmaceutical, animal health and food and beverage use.

[91] The following numbered paragraphs further describe various aspects and embodiments of the present compositions and methods. The subject matter of each of the numbered paragraphs can be used alone or in combination with the subject matter of any other numbered paragraph, as indicated.

1. In one aspect, a variant strain of filamentous fungus derived from a parental strain is provided, the variant strain comprising a genetic alteration that causes cells of the variant strain to produce an altered amount of functional Tps2 protein compared to cells of the parental strain, wherein the cells of the variant strain produce during aerobic fermentation in submerged culture a cell broth that (i) requires an altered amount of agitation to maintain a preselected dissolved oxygen content compared to the cells of the parental strain, and/or (ii) maintains an altered dissolved oxygen content at a preselected amount of agitation, compared to the cells of the parental strain.

2. In some embodiments of the variant strain of paragraph 1, the altered amount of functional Tps2 protein is a reduced amount, and the variant strain produces during aerobic fermentation in submerged culture a cell broth that (i) requires reduced agitation to maintain a preselected dissolved oxygen content compared to the cells of the parental strain, and/or (ii) maintains an increased dissolved oxygen content at a preselected amount of agitation, compared to the cells of the parental strain.

3. In some embodiments of the variant strain of paragraphs 1 or 2, the genetic alteration comprises a disruption of the *tps2* gene present in the parental strain.

4. In some embodiments of the variant strain of paragraph 3, disruption of the *tps2* gene is the result of deletion of all or part of the *tps2* gene.

5. In some embodiments of the variant strain of paragraph 3, disruption of the *tps2* gene is the result of deletion of a portion of genomic DNA comprising the *tps2* gene.

6. In some embodiments of the variant strain of paragraph 3, disruption of the *tps2* gene is the result of mutagenesis of the *tps2* gene.

7. In some embodiments of the variant strain of any of paragraphs 3-6, disruption of the *tps2* gene is performed using site-specific recombination.

8. In some embodiments of the variant strain of any of paragraphs 3-7, disruption of the *tps2* gene is performed in combination with introducing a selectable marker at the genetic locus of the *tps2* gene.

9. In some embodiments of the variant strain of any of paragraphs 1-8, the variant strain does not produce functional Tps2 protein.

10. In some embodiments of the variant strain of any of paragraphs 1-8, the variant strain does not produce Tps2 protein.

11. In some embodiments of the variant strain of any of paragraphs 1-10, the variant strain further comprises a gene encoding a protein of interest.

12. In some embodiments of the variant strain of any of paragraphs 1-11, further comprising a disruption of the *sfb3* gene.

13. In some embodiments of the variant strain of any of paragraphs 1-12, further comprising a disruption of at least one gene selected from the group consisting of the *sfb3* gene, the *seb1* gene, the *mpg1* gene, the *gas1* gene, and the *crz1* gene.

14. In some embodiments of the variant strain of any of paragraphs 1-13, the variant strain produces substantially the same amount of, or more, protein per unit amount of biomass as the parental strain.

15. In some embodiments of the variant strain of any of paragraphs 1-14, the filamentous fungus is a Pezizomycotina species.

16. In some embodiments of the variant strain of any of paragraphs 1-15, the filamentous fungus is a *Trichoderma* spp.

17. In some embodiments of the variant strain of any of paragraphs 1-16, the filamentous fungus is *Trichoderma reesei.*

18. In another aspect, a method for producing a variant strain of filamentous fungus cells is provided, comprising: introducing a genetic alteration into a parental strain of filamentous fungal cell, which genetic alteration alters the production of functional Tps2 protein compared to the cells of the parental strain, thereby producing a variant filamentous fungal cell that produces during aerobic fermentation in submerged culture a cell broth that (i) requires an altered amount of agitation to maintain a preselected dissolved oxygen content, compared to the cells of the parental strain, and/or (ii) maintains an altered dissolved oxygen content at a preselected amount of agitation, compared to the cells of the parental strain.

19. In some embodiments of the method of paragraph 18, the genetic alteration reduces or prevents the production of functional Tps2 protein, thereby producing a variant filamentous fungal cell that produces during aerobic fermentation in submerged culture a cell broth that (i) requires reduced agitation to maintain a preselected dissolved oxygen content, compared to the cells of the parental strain, and/or (ii) maintains an increased dissolved oxygen content at a preselected amount of agitation, compared to the cells of the parental strain.

20. In some embodiments of the method of paragraph 18 or 19, the genetic alteration comprises disrupting the *tps2* gene in a parental filamentous fungal cell using genetic manipulation.

21. In some embodiments of the method of any of paragraphs 18-20, the genetic alteration comprises deleting the *tps2* gene in a parental filamentous fungal cell using genetic manipulation.

22. In some embodiments of the method of any of paragraphs 18-21, the genetic alteration is performed using site-specific genetic recombination.

23. In some embodiments of the method of any of paragraphs 18-22, disruption of the *tps2* gene is performed in combination with introducing a selectable marker at the genetic locus of the *tps2* gene.

24. In some embodiments of the method of any of paragraphs 18-23, disruption of the *tps2* gene is performed in combination with disrupting the *sfb3* gene.

25. In some embodiments of the method of any of paragraphs 18-24, disruption of the *tps2* gene is performed in combination with disruption of at least one gene selected from the group consisting of the *sbf1* gene, the *seb1* gene, the *mpg1* gene, the *gas1* gene, and the *crz1* gene.

26. In some embodiments of the method of any of paragraphs 18-25, the variant strain produces substantially the same amount of, or more, protein per unit amount of biomass as the parental strain.

27. In some embodiments of the method of any of paragraphs 18-26, the filamentous fungus is a Pezizomycotina species.

28. In some embodiments of the method of any of paragraphs 18-27, the filamentous fungus is a *Trichoderma* spp.

29. In some embodiments of the method of any of paragraphs 18-28, the filamentous fungus is *Trichoderma reesei*.

30. In some embodiments of the method of any of paragraphs 18-29, the parental strain further comprises a gene encoding a protein of interest.

31. In some embodiments of the method of paragraph 30, the gene encoding the protein of interest is present in the parental strain prior to introducing the genetic alteration that reduces or prevents the production of functional Tps2 protein.

32. In another aspect, a protein of interest produced by the variant strain of paragraph 11 is provided.

33. In another aspect, a variant strain of filamentous fungus produced by the method of any of paragraphs 18-31 is provided.

34. In another aspect, a variant strain of filamentous fungus derived from a parental strain is provided, the variant strain comprising:

(a) a genetic alteration that results in (i) a requirement for reduced agitation in submerged culture to maintain a preselected dissolved oxygen content, compared to the cells of the parental strain, and/or (ii) maintenance of an increased dissolved oxygen content in submerged culture at a preselected amount of agitation, compared to the cells of the parental strain, and

(b) a gene encoding a protein of interest, wherein the gene encoding the protein of interest is present in the variant strain prior to the genetic alteration in (a).

35. In some embodiments of the variant strain of paragraph 34, the genetic alteration comprises a disruption of the *tps2* gene present in the parental strain.

36. In some embodiments of the variant strain of paragraph 35, disruption of the *tps2* gene is performed in combination with introducing a selectable marker at the genetic locus of the *tps2* gene.

37. In some embodiments of the variant strain of paragraph 35 or 36, disruption of the *tps2* gene is performed in combination with disrupting at least one gene selected from the group consisting of the *sfb3* gene, the *seb1* gene, the *mpg1* gene, the *gas1* gene, and the *crz1* gene.

38. In some embodiments of the variant strain of any of paragraphs 35-37, disruption of the *tps2* gene is performed in combination with disrupting the *seb1* gene.

[92] These and other aspects and embodiments of the present strains and methods will be apparent to the skilled person in view of the present description. The following examples are intended to further illustrate, but not limit, the strains and methods.

EXAMPLES

Example 1. Deletion of the *tps2* gene from *T. reesei* mutant Morph 77B7

[93] A *Trichoderma reesei* Morph strain was deleted for four major cellulase genes, including *cbhI, cbhII, egII,* and *egIV,* which makes it particular suitable for expressing other proteins in the absence of or in reduced cellulase background. *See,* WO 05/001036.

A. TrGA producing strain Morph 77B7

[94] The Morph strain, described above, was previously transformed with a native *Trichoderma* glucoamylase gene (TrGA) under control of the CBH1 promoter, using *amdS* as a marker. A transformant containing two tandem copies of glucoamylase (TrGA 29-9) was subsequently isolated, and random chemical mutagenesis was used to produce a mutant (77B7). A spontaneous *pyr2* mutant derivative was subsequently isolated by *5-fluoro*-orotic acid (FOA) selection.

B. Generation of a *tps2* disruption cassette

[95] The *Trichoderma reesei tps2* (PID 48707) was deleted from mutant Morph 77B7.

[96] The *tps2* disruption cassette plasmid pRATT251 (Figure 1) was prepared using standard molecular biology procedures. This plasmid included a DNA sequence having a 2.8 Kb region homologous to the DNA sequence spanning part of the 5′ untranslated region and contiguous upstream sequences (Left Flank). Also included within the plasmid was a DNA sequence having a 2.5 Kb region homologous to the DNA sequence spanning part of the second exon of the *tps2* gene and contiguous downstream sequences (Right Flank). These sequences were designed to target the *tps2* gene and replace the regions of the genome between the Left and Right Flanks, region 1135546 to 1136670 on Scaffold 10 (JGI *T. reesei* genomic database v2), with the intervening cassette sequences. These intervening sequences included a *pyr2* selection marker from *Trichoderma atroviride* intended to minimize homology to the endogenous *T. reesei pyr2* in the genome of the strain to be transformed. Immediately upstream of the *pyr2*

selection marker was a directly repeated duplication of the 3′end of the marker, which facilitated the subsequent loss of the marker and isolation of useful *pyr2* mutant derivatives of the transformants/disruptants. This full *tps2* disruption cassette was amplified by PCR using primers RPG398 and RPG401. Multiple PCR reactions were pooled and cleaned using standard molecular biology procedures for use in the subsequent steps.

[97] The nucleic acid sequence of the *tps2* gene was obtained from the JGI data base: Protein ID: 48707, Name: estExt_Genewise1.C_100658, (The Genome Portal of the Department of Energy Joint Genome Institute I. V. Grigoriev, H. Nordberg, I. Shabalov, A. Aerts, M. Cantor, D. Goodstein, A. Kuo, S. Minovitsky, R. Nikitin, R. A. Ohm, R. Otillar, A. Poliakov, I. Ratnere, R. Riley, T. Smirnova, D. Rokhsar, and I. Dubchak. Nucleic Acids Res 2011 0: gkr947v1-gkr947) as disclosed below. The untranslated region is italicized and flanked 5' and 3' by upstream or downstream sequence, coding regions are in bold and introns are in lower case (SEQ ID NO: 14):

AGCTCCCGATAGGGCGGCGGCCAAGTCACAGGCCATCTCAGCAAAGACGAGGCCGAGA
ACATCAATCGACGGAAAGGAGATAAACGTTGCGCCCCCCCAGAATACTAGCCGTCGTCT
TAAGCCACACTCCTTCTCACCCTTCCCTCCTCCTGCCCATTCTCTCCCTGAACCCGCACA
ACTCCAGGAGCAGCTGTGACTCC*TCCTGCCTCTCCTCTTCTCCTCGTCGTCCACACGTAGCA*
GGTGCTCGCTGTCGGTCAACAGTTTGAGCCTTCCCTCAGCAGCCGGCACCAAGCCTCCGAAG
CGCTTGGCACCACAGTACGAGGCCAAGCACCGCCTAACGCCCTTCTGCCAGCCGCTGACCTT
GTACCCCTCCCCCTCCTCCAGCACAGGTTCCTCGAGACTTTGCAAGCACCGACCGACGTCGA
*CAAGACACAAACACAAAACCATCCGGGAACCTCGCGCGCAACCGGCACA***ATGGCGCGCCG**
TGAGTCTCTGTCTGAGATTCGCGCCGCCAACCCCGAGCTCTTCCTGACGGGCAAC
ATCATCTCGGCGACCTTCAACATCCCCCATGCTGTGACATACCACAAGGGCGGTGC
TTGGgtgagtgcattttcctggctgggcatcgctttgaggacgtcctaagcttgtctgtgcttgaagcacttggcactggtcgaggcgagatca
aggcagacgagctttgtttgattttctgagacatctccctcctccctctgcgttgattgcctcattctgctgctctcctccgtcgccccccgcccgtggga
agccatcattctgactgactttggctgcgcag**GATCTGAAGCCCCGCCGTGGCCAGTCGGCCCTCATCG**
ACTCCTTCGCCTATCTCTCGTCCGACGCGACGCCCTGGAATCACACAGTCGTGGCC
TGGACAGGCGAAATTGCCAACCCCGACAACGACCCGCTGTCTCCTCCAGATACCC
CCTCAGCCGCGGCCACCACCATCGGTGCTGCCAACTCGCTGTCGGCTCCCGTCCC
GATCGATGCCACCACTCGGCTGCCCACGCCTCCCCCAGTCGACGGGCTCTGGATC
CCCAAGGCAGACCAGACGCGGCTGGAGCACCAGCTGTCCCACAGCACAACCATTC
GCACCGTGCCTGTCTGGCTGGCTGACCAGAGCGAGGCCACCGATGATGGCATCAT
GCTCAAGGACCAGGCTCGCTGGAGGCGCTATGCTGAGCACGATCTCTACACACTC

TTCCACTACAAGCAGCACGAGCCCACGGATGGCCGCAAGGAGCGGGCGCAGTGG
GCCGACTACTACCGCATGAACCAGAAGTTCGCCAACAAGATCATTGAGATCTACAA
GCCTGGTGACGTTGTCATCGTTCATGATTACTATCTGATGCTGCTGCCCAGCATGC
TCCGCCAGCGGGCTCCCAAGATGTACATCTCCTTCTTCCTCCACTCGCCCTTCCCC
AGCAGCGAGTTCCTCCGTTGCCTGCCCCGCCGCAAGGAGGTGCTTGAGGGTGTCC
TGGGCGCCAATCTCGTGGGCTTCCAGTCTTACAGCTACTCGCGCCACTTCCTCAGC
TGCTGCACCCGCATCCTCGGTTTCCCCTCTGACACTCTTGGCATCGACGCCTATGG
CTCCAGGGTGCAGGTCGGAGTGTTTCCCATTGGCATCGACGCCGCCAAGGTGGAG
ACCGCCGCCTGGGCGGACACCGTCAACGAGAAGCACGCTGCCGTCCTGAAGATGT
ACGAAGGCAAGAAGATCATCGTCGGCCGAGATCGTTTGGACAGCGTGAGGGGCGT
TGCTCAAAAGCTGCAGGCGTTTGAGCGCTTCCTGGAGCTGTACCCTCACTGGCGC
GAGAAGGTGGTCCTGATCCAGGTCACGTCGCCCACCAGCATCGAGGCTGAGAAGG
GTGACCCGGAGAACAAGAACGCCAGTCGAGTCAACGAGCTCATCACCAAGATCAA
TGGCGAATACGGCAGTCTCGGCTTTTCGCCTGTGCAGCACTACCCCCAGTACCTCA
GCCAGGCCGAGTACTTTGCCTTGCTCCGGGCCGCAGACATTGGCCTCATCACCTC
GGTGCGAGATGGAATGAACACGACAAGTCTCGAGTACGTTGTCTGCCAGAAGGAT
AGCAACGGCCCACTCATTCTCTCCGAGTTCAGCGGCACCGCGGGTAGTCTCCGCG
ACGCCATCCACATCAACCCCTGGGATCTGACGGGCGTGGCGGAAAAGATCAACGC
GGCTCTGGAGATGTCTGAGGAGGAGCGCGTCAAGATGCAGACAAGCCTCTACACC
CACGTCACGACGCAGAATGTCCAGTCGTGGATCACCAAGTTCATCCGCAAGTTCCA
CGCGGCGCTGAGCGAGACCAACTCAGTCACATCGACACCCCTTCTCGACCGCGCG
CTCTTGCTGTCCCGTTACCGCGCCGCCAAGAAGCGCCTGTTCATGTTTGACTACGA
CGGCACCCTCACGCCCATTGTGCGCGAACCGAGCGCCGCTGTTCCTTCGGAGCGC
ATCATCCGCTACCTGCAGTCGCTTGCATCGGACCCCAGGAACGCGGTCTGGATCA
TCTCTGGCCGAGACCAAGAGTTCCTTCAGCAACATCTCGGCCACATCCCCCGGATC
GGATTCTCTGCCGAGCATGGTAGTTTCATGCGAGACCCCGGCAGCGACGAGTGGG
TTAACCTGGCAGAGAAGTTTGACATGGGCTGGCAGGCAGAGGTCATGGAGGTGTT
CCAGCGTTACACGGACAAGGTTCCAGgtgagttgctgtctatcccgagtttgagttgcctcaaagaacaatcctatcac
gggttaaggcaagacaagacagaaagcagaagctaacacactatccttagGTTCCTTCATCGAGCGAAAACGCTGC
GCCCTGACCTGGCATTATCGACTGGCCGAGCCGGAGCAAGGCCTCCACATGTCAC
GCGAGTGTCACCGAGAGCTCGAGACCGGCATTGCCCAGCGATGGGAGGTCGAGGT
GATGCCTGGCAAGGCCAACATCGAGGTGCGCCCTACGTTCATCAACAAGGGTGAG
ATCGCCAAGCGACTGGTGGCCACTTATCACAACCCGGGAGCCGCCCCGACCGACA
AGGACCCTTACCCCGGAAAGATTGAGTTTGCTCTCTGCTCTGGAGACGACTTTACC
GACGAGGACATGTTCCGCAGCCTCAACGGAGCATGTGGCACGATCCTGGAAGACC
AGCACGTCTTCACCGTCACTGTGGGAGCCAGCACCAAGGTGACGCTGGCCAAATG

GCATCTCCTGGAGCCCGAGGACGTGATTGAGTGCGTGGGTCTGCTGGCTGGTGCT GGCGACCCGGCCAGCCTCGAGCGTGTTGGAGAGGTGAACCTGGCCGCTTTGAGCC AGGTGGAGGGTCACATTCCCGCCGAGGAGCTGTAA*AGGACATTCGTTTGTCCCAGTGC TTTCAGGCGTGGAATGGCCTCTTGATGGGAAACCACGAGGCTTTCTCCAGATGCTGAACTTGA GTGTTTGGCAAAGTCTGGGGGTGATTCTTTTCCTTTTGACGACTTGCACATTTGAGATGAAGAG AGCGAAAACGGACGCATAGAACGGTAATAGAAACGAAGGATGGCGCGTGGCGTACGGGCTA GTAATGACCTTGTGGCACAGAATCTGTGATAAAGAGTAAAAAAAAAGGAAACAAAGGCCCCCC CGAGAGACAGAAAAATATCAAAAGAAGAAG***AAAAAAAAAAAAAAAAATCACGGCAGCAAAT ACGGGACAAAAAGGGGATTGCGGACGCTCTGGTAATCTGACATGGAAAGTTCCGCGAT GAACTGGCAAAGTATACAGAGTAGCATCAGTCGCGATTCATTTAGCGTGGAGAATAAG ACAAAGTTTAACCTCGACTTGTGTTGCACTGCAGCTGAGTTTCAATCTTGAATC

C. Generation of strain Morph 77B7 Δ*tps2*

[98] Strain Morph TrGA 77B7 Δ*pyr2* was transformed with the *tps2* disruption cassette using PEG-mediated transformation, and plated on Vogel's minimal medium containing sorbitol to select for candidates based on uridine prototrophy acquired by the *pyr2* marker. Individual transformants were isolated and propagated by transfer to Vogel's minimal medium. PCR analysis was used to identify transformants in which the *tps2* disruption cassette integrated at the *tps2* locus by homologous recombination. Homologous integration of the Δ*tps2* disruption cassette at the *tps2* locus was verified by amplifying DNA fragments of the expected sizes using two primer pairs. Primer pair RPG408 and RPG253 amplified a DNA fragment starting outside the 5′ end of the disruption cassette region and ending within 3′ region. Primer pair RPG409 and RPG273 amplified a DNA fragment starting within the 5′ region of the disruption cassette and ending outside the 3′ end of the disruption cassette region. The generated strain with confirmed homologous integration of the *tps2* disruption cassette was named Morph 77B7 Δ*tps2*.

Table 1. Primers used in example 1

Primer	Sequence	SEQ ID NO:
RPG398	5′- CCCCTCCGGAGCGTGGGTGGTCACGTGATACTG -3′	8
RPG401	5′- GGCCTCTAGACCGGAGACGCACGATTCAAGATT -3′	9
RPG408	5′- GTCGCGATCGATGGCTGATTGTC -3′	10
RPG253	5′- TTCCTGACAACGAGGACATCTCAAGCTGT-3′	11
RPG409	5′- TCACCGGTCGGATTCGCCTAGTT -3′	12
RPG273	5′- GGTCAGTAACATAGCAGGACTATAGTAGTGGCTCAC-3′	13

[99] Morph 77B7 Δ*tps2* obtained from the above procedure was observed to have altered morphology in liquid culture having shorter filaments than the Morph 77B7 parent. In liquid medium, cultures containing the Morph 77B7 Δ*tps2* mutant also showed a higher level of dissolved oxygen during growth compared to cultures containing the Morph 77B7 parent (Table 2).

[100] Strains Morph 77B7 and Morph 77B7 Δ*tps2* were grown under similar conditions in submerged (liquid) culture, and their growth phenotypes were compared. Briefly, spores of each strain were added separately to 500-mL of minimal medium in a 3-L flask with both side and bottom baffles. After autoclaving for 30 minutes, sterile 60% glucose was added to a final concentration of 27.5 g/L. The cultures were grown for 48 hrs at 34°C in a shaking incubator.

[101] After 48 hrs, the contents of each flask were added separately to 14-L fermentors containing 9.5 L of medium containing 4.7 g/L KH_2PO_4, 1.0 g/L $MgSO_4 \cdot 7 \cdot H_2O$, 4.3 g/L $(NH_4)_2SO_4$ and 2.5 mL/L of the same trace element solution. These components were heat sterilized together at 121°C for 30 minutes. A solution of 60% glucose and 0.48% $CaCl_2 \cdot 2 \cdot H_2O$ was separately autoclaved, cooled, and added to the fermentor to a final concentration of 75 g/L glucose and 0.6 g/L $CaCl_2 \cdot 2 \cdot H_2O$. The medium was adjusted to pH 3.5 with 28% NH_3 and the temperature was maintained at 34°C for the entire growth period.

[102] A dissolved oxygen (DO) probe was calibrated to 100% when there was no added pressure in the headspace (*i.e.*, 0 bar gauge, 1 bar absolute). The pressure in the headspace was then set to 0.7 bar (gauge), after which the oxygen probe read 170% before the seed culture was added. The fermentor contained two, four-blade turbines that provided mixing via a variable speed motor that was initially set at 500 rpm.

[103] As the cultures grew, DO content levels dropped, at least partly as a consequence of the increased viscosity of the broth due to the proliferation of filamentous fungus hyphae. When DO content level fell below 40%, the agitation rate was increased to maintain the DO content level at 40%. Upon reaching 750 rpm agitation, DO content level would be allowed to drop below 40%. If the DO content level did not fall below 40%, then it was unnecessary to increase the agitation rate during the fermentation run, and the initial agitation rate was higher than necessary. When the glucose was completely consumed, the amount of biomass produced in each fermentor was measured, and found to be substantially the same for both strains.

[104] The DO content level in each fermentor at a given level of agitation, and the amount of agitation required to maintain a given DO content level are indirect measures of the viscosity of the different broths, due to the different strain growth phenotypes. Although it would be ideal to vary only one variable (*e.g.*, DO content or agitation) and measure the other, it is desirable to

prevent the DO content level from falling below 40% to ensure the production of sufficient biomass in each fermentor, thereby permitting a more meaningful comparison among the growth characteristics of the different strains.

[105] Generally, where it is necessary to increase the agitation rate to maintain a target DO content level, the amount of agitation can be estimated by the amount of power supplied to the motor driving the fermentor turbine, which provides a metric that correlates with the viscosity of the broth. In particular, the extra power required to agitate the suspended culture is proportional to the agitation rate raised to the 3rd power.

[106] As shown in Table 2, Morph 77B7 Δ*tps2* has a reduction in broth viscosity compared to the parent Morph 77B7. At the end of the batch growth phase, when all the glucose has been consumed, both strains had achieved a similar biomass concentration. To arrive at the end of the batch growth phase, the Morph 77B7 control strain saw agitation increased to 616 rpm and then saw DO content level drop down to as low as 40%. The strain Morph 77B7 Δ*tps2* did not require as much energy to achieve the same biomass concentration. Agitation rate never increased above 500 rpm and the % DO never dropped below 110.

Table 2. Broth viscosity of Morph 77B7 compared to Morph 77b7 Δ*tps2*

Strain	Deletion	DO (%)	Agitation (rpm)	Biomass (g/kg)	CER (mmol/L/hr)
Morph 77b7	none	40	616	38	141
Morph 77b7Δ*tps2*	*tps2*	110	500	41	94

Example 2. Additive effect produced by altering at least one of Sfb3, Seb1, Mpg1, Gas1, and Crz1 production

A. Viscosity reduction in disrupted *shf3*

[107] The *Sfb3* gene (also known as *Lst1*) has previously only been characterized in budding yeast (*i.e.*, *Saccharomyces cerevisiae*), where it encodes a protein associated with the COPII protein coat surrounding transport vesicles that carry proteins from the endoplasmic reticulum to the Golgi apparatus. *Sfb3*, as well as *Sfb2*, are homologs of *Sec24*, all of which genes are involved with packaging specific cargo proteins into the vesicles.

[108] As shown in Table 3, disrupting the *sfb3* gene from strain 29-9 Δ*sfb3* resulted in a strain having a reduction in the highest agitation rate required to maintain the dissolved oxygen at 40% at the end of the growth phase. Under these growth conditions, the original strain, 29-9, required 2.6 times more power than either the 70H2 (chemically mutagenized 29-9) or 29-9 Δ*sfb3* strains in order to maintain a DO of 40% and produce the amount of biomass. Strains 70H2 and 29-9 Δ*sfb3* had similar viscosity properties, and produced similar levels of a protein of interest (TrGA) in suspended culture, demonstrating that a reduced viscosity growth phenotype

can be imparted to a filamentous fungus by disrupting the *sfb3* gene. Alterations in the Sfb3 protein resulting in alterations in viscosity are further described in PCT Publication No. WO 2012/027580 A1, published 1, March 2012, filed as International Application No. PCT/US2011/049164, filed 25, August 2011.

Table 3. Agitation rate required to maintain a DO of 40% at the end of the growth phase

Strain	Agitation rate	Relative power increase from baseline at 500 rpm
29-9	750	$(750/500)^3 = 3.4$
70H2	539	$(539/500)^3 = 1.3$
29-9 Δ*sfb3*	540	$(540/500)^3 = 1.3$

B. **Viscosity reduction in disrupted *seb1***

[109] Seb1 from *Trichoderma atroviride* is a STRE-element-binding protein, and the *seb1* gene is believed to be an orthologue of the yeast *msn2/4* gene and the *Aspergillus nidulans msnA* gene. Notably, the *seb1* gene cannot complement the *msn2/4* gene in yeast, so is probably not a functional homologue (Peterbauer, C. *et al.* ((2002) *Molecular Genetics and Genomics* 268:223-31). Seb1 is involved with but not essential in the osmotic stress response but has been found to be associated with altered morphology, particularly those giving rise to a low viscosity phenotype when *seb1* is disrupted.

[110] As shown in Table 4, deletion of the *seb1* gene from strain Morph1/1 Δ*ku80* resulted in a strain having a reduction in broth viscosity. At the end of the batch growth phase, when all the glucose has been consumed, both strains had achieved a similar biomass concentration. To get there, the control strain saw agitation increased to the maximum of 750 rpm and then saw DO drop down to as low as 29%. The *seb1* deleted strain did not require as much energy to achieve the same biomass concentration. Agitation rate was never increased above 500 rpm and DO dropped only as low as 55%.

Table 4. Broth viscosity in Morph1/1 Δku80 with and without the *seb1* gene

Strain	**Deletion**	**DO (%)**	**Agitation (rpm)**	**Biomass (g/kg)**	**CER (mmol/L/hr)**
Morph1.1Δ*ku80*	none	29	750	38	157
Morph1.1Δ*ku80*,Δ*pyr4*,Δ*seb1*	seb1	55	500	37	138

C. **Viscosity reduction in disrupted *mpg1***

[111] The *mpg1* gene encodes a GTP:alpha-D-mannose-1-phoshate guanyltransferase. Over-expression of the *mpg1* gene increases GDP-mannose levels, which can play a major regulatory role in early stages of protein glycosylation.

[112] As shown in Table 5, MAGI 10-8g, the *mpg1* deletion variant strain, has a reduction in broth viscosity compared to the parent MAGI. At the end of the batch growth phase, when all the glucose has been consumed, both strains had achieved a similar biomass concentration. To get there, the MAGI control strain saw agitation increased to the maximum of 750 rpm and then saw DO drop down to as low as 35%. The strain MAGI 10-8g did not require as much energy to achieve the same biomass concentration. Agitation rate was increased slightly to 513 rpm when the % DO dropped to 40%. Protein production was not adversely affected in MAGI 10-8g compared to MAGI (not shown).

Table 5. Broth viscosity of MAGI compared to MAGI 10-8 g

Strain	Deletion	DO (%)	Agitation (rpm)	Biomass (g/kg)	CER (mmol/L/hr)
MAGI	none	35	750	39	125
MAGI 10-8g	*mpg1*	40	513	40	128

D. **Viscosity reduction in disrupted *gas1***

[113] The Gel/Gas/Phr family of fungal β(1,3)-glucanosyltransferases plays an important role in cell wall biogenesis by processing the main component β(1,3)-glucan (Popolo *et* al., 2008). *gas1* (PID 22914) encodes a beta-1,3-glucanosyltransferase that is a GPI (and/or glucan)-anchored protein capable of breaking and joining beta-1,3-glucans. There are multiple paralogs in many fungal genomes including *T. reesei,* which has five. Separate studies have shown that mutation of the *gas1* gene (or the *gel1* gene as it is known in *Aspergillus fumigatus*) affects fungal cell wall structure, and can lead to morphological changes as well as hypersensitivity to Calcofluor White, Congo Red and sodium dodecyl sulfate (Schirawski, J. *et al.* 2005, Mouyna, I. *et al.* 2005).

[114] A *Trichoderma reesei* Morph strain was deleted for four major cellulase genes, including *cbhI, cbhII, egII,* and *egIV,* which makes it particular suitable for expressing other proteins in the absence of or in reduced cellulase background. *See,* WO 05/001036. The Morph strain had been previously transformed with a native *Trichoderma* glucoamylase gene (TrGA) under control of the CBH1 promoter, using *amdS* as a marker. A transformant containing two tandem copies of

glucoamylase (TrGA 29-9) was subsequently isolated, and random chemical mutagenesis was used to produce a mutant (77B7). A spontaneous *pyr2* mutant derivative was subsequently isolated by *5-fluoro*-orotic acid (FOA) selection. The *Trichoderma reesei gas1* (PID 22914) was deleted from mutant Morph 77B7.

[115] Strain Morph TrGA 77B7 Δ*pyr2* was transformed with a *gas1* disruption cassette using PEG-mediated transformation, and plated on Vogel's minimal medium containing sorbitol to select for candidates based on uridine prototrophy acquired by the *pyr2* marker. As shown in Table 6, Morph 77B7 Δ*gas1* has a reduction in broth viscosity compared to the parent Morph 77B7. At the end of the batch growth phase, when all the glucose has been consumed, both strains had achieved a similar biomass concentration. To arrive at the end of the batch growth phase, the Morph 77B7 control strain saw agitation increased to 616 rpm and then saw DO content level drop down to as low as 40%. The strain Morph 77B7 Δ*gas1* did not require as much energy (*i.e.*, rpm increase in agitation) to achieve the same biomass concentration. Agitation rate never increased above 500 rpm and the % DO never dropped below 115. Protein production was not adversely affected in Morph 77B7 Δ*gas1* compared to Morph 77B7 (data not shown).

Table 6. Broth viscosity of Morph 77B7 compared to Morph 77b7 Δ*gas1*

Strain	Deletion	DO (%)	Agitation (rpm)	Biomass (g/kg)	CER (mmol/L/hr)
Morph 77b7	none	40	616	38	141
Morph 77b7Δ*gas1*	*gas1*	115	500	39	147

E. **Viscosity reduction in disrupted *crz1***

[116] In fungi, calcineurin mediated Ca^{2+} signaling has been shown to be required for growth, development, and virulence in many organisms. It is necessary for adaption to diverse environmental conditions including high cation levels and alkaline pH. The gene *crz*1 encodes a calcineurin-regulated transcription factor. The Crz1p transcription factor is dephosphorylated when the phosphatase calcineurin is activated by Ca^{2+}/calmodulin. It then enters the nucleus and induces expression of a number of genes, many of which encode proteins with cell wall-related functions (Yoshimoto *et al.*, 2002; Lagorce *et al.*, 2003; Garcia *et al.*, 2004; Karababa *et al.*, 2006; Pardini *et al.*, 2006, Munro, C. *et al.* 2009). Deletion of *crz*1 or a homolog can result in alterations in hyphal morphology (Kothe, G. and Free, S. 1998, Prokisch, H. *et al.* 1997).

[117] A *Trichoderma reesei* Morph strain was prepared as described above. The *Trichoderma reesei crz1* (PID 36391) was deleted from mutant Morph 77B7. Strain Morph TrGA 77B7 Δ*pyr2* was transformed with the *crz1* disruption cassette using PEG-mediated transformation, and plated on Vogel's minimal medium containing sorbitol to select for candidates based on uridine prototrophy acquired by the *pyr2* marker. As shown in Table 6, Morph 77B7 Δ*crz1* has a reduction in broth viscosity compared to the parent Morph 77B7. At the end of the batch growth phase, when all the glucose has been consumed, both strains had achieved a similar biomass concentration. To arrive at the end of the batch growth phase, the Morph 77B7 control strain saw agitation increased to 616 rpm and then saw DO content level drop down to as low as 40%. The strain Morph 77B7 Δ*crz1* did not require as much energy to achieve the same biomass concentration. Agitation rate never increased above 500 rpm and the % DO never dropped below 100.

Table 6. Broth viscosity of Morph 77B7 compared to Morph 77b7 Δ*crz1*

Strain	**Deletion**	**DO (%)**	**Agitation (rpm)**	**Biomass (g/kg)**	**CER (mmol/L/hr)**
Morph 77b7	none	40	616	38	141
Morph 77b7Δ*crz1*	*crz1*	100	500	39	120

[118] Although the foregoing compositions and methods have been described in some detail by way of illustration and examples for purposes of clarity of understanding, it will be apparent to those skilled in the art that certain changes and modifications can be made. Therefore, the description should not be construed as limiting the scope of the invention, which is delineated by the appended claims.

[119]

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CLAIMS

What is claimed is:

1. A variant strain of filamentous fungus derived from a parental strain, the variant strain including a genetic alteration comprising a disruption of the *tps2* gene that causes cells of the variant strain to produce a reduced amount of functional Tps2 protein compared to cells of the parental strain, wherein the cells of the variant strain produce during aerobic fermentation in submerged culture a cell broth that (i) requires a reduced amount of agitation to maintain a preselected dissolved oxygen content compared to the cells of the parental strain, and/or (ii) maintains an increased dissolved oxygen content at a preselected amount of agitation, compared to the cells of the parental strain, wherein the variant strain further comprises a disruption of at least one gene selected from the group consisting of the *sfb3* gene, the *seb1* gene, the *mpg1* gene, the *gas1* gene and the *crz1* gene.

2. The variant strain of claim 1, wherein the genetic alteration comprising disruption of the *tps2* gene is the result of deletion of all or part of the *tps2* gene.

3. The variant strain of claim 1, wherein the genetic alteration comprising disruption of the *tps2* gene is the result of deletion of a portion of genomic DNA comprising the *tps2* gene.

4. The variant strain of claim 1, wherein the genetic alteration comprising disruption of the *tps2* gene is the result of mutagenesis of the *tps2* gene.

5. The variant strain of any one of claims 1-4, wherein disruption of the *tps2* gene is performed using site-specific recombination.

6. The variant strain of any one of claims 1-5, wherein disruption of the *tps2* gene is performed in combination with introducing a selectable marker at the genetic locus of the *tps2* gene.

7. The variant strain of any one of claims 1-6, wherein the variant strain does not produce functional Tps2 protein.

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8. The variant strain of any one of claims 1-6, wherein the variant strain does not produce Tps2 protein.

9. The variant strain of any one of claims 1-8, comprising a disruption of the *sfb3* gene.

10. The variant strain of any one of claims 1-8, wherein the variant strain comprises a disruption of at least one gene selected from the group consisting of the *sfb3* gene, the *seb1* gene, the *mpg1* gene, and the *gas1* gene.

11. The variant strain of any one of claims 1-10, wherein the variant strain produces the same amount of, or more, protein per unit amount of biomass as the parental strain.

12. The variant strain of any one of claims 1-11, wherein the filamentous fungus is a Pezizomycotina species.

13. The variant strain of any one of claims 1-12, wherein the filamentous fungus is a *Trichoderma* spp.

14. The variant strain of claim 13, wherein the filamentous fungus is *Trichoderma reesei*.

15. A method for producing a variant strain of filamentous fungus cells comprising: introducing a genetic alteration into a parental strain of filamentous fungal cell, which genetic alteration comprises disrupting the *tps2* gene using genetic manipulation to reduce or prevent the production of functional Tps2 protein compared to the cells of the parental strain wherein disrupting the *tps2* gene is performed in combination with disruption of at least one gene selected from the group consisting of the *sfb3* gene, the *seb1* gene, the *mpg1* gene, the *gas1* gene and the *crz1* gene, thereby producing a variant filamentous fungal cell that produces during aerobic fermentation in submerged culture a cell broth that (i) requires a reduced amount of agitation to maintain a preselected dissolved oxygen content, compared to the cells

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of the parental strain, and/or (ii) maintains an increased dissolved oxygen content at a preselected amount of agitation, compared to the cells of the parental strain.

16. The method of claim 15, wherein the genetic alteration comprises deleting the *tps2* gene in a parental filamentous fungal cell using genetic manipulation.

17. The method of claim 15 or 16, wherein the genetic alteration is performed using site-specific genetic recombination.

18. The method of any one of claims 15-17, wherein disrupting the *tps2* gene is performed in combination with introducing a selectable marker at the genetic locus of the *tps2* gene.

19. The method of any one of claims 15-18, wherein disrupting the *tps2* gene is performed in combination with disrupting the *sfb3* gene to reduce or prevent the production of functional Sfb3 protein compared to cells of the parental strain.

20. The method of any one of claims 15-18, wherein disrupting the *tps2* gene is performed in combination with disruption of at least one gene selected from the group consisting of the *sfb3* gene, the *seb1* gene, the *mpg1* gene, and the *gas1* gene.

21. The method of any one of claims 15-18, wherein disrupting the *tps2* gene is performed in combination with disruption of at least two genes selected from the group consisting of the *sfb3* gene, the *seb1* gene, the *mpg1* gene, and the *gas1* gene.

22. The method of any one of claims 15-21, wherein the variant strain produces the same amount of, or more, protein per unit amount of biomass as the parental strain.

23. The method of any one of claims 15-22, wherein the filamentous fungus is a Pezizomycotina species.

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24. The method of any one of claims 15-23, wherein the filamentous fungus is a *Trichoderma* spp.

25. The method of claim 24, wherein the filamentous fungus is *Trichoderma reesei*.

26. The method of any one of claims 15-25, wherein the variant strain further comprises a gene encoding a protein of interest for expressing the protein of interest in filamentous fungus cells.

27. The method of claim 26, wherein the gene encoding the protein of interest is present in the parental strain prior to introducing the genetic alteration that reduces or prevents the production of functional Tps2 protein.

28. The variant strain of any one of claims 1 to 14, wherein the variant strain further comprises a gene encoding a protein of interest for expressing the protein of interest in filamentous fungus cells.

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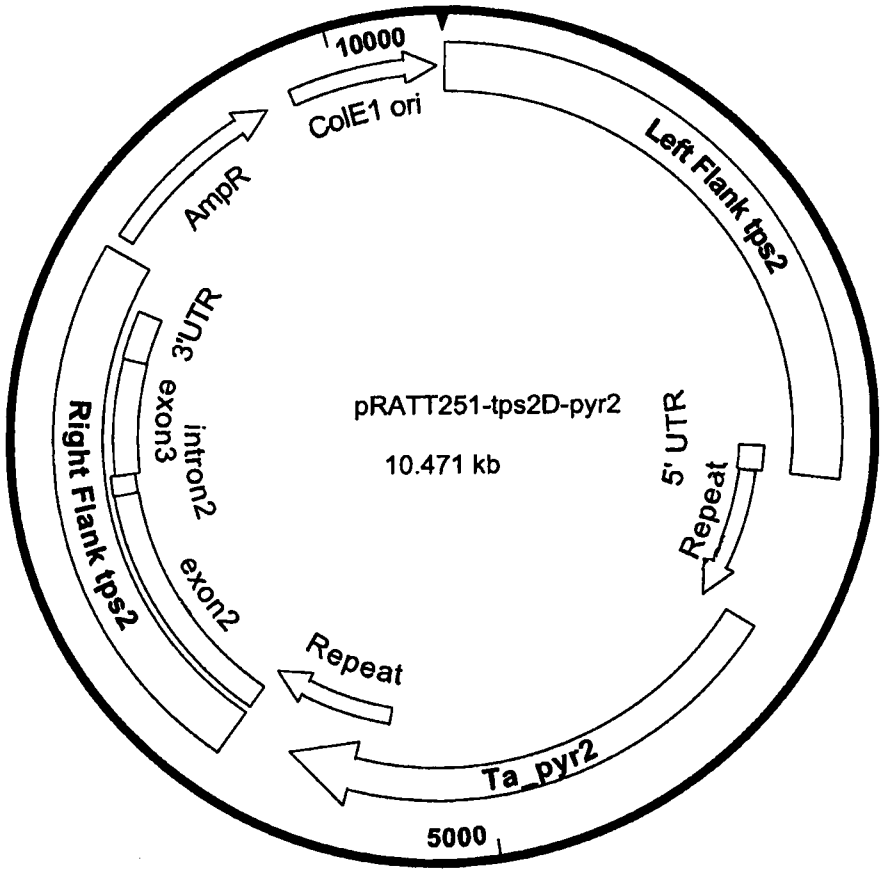
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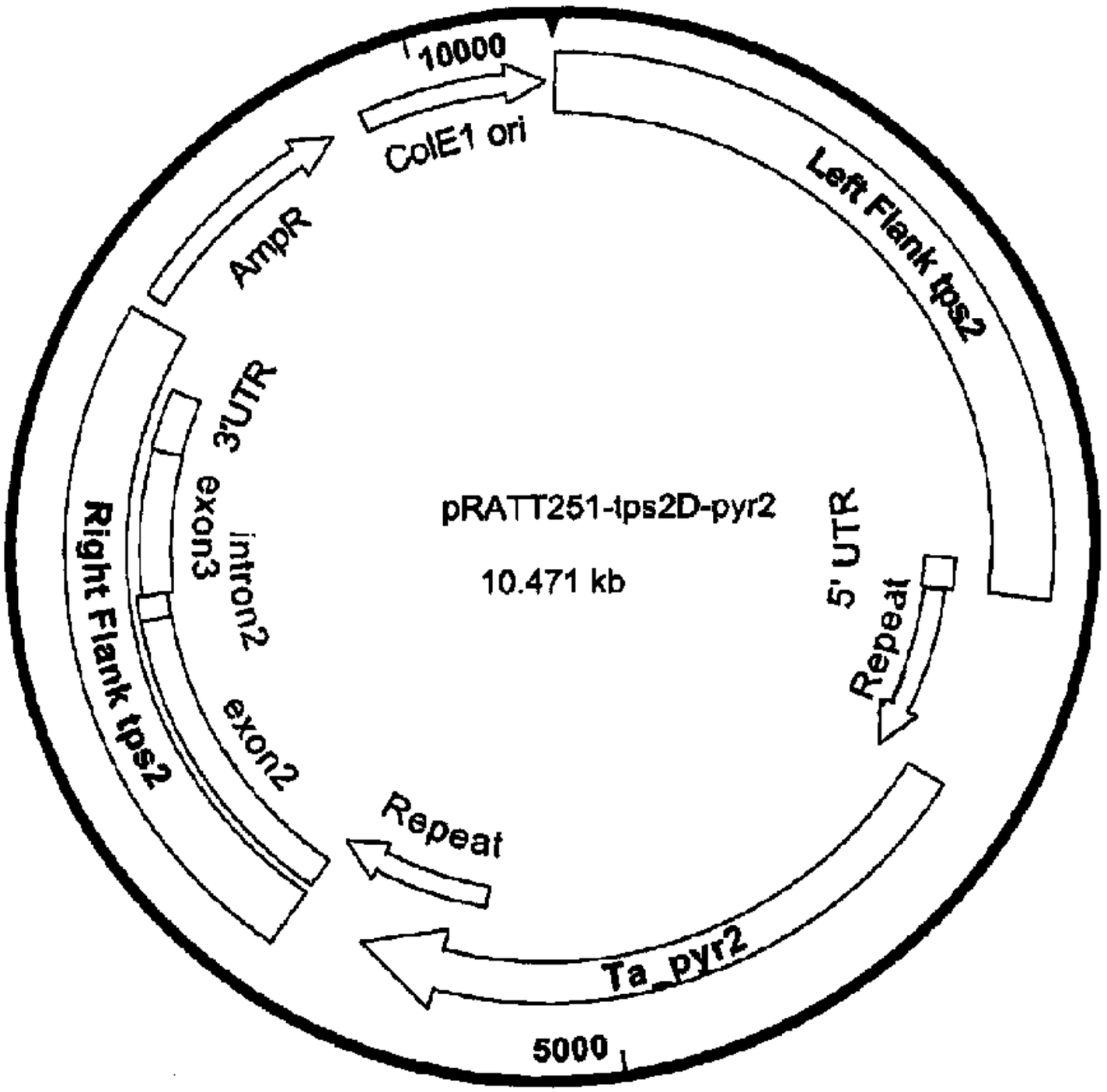
FIG. 1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