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(54) Title: METHODS FOR PRODUCING HETEROLOGOUS POLYPEPTIDES IN MUTANTS OF TRICHODERMA

(57) Abstract: The present invention relates to mutants of a parent *Trichoderma* strain, comprising a polynucleotide encoding a polypeptide and one or more genes selected from the group consisting of a peptaibol synthetase gene, a paracelsin synthetase gene, a first terpene cyclase gene, a second terpene cyclase gene, and a third terpene cyclase gene, wherein one or more of the genes are modified rendering the mutant strain deficient in the production of one or more of the enzymes selected from the group consisting of a peptaibol synthetase, a paracelsin synthetase, a first terpene cyclase, a second terpene cyclase, and a third terpene cyclase compared to the parent *Trichoderma* strain when cultivated under identical conditions. The present invention also relates to methods of producing a polypeptide in such mutants and methods for producing such mutants.



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METHODS FOR PRODUCING HETEROLOGOUS POLYPEPTIDES IN MUTANTS OF *TRICHODERMA*

Reference to a Sequence Listing

5 This application contains a Sequence Listing in computer readable form, which is incorporated herein by reference.

Background of the Invention

10 Field of the Invention

The present invention relates to *Trichoderma* mutant strains deficient in the production of secondary metabolites, methods of obtaining the *Trichoderma* mutant strains, and methods of producing heterologous polypeptides in the *Trichoderma* mutant strains.

15 Description of the Related Art

Trichoderma has been shown to be useful as a host cell for the recombinant production of polypeptides having biological activity (WO 96/00787 and WO 97/26330). *Trichoderma* hosts with the desirable traits of increased protein expression and secretion may not necessarily have the most desirable characteristics for successful fermentation. The fermentation may not be optimal because of the production of biological substances detrimental to the production, recovery, or application of a particular polypeptide of interest.

20 Peptaibols are synthesized by large multidomain enzymes known as non-ribosomal peptide synthetases that assemble compounds from a range of precursors (including nonproteinogenic amino acids and hydroxy or carboxyl acids), which can be N-methylated, acylated, reduced, or epimerized (Marahiel *et al.*, 1997, *Chem. Rev.* 97: 2651–2674; Zocher and Keller, 1997, *Adv. Microb. Physiol.* 38: 85–131). The synthetases have a modular structure in which each module is a semiautonomous unit that recognizes, activates, and modifies a single residue of the final peptide. Each module can be further partitioned into distinct adenylation, thiolation, and condensation domains, which together represent a minimal repeating unit of such a synthetase (Stein *et al.*, 1996, *Journal of Biological Chemistry* 271: 15428-15435).

30 Mukherjee *et al.*, 2012, *Microbiology* 158: 35-45, describe secondary metabolism in *Trichoderma*. Mukherjee *et al.*, 2011, *Journal of Biological Chemistry* 286: 4544-4554, disclose two classes of new peptaibols synthesized by a single non-ribosomal peptide synthetase of *Trichoderma virens*. Kubicek *et al.*, 2011, *Genome Biology* 12: R40, describe a comparative genome sequence analysis underscoring mycoparasitism as the ancestral life

style of *Trichoderma*. Neohof *et al.*, 2007, *Microbiology* 153: 3417-3437, describe intact-cell MALDI-TOF mass spectrometry analysis of peptaibol formation by the genus *Trichoderma*.

Mukherjee *et al.*, 2012, *supra*, also describe that several *Trichoderma* spp. strains produce terpenoids synthesized from five-carbon isopentenyl units by the action of terpene cyclases. The *T. virens* genome harbors six terpene cyclases, while *T. atroviride* and *T. reesei* have three each.

The present invention relates to improved *Trichoderma* hosts that combine the capacity for expression of commercial quantities of a polypeptide of interest while being deficient in the production of peptaibol(s) and terpene(s) that can complicate production, recovery, or application of the polypeptide.

Summary of the Invention

The present invention relates to mutants of a parent *Trichoderma* strain, comprising a polynucleotide encoding a heterologous polypeptide and one or more (*e.g.*, several) genes selected from the group consisting of a peptaibol synthetase gene, a paracelsin synthetase gene, a first terpene cyclase gene, a second terpene cyclase gene, and a third terpene cyclase gene, wherein one or more of the genes are modified rendering the mutant strain deficient in the production of one or more (*e.g.*, several) enzymes selected from the group consisting of a peptaibol synthetase, a paracelsin synthetase, a first terpene cyclase, a second terpene cyclase, and a third terpene cyclase compared to the parent *Trichoderma* strain when cultivated under identical conditions.

The present invention also relates to methods of producing a heterologous polypeptide, comprising:

(a) cultivating a mutant of a parent *Trichoderma* strain in a medium for the production of the heterologous polypeptide, wherein the mutant strain comprises a polynucleotide encoding the heterologous polypeptide and one or more (*e.g.*, several) genes selected from the group consisting of a peptaibol synthetase gene, a paracelsin synthetase gene, a first terpene cyclase gene, a second terpene cyclase gene, and a third terpene cyclase gene, wherein one or more of the genes are modified rendering the mutant strain deficient in the production of one or more (*e.g.*, several) enzymes selected from the group consisting of a peptaibol synthetase, a paracelsin synthetase, a first terpene cyclase, a second terpene cyclase, and a third terpene cyclase compared to the parent *Trichoderma* strain when cultivated under identical conditions; and optionally

(b) recovering the heterologous polypeptide from the cultivation medium.

The present invention further relates to methods of obtaining mutants of a parent *Trichoderma* strain, comprising:

(a) modifying one or more (e.g., several) genes selected from the group consisting of a peptaibol synthetase gene, a paracelsin synthetase gene, a first terpene cyclase gene, a second terpene cyclase gene, and a third terpene cyclase gene; and

(b) identifying a mutant strain from step (a) wherein one or more of the genes are modified rendering the mutant strain deficient in the production of one or more (e.g., several) enzymes selected from the group consisting of a peptaibol synthetase, a paracelsin synthetase, a first terpene cyclase, a second terpene cyclase, and a third terpene cyclase compared to the parent *Trichoderma* strain when cultivated under identical conditions.

Brief Description of the Figures

Figure 1 shows a restriction map of pJyHi002.

Figure 2 shows a restriction map of pJyHi001.

Definitions

Allelic variant: The term "allelic variant" means any of two or more alternative forms of a gene occupying the same chromosomal locus. Allelic variation arises naturally through mutation, and may result in polymorphism within populations. Gene mutations can be silent (no change in the encoded polypeptide) or may encode polypeptides having altered amino acid sequences. An allelic variant of a polypeptide is a polypeptide encoded by an allelic variant of a gene.

Aspartic protease: The term "aspartic protease" means a protease that uses an aspartate residue(s) for catalyzing the hydrolysis of peptide bonds in peptides and proteins. Aspartic proteases are a family of protease enzymes that use an aspartate residue for catalysis of their peptide substrates. In general, they have two highly-conserved aspartates in the active site and are optimally active at acidic pH (Szecsi, 1992, *Scand. J. Clin. Lab. Invest. Suppl.* 210: 5–22). For purposes of the present invention, aspartic protease activity is determined according to the procedure described in WO 2011/075677.

cDNA: The term "cDNA" means a DNA molecule that can be prepared by reverse transcription from a mature, spliced, mRNA molecule obtained from a eukaryotic cell. cDNA lacks intron sequences that may be present in the corresponding genomic DNA. The initial, primary RNA transcript is a precursor to mRNA that is processed through a series of steps, including splicing, before appearing as mature spliced mRNA.

Coding sequence: The term "coding sequence" means a polynucleotide, which directly specifies the amino acid sequence of a polypeptide. The boundaries of the coding sequence are generally determined by an open reading frame, which begins with a start

codon such as ATG, GTG, or TTG and ends with a stop codon such as TAA, TAG, or TGA. The coding sequence may be a genomic DNA, cDNA, synthetic DNA, or a combination thereof.

Control sequences: The term “control sequences” means nucleic acid sequences necessary for expression of a polynucleotide encoding a polypeptide. Each control sequence may be native (*i.e.*, from the same gene) or foreign (*i.e.*, from a different gene) to the polynucleotide encoding the polypeptide or native or foreign to each other. Such control sequences include, but are not limited to, a leader, polyadenylation sequence, propeptide sequence, promoter, signal peptide sequence, and transcription terminator. At a minimum, the control sequences include a promoter, and transcriptional and translational stop signals. The control sequences may be provided with linkers for the purpose of introducing specific restriction sites facilitating ligation of the control sequences with the coding region of the polynucleotide encoding a polypeptide.

Deficient: The term “deficient” means a *Trichoderma* mutant strain that produces no detectable activity of one or more (*e.g.*, several) enzymes selected from the group consisting of a peptaibol synthetase, a paracelsin synthetase, a first terpene cyclase, and a third terpene cyclase compared to the parent *Trichoderma* strain when cultivated under identical conditions, or, in the alternative, produces preferably at least 25% less, more preferably at least 50% less, even more preferably at least 75% less, and most preferably at least 95% less of one or more (*e.g.*, several) enzymes selected from the group consisting of a peptaibol synthetase, a paracelsin synthetase, a first terpene cyclase, a second terpene cyclase, and a third terpene cyclase than the parent *Trichoderma* strain when cultivated under identical conditions. The level of peptaibol, paracelsin, or terpenoid produced by a *Trichoderma* mutant strain of the present invention may be determined using methods described by Neuhoef *et al.*, 2007, *Microbiology* 153: 3417-3437 or Degenkolb *et al.*, 2012, *Chemistry & Biodiversity* 9: 499-535.

Expression: The term “expression” includes any step involved in the production of a polypeptide including, but not limited to, transcription, post-transcriptional modification, translation, post-translational modification, and secretion.

Expression vector: The term “expression vector” means a linear or circular DNA molecule that comprises a polynucleotide encoding a polypeptide and is operably linked to control sequences that provide for its expression.

High stringency conditions: The term “high stringency conditions” means for probes of at least 100 nucleotides in length, prehybridization and hybridization at 42°C in 5X SSPE, 0.3% SDS, 200 micrograms/ml sheared and denatured salmon sperm DNA, and 50% formamide, following standard Southern blotting procedures for 12 to 24 hours. The carrier

material is finally washed three times each for 15 minutes using 0.2X SSC, 0.2% SDS at 65°C.

Host cell: The term "host cell" means any cell type that is susceptible to transformation, transfection, transduction, or the like with a nucleic acid construct or expression vector comprising a polynucleotide. The term "host cell" encompasses any progeny of a parent cell that is not identical to the parent cell due to mutations that occur during replication.

Isolated: The term "isolated" means a substance in a form or environment that does not occur in nature. Non-limiting examples of isolated substances include (1) any non-naturally occurring substance, (2) any substance including, but not limited to, any enzyme, variant, nucleic acid, protein, peptide or cofactor, that is at least partially removed from one or more or all of the naturally occurring constituents with which it is associated in nature; (3) any substance modified by the hand of man relative to that substance found in nature; or (4) any substance modified by increasing the amount of the substance relative to other components with which it is naturally associated (e.g., recombinant production in a host cell; multiple copies of a gene encoding the substance; and use of a stronger promoter than the promoter naturally associated with the gene encoding the substance).

Low stringency conditions: The term "low stringency conditions" means for probes of at least 100 nucleotides in length, prehybridization and hybridization at 42°C in 5X SSPE, 0.3% SDS, 200 micrograms/ml sheared and denatured salmon sperm DNA, and 25% formamide, following standard Southern blotting procedures for 12 to 24 hours. The carrier material is finally washed three times each for 15 minutes using 0.2X SSC, 0.2% SDS at 50°C.

Mature polypeptide: The term "mature polypeptide" means a polypeptide in its final form following translation and any post-translational modifications, such as N-terminal processing, C-terminal truncation, glycosylation, phosphorylation, etc.

Mature polypeptide coding sequence: The term "mature polypeptide coding sequence" means a polynucleotide that encodes a mature polypeptide having enzyme activity.

Medium stringency conditions: The term "medium stringency conditions" means for probes of at least 100 nucleotides in length, prehybridization and hybridization at 42°C in 5X SSPE, 0.3% SDS, 200 micrograms/ml sheared and denatured salmon sperm DNA, and 35% formamide, following standard Southern blotting procedures for 12 to 24 hours. The carrier material is finally washed three times each for 15 minutes using 0.2X SSC, 0.2% SDS at 55°C.

Medium-high stringency conditions: The term "medium-high stringency conditions" means for probes of at least 100 nucleotides in length, prehybridization and

hybridization at 42°C in 5X SSPE, 0.3% SDS, 200 micrograms/ml sheared and denatured salmon sperm DNA, and 35% formamide, following standard Southern blotting procedures for 12 to 24 hours. The carrier material is finally washed three times each for 15 minutes using 0.2X SSC, 0.2% SDS at 60°C.

Modification: The term “modification” means introduction, substitution, or removal of one or more (e.g., several) nucleotides in a gene or a control sequence required for the transcription or translation thereof, or gene disruption, gene conversion, gene deletion, or random or specific mutagenesis of a gene, e.g., a peptaibol synthetase gene, a paracelsin synthetase gene, a first terpene cyclase gene, a second terpene cyclase gene, a third terpene cyclase gene, or a combination thereof. The deletion of one or more (e.g., several) of the peptaibol synthetase gene, the paracelsin synthetase gene, the first terpene cyclase gene, the second terpene cyclase gene, and the third terpene cyclase gene may be partial or complete. The modification results in a decrease in or elimination (inactivation) of expression of the peptaibol synthetase, the paracelsin synthetase, the first terpene cyclase, the second terpene cyclase, the third terpene cyclase, or a combination thereof. In a preferred aspect, one or more (e.g., several) of the peptaibol synthetase gene, the paracelsin synthetase gene, the first terpene cyclase gene, the second terpene cyclase gene, and the third terpene cyclase gene are inactivated.

Non-ribosomal peptide synthetases: The term “non-ribosomal peptide synthetases” means enzymes involved in the biosynthesis of a class of non-ribosomally synthesized peptides known as peptaibols, containing non-proteinogenic amino acids (particularly alpha-aminoisobutyric acid).

Nucleic acid construct: The term “nucleic acid construct” means a nucleic acid molecule, either single- or double-stranded, which is isolated from a naturally occurring gene or is modified to contain segments of nucleic acids in a manner that would not otherwise exist in nature or which is synthetic, which comprises one or more control sequences.

Operably linked: The term “operably linked” means a configuration in which a control sequence is placed at an appropriate position relative to the coding sequence of a polynucleotide such that the control sequence directs expression of the coding sequence.

Paracelsin: The term “paracelsin” means a peptide antibiotic containing alpha-aminoisobutyric acid. Paracelsin is a peptaibol characterized by the presence of phenylalaninol as the C-terminal amino alcohol and by the specific amino acid content and sequence of peptaibols (Przybylski *et al.*, 1984, *Biomed. Mass Spectrometry* 11, 569; Brückner *et al.*, 1983, *Experientia* 39: 528-530; Brückner *et al.*, 1984, *Experientia* 40: 1189-1197; Ritieni *et al.*, 1995, *Journal of Natural Products* 58: 1745-1748; Pócsfalvi *et al.*, 1997, *Rapid Commun. Mass Spectrometry* 11: 922-930).

Paracelsin synthetase: The term “paracelsin synthetase” means a peptaibol

synthetase that catalyzes the formation of paracelsin.

Peptaibol: The term “peptaibol” means peptides characterized by short linear chain lengths (≤ 20 residues), C-terminal alcohol residues, and high levels of non-standard amino acids, principally alpha-aminoisobutyric acid, isovaline, and the imino acid hydroxyproline. Peptaibol subfamilies 1, 4, 5 and 9 have been described (Szekeres *et al.*, 2005, *Acta Microbiol. Immunol. Hung.* 52: 137-168). Subfamily 1 (SF1) comprises about half of the known structures and includes peptides ranging from 18 to 20 residues in length. All of these peptides have partial sequence identities or similarities. Subfamily 4 (SF4) is comprised of peptides of 11–14 residues, also sharing sequence similarities, but having no sequence relationship to SF1. Subfamilies 5 and 9 (SF5 and SF9) have only a few members and comprise peptides with 11 or 6 and 7 residues, respectively, again with no sequence similarities to the other subfamilies (Neuhof *et al.*, 2007, *Microbiology* 153: 3417-3427; Whitmore *et al.*, 2004, *Nucleic Acids Research* 32 D593-D594; Mukherjee *et al.*, 2011, *J. Biol. Chem.* 286: 4544-4554).

Peptaibol synthetase: The term “peptaibol synthetase” means a non-ribosomal peptide synthetase that is involved in the synthesis of one or more peptaibol(s).

Polypeptide fragment: The term “polypeptide fragment” means a polypeptide having one or more (*e.g.*, several) amino acids deleted from the amino and/or carboxyl terminus of a polypeptide, wherein the fragment has enzyme activity, *e.g.*, paracelsin synthetase, peptaibol synthetase, or terpene cyclase activity. In one aspect, a fragment contains at least 85% of the amino acid residues, *e.g.*, at least 90% of the amino acid residues or at least 95% of the amino acid residues of a peptaibol synthetase, a paracelsin synthetase, or a terpene cyclase, such as SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 8, or SEQ ID NO: 10; or homologous sequences thereof.

Sequence identity: The relatedness between two amino acid sequences or between two nucleotide sequences is described by the parameter “sequence identity”.

For purposes of the present invention, the sequence identity between two amino acid sequences is determined using the Needleman-Wunsch algorithm (Needleman and Wunsch, 1970, *J. Mol. Biol.* 48: 443-453) as implemented in the Needle program of the EMBOSS package (EMBOSS: The European Molecular Biology Open Software Suite, Rice *et al.*, 2000, *Trends Genet.* 16: 276-277), preferably version 5.0.0 or later. The parameters used are a gap open penalty of 10, a gap extension penalty of 0.5, and the EBLOSUM62 (EMBOSS version of BLOSUM62) substitution matrix. The output of Needle labeled “longest identity” (obtained using the *–nobrief* option) is used as the percent identity and is calculated as follows:

$$(\text{Identical Residues} \times 100) / (\text{Length of Alignment} - \text{Total Number of Gaps in Alignment})$$

For purposes of the present invention, the sequence identity between two deoxyribonucleotide sequences is determined using the Needleman-Wunsch algorithm (Needleman and Wunsch, 1970, *supra*) as implemented in the Needle program of the EMBOSS package (EMBOSS: The European Molecular Biology Open Software Suite, Rice *et al.*, 2000, *supra*), preferably version 5.0.0 or later. The parameters used are a gap open penalty of 10, a gap extension penalty of 0.5, and the EDNAFULL (EMBOSS version of NCBI NUC4.4) substitution matrix. The output of Needle labeled "longest identity" (obtained using the -nobrief option) is used as the percent identity and is calculated as follows:

$$(\text{Identical Deoxyribonucleotides} \times 100) / (\text{Length of Alignment} - \text{Total Number of Gaps in Alignment})$$

Subsequence: The term "subsequence" means a nucleotide sequence having one or more (e.g., several) nucleotides deleted from the 5' and/or 3' end of a polypeptide coding sequence, wherein the subsequence encodes a polypeptide fragment having enzyme activity, e.g., paracelsin synthetase, peptaibol synthetase, or terpene synthase activity. In one aspect, a subsequence contains at least 85% of the nucleotides, e.g., at least 90% of the nucleotides or at least 95% of the nucleotides of a polynucleotide encoding a peptaibol synthetase, a paracelsin synthetase, or a terpene cyclase, such as SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 7, or SEQ ID NO: 9; or homologous sequences thereof.

Subtilisin-like serine protease: The term "subtilisin-like serine protease" means a protease with a substrate specificity similar to subtilisin that uses a serine residue for catalyzing the hydrolysis of peptide bonds in peptides and proteins. Subtilisin-like proteases (subtilases) are serine proteases characterized by a catalytic triad of the three amino acids aspartate, histidine, and serine. The arrangement of these catalytic residues is shared with the prototypical subtilisin from *Bacillus licheniformis* (Siezen and Leunissen, 1997, *Protein Science* 6: 501-523). For purposes of the present invention, subtilisin-like serine protease activity is determined according to the procedure described in WO 2011/075677.

Terpene cyclase: The term "terpene cyclase" means an enzyme that catalyzes the formation of cyclic terpenes through the cyclization of linear terpenes (e.g., isopentenyl-pyrophosphate, geranyl-pyrophosphate, farnesyl-pyrophosphate, and geranylgeranyl-pyrophosphate) containing varying numbers of isoprene units. The *T. virens* genome harbors six terpene cyclases, while *T. atroviride* and *T. reesei* have three each.

Terpene: The term "terpene" means a group of natural products composed of several isoprene units, which are synthesized from isopentenyl pyrophosphate. A terpene is also known as a terpenoid or an isoprenoid.

Trypsin-like serine protease: The term "trypsin-like serine protease" means a protease with a substrate specificity similar to trypsin that uses a serine residue for catalyzing the hydrolysis of peptide bonds in peptides and proteins. For purposes of the

present invention, trypsin-like serine protease activity is determined according to the procedure described by WO 2011/075677.

Very high stringency conditions: The term “very high stringency conditions” means for probes of at least 100 nucleotides in length, prehybridization and hybridization at 42°C in 5X SSPE, 0.3% SDS, 200 micrograms/ml sheared and denatured salmon sperm DNA, and 50% formamide, following standard Southern blotting procedures for 12 to 24 hours. The carrier material is finally washed three times each for 15 minutes using 0.2X SSC, 0.2% SDS at 70°C.

Very low stringency conditions: The term “very low stringency conditions” means for probes of at least 100 nucleotides in length, prehybridization and hybridization at 42°C in 5X SSPE, 0.3% SDS, 200 micrograms/ml sheared and denatured salmon sperm DNA, and 25% formamide, following standard Southern blotting procedures for 12 to 24 hours. The carrier material is finally washed three times each for 15 minutes using 0.2X SSC, 0.2% SDS at 45°C.

Detailed Description of the Invention

The present invention relates to mutants of a parent *Trichoderma* strain, comprising a polynucleotide encoding a heterologous polypeptide and one or more (e.g., several) genes selected from the group consisting of a peptaibol synthetase gene, a paracelsin synthetase gene, a first terpene cyclase gene, a second terpene cyclase gene, and a third terpene cyclase gene, wherein one or more of the genes are modified rendering the mutant strain deficient in the production of one or more (e.g., several) enzymes selected from the group consisting of a peptaibol synthetase, a paracelsin synthetase, a first terpene cyclase, a second terpene cyclase, and a third terpene cyclase compared to the parent *Trichoderma* strain when cultivated under identical conditions.

The present invention also relates to methods of producing a heterologous polypeptide, comprising: (a) cultivating a mutant of a parent *Trichoderma* strain in a medium for the production of the heterologous polypeptide, wherein the mutant strain comprises a polynucleotide encoding the heterologous polypeptide and one or more (e.g., several) genes selected from the group consisting of a peptaibol synthetase gene, a paracelsin synthetase gene, a first terpene cyclase gene, a second terpene cyclase gene, and a third terpene cyclase gene, wherein one or more of the genes are modified rendering the mutant strain deficient in the production of one or more (e.g., several) enzymes selected from the group consisting of a peptaibol synthetase, a paracelsin synthetase, a first terpene cyclase, a second terpene cyclase, and a third terpene cyclase compared to the parent *Trichoderma* strain when cultivated under identical conditions; and optionally (b) recovering the

heterologous polypeptide from the cultivation medium.

The present invention further relates to methods of obtaining mutants of a parent *Trichoderma* strain, comprising: (a) modifying one or more (e.g., several) genes selected from the group consisting of a peptaibol synthetase gene, a paracelsin synthetase gene, a first terpene cyclase gene, a second terpene cyclase gene, and a third terpene cyclase gene; and (b) identifying a mutant strain from step (a) wherein one or more of the genes are modified rendering the mutant strain deficient in the production of one or more (e.g., several) enzymes selected from the group consisting of a peptaibol synthetase, a paracelsin synthetase, a first terpene cyclase, a second terpene cyclase, and a third terpene cyclase compared to the parent *Trichoderma* strain when cultivated under identical conditions.

The terms "a peptaibol synthetase gene" and "a paracelsin synthetase gene" can also be referred to herein as "a first peptaibol synthetase gene" and "a second peptaibol synthetase gene", respectively. The terms "a peptaibol synthetase" and "a paracelsin synthetase" can also be referred to herein as "a first peptaibol synthetase" and "a second peptaibol synthetase", respectively.

An advantage of the present invention is elimination or reduction of one or more (e.g., several) enzyme activities, which may be detrimental to the production, recovery, and/or application of a particular polypeptide of interest.

In the methods of the present invention, the parent *Trichoderma* strain may be any *Trichoderma* strain such as a wild-type *Trichoderma* strain or a mutant thereof. The parent *Trichoderma* strain may be *Trichoderma harzianum*, *Trichoderma koningii*, *Trichoderma longibrachiatum*, *Trichoderma reesei*, or *Trichoderma viride*; or the alternative sexual form thereof, i.e., *Hypocrea*.

In another aspect, the parent *Trichoderma* strain is *Trichoderma harzianum*. In another aspect, the parent *Trichoderma* strain is *Trichoderma koningii*. In another aspect, the parent *Trichoderma* strain is *Trichoderma longibrachiatum*. In another aspect, the parent *Trichoderma* strain is *Trichoderma reesei*. In another aspect, the parent *Trichoderma* strain is *Trichoderma viride*.

In another aspect, the parent *Trichoderma reesei* strain is *Trichoderma reesei* RutC30. In another aspect, the parent *Trichoderma reesei* strain is a mutant of *Trichoderma reesei*. In another aspect, the parent *Trichoderma reesei* strain is a mutant of *Trichoderma reesei* RutC30. In another aspect, the parent *Trichoderma reesei* strain is a morphological mutant of *Trichoderma reesei* (WO 97/26330).

A *Trichoderma* mutant strain of the present invention may be constructed by reducing or eliminating expression of one or more (e.g., several) genes selected from the group consisting of a peptaibol synthetase gene, a paracelsin synthetase gene, a first terpene cyclase gene, a second terpene cyclase gene, and a third terpene cyclase gene using

methods well known in the art, such as insertions, disruptions, replacements, or deletions. A portion of the gene can be modified such as the coding region or a control sequence required for expression of the coding region. Such a control sequence of the gene may be a promoter sequence or a functional part thereof, *i.e.*, a part that is sufficient for affecting expression of the gene. For example, a promoter sequence may be inactivated resulting in no expression or a weaker promoter may be substituted for the native promoter sequence to reduce expression of the coding sequence. Other control sequences for possible modification include, but are not limited to, a leader, propeptide sequence, signal sequence, transcription terminator, and transcriptional activator.

The *Trichoderma* mutant strains may be constructed by gene deletion techniques to eliminate or reduce expression of the genes. Gene deletion techniques enable the partial or complete removal of the gene thereby eliminating their expression. In such methods, deletion of the gene is accomplished by homologous recombination using a plasmid that has been constructed to contiguously contain the 5' and 3' regions flanking the gene.

The *Trichoderma* mutant strains may also be constructed by introducing, substituting, and/or removing one or more (*e.g.*, several) nucleotides in the gene or a control sequence thereof required for the transcription or translation thereof. For example, nucleotides may be inserted or removed, *e.g.*, for the introduction of a stop codon, the removal of the start codon, or a frame-shift of the open reading frame. Such a modification may be accomplished by site-directed mutagenesis or PCR generated mutagenesis in accordance with methods known in the art. See, for example, Botstein and Shortle, 1985, *Science* 229: 4719; Lo *et al.*, 1985, *Proceedings of the National Academy of Sciences USA* 81: 2285; Higuchi *et al.*, 1988, *Nucleic Acids Research* 16: 7351; Shimada, 1996, *Meth. Mol. Biol.* 57: 157; Ho *et al.*, 1989, *Gene* 77: 61; Horton *et al.*, 1989, *Gene* 77: 61; and Sarkar and Sommer, 1990, *BioTechniques* 8: 404.

The *Trichoderma* mutant strains may also be constructed by gene disruption techniques by inserting into the gene a disruptive nucleic acid construct comprising a nucleic acid fragment homologous to the gene that will create a duplication of the region of homology and incorporate construct DNA between the duplicated regions. Such a gene disruption can eliminate gene expression if the inserted construct separates the promoter of the gene from the coding region or interrupts the coding sequence such that a non-functional gene product results. A disrupting construct may be simply a selectable marker gene accompanied by 5' and 3' regions homologous to the gene. The selectable marker enables identification of transformants containing the disrupted gene.

The *Trichoderma* mutant strains may also be constructed by the process of gene conversion (see, for example, Iglesias and Trautner, 1983, *Molecular General Genetics* 189: 73-76). For example, in the gene conversion method, a nucleotide sequence corresponding

to the gene is mutagenized *in vitro* to produce a defective nucleotide sequence, which is then transformed into the parent *Trichoderma* strain to produce a defective gene. By homologous recombination, the defective nucleotide sequence replaces the endogenous gene. It may be desirable that the defective nucleotide sequence also comprises a marker
5 for selection of transformants containing the defective gene.

The *Trichoderma* mutant strains may also be constructed by established anti-sense techniques using a nucleotide sequence complementary to the nucleotide sequence of the gene (Parish and Stoker, 1997, *FEMS Microbiology Letters* 154: 151-157). More specifically, expression of the gene by a *Trichoderma* strain may be reduced or inactivated by
10 introducing a nucleotide sequence complementary to the nucleotide sequence of the gene, which may be transcribed in the strain and is capable of hybridizing to the mRNA produced in the strain. Under conditions allowing the complementary anti-sense nucleotide sequence to hybridize to the mRNA, the amount of protein translated is thus reduced or eliminated.

The *Trichoderma* mutant strains may also be constructed by established RNA
15 interference (RNAi) techniques (see, for example, WO 2005/056772 and WO 2008/080017).

The *Trichoderma* mutant strains may be further constructed by random or specific mutagenesis using methods well known in the art, including, but not limited to, chemical mutagenesis (see, for example, Hopwood, *The Isolation of Mutants in Methods in Microbiology* (J.R. Norris and D.W. Ribbons, eds.) pp. 363-433, Academic Press, New York,
20 1970). Modification of the gene may be performed by subjecting the parent strain to mutagenesis and screening for mutant strains in which expression of the gene has been reduced or inactivated. The mutagenesis, which may be specific or random, may be performed, for example, by use of a suitable physical or chemical mutagenizing agent, use of a suitable oligonucleotide, or subjecting the DNA sequence to PCR generated
25 mutagenesis. Furthermore, the mutagenesis may be performed by use of any combination of these mutagenizing methods.

Examples of a physical or chemical mutagenizing agent suitable for the present purpose include ultraviolet (UV) irradiation, hydroxylamine, N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), N-methyl-N'-nitrosoguanidine (NTG) O-methyl hydroxylamine,
30 nitrous acid, ethyl methane sulphonate (EMS), sodium bisulphite, formic acid, and nucleotide analogues. When such agents are used, the mutagenesis is typically performed by incubating the parent strain to be mutagenized in the presence of the mutagenizing agent of choice under suitable conditions, and selecting for mutants exhibiting reduced or no expression of the gene.

In one aspect, the modification results in the inactivation of one or more (e.g.,
35 several) genes selected from the group consisting of a peptaibol synthetase gene, a paracelsin synthetase gene, a first terpene cyclase gene, a second terpene cyclase gene,

and a third terpene cyclase gene. In another aspect, the modification results in a decrease in expression of one or more (*e.g.*, several) genes selected from the group consisting of a peptaibol synthetase gene, a paracelsin synthetase gene, a first terpene cyclase gene, a second terpene cyclase gene, and a third terpene cyclase gene. In another aspect, the modification results in expression of one or more (*e.g.*, several) genes selected from the group consisting of a peptaibol synthetase gene, a paracelsin synthetase gene, a first terpene cyclase gene, a second terpene cyclase gene, and a third terpene cyclase gene being decreased, inactivated, or a combination thereof.

In another aspect, the mutant comprises a modification of a peptaibol synthetase gene. In another aspect, the mutant comprises a modification of a paracelsin synthetase gene. In another aspect, the mutant comprises a modification of a first terpene cyclase gene. In another aspect, the mutant comprises a modification of a second terpene cyclase gene. In another aspect, the mutant comprises a modification of a third terpene cyclase gene.

In another aspect, the mutant comprises a modification of a peptaibol synthetase gene and a paracelsin synthetase gene. In another aspect, the mutant comprises a modification of a peptaibol synthetase gene and a first terpene cyclase gene. In another aspect, the mutant comprises a modification of a peptaibol synthetase gene and a second terpene cyclase gene. In another aspect, the mutant comprises a modification of a peptaibol synthetase gene and a third terpene cyclase gene. In another aspect, the mutant comprises a modification of a paracelsin synthetase gene and a first terpene cyclase gene. In another aspect, the mutant comprises a modification of a paracelsin synthetase gene and a second terpene cyclase gene. In another aspect, the mutant comprises a modification of a paracelsin synthetase gene and a third terpene cyclase gene. In another aspect, the mutant comprises a modification of a first terpene cyclase gene and a second terpene cyclase gene. In another aspect, the mutant comprises a modification of a first terpene cyclase gene and a third terpene cyclase gene. In another aspect, the mutant comprises a modification of a second terpene cyclase gene and a third terpene cyclase gene.

In another aspect, the mutant comprises a modification of a peptaibol synthetase gene, a paracelsin synthetase gene, and a first terpene cyclase gene. In another aspect, the mutant comprises a modification of a paracelsin synthetase gene, a first terpene cyclase gene, and a second terpene cyclase gene. In another aspect, the mutant comprises a modification of a paracelsin synthetase gene, a second terpene cyclase gene, and a third terpene cyclase gene. In another aspect, the mutant comprises a modification of a peptaibol synthetase gene, a paracelsin synthetase gene, and a second terpene cyclase gene. In another aspect, the mutant comprises a modification of a peptaibol synthetase gene, a paracelsin synthetase gene, and a third terpene cyclase gene. In another aspect, the mutant comprises a modification of a paracelsin synthetase gene, a first terpene cyclase gene, and

a third terpene cyclase gene. In another aspect, the mutant comprises a modification of a peptaibol synthetase gene, a first terpene cyclase gene, and a second terpene cyclase gene. In another aspect, the mutant comprises a modification of a peptaibol synthetase gene, a second terpene cyclase gene, and a third terpene cyclase gene. In another aspect, the mutant comprises a modification of a peptaibol synthetase gene, a first terpene cyclase gene, and a third terpene cyclase gene. In another aspect, the mutant comprises a modification of a first terpene cyclase gene, a second terpene cyclase gene, and a third terpene cyclase gene.

In another aspect, the mutant comprises a modification of a peptaibol synthetase gene, a paracelsin synthetase gene, a first terpene cyclase gene, and a second terpene cyclase gene. In another aspect, the mutant comprises a modification of a paracelsin synthetase gene, a first terpene cyclase gene, a second terpene cyclase gene, and a third terpene cyclase gene. In another aspect, the mutant comprises a modification of a peptaibol synthetase gene, a first terpene cyclase gene, a second terpene cyclase gene, and a third terpene cyclase gene. In another aspect, the mutant comprises a modification of a peptaibol synthetase gene, a paracelsin synthetase gene, a second terpene cyclase gene, and a third terpene cyclase gene. In another aspect, the mutant comprises a modification of a peptaibol synthetase gene, a paracelsin synthetase gene, a first terpene cyclase gene, and a third terpene cyclase gene.

In another aspect, the mutant comprises a modification of a peptaibol synthetase gene, a paracelsin synthetase gene, a first terpene cyclase gene, a second terpene cyclase gene, and a third terpene cyclase gene.

In one aspect, the peptaibol synthetase gene encodes a polypeptide having peptaibol synthetase activity comprising or consisting of an amino acid sequence having at least 60%, *e.g.*, at least 65%, at least 70%, at least 75%, at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to SEQ ID NO: 2. In another aspect, the peptaibol synthetase gene encodes a polypeptide having peptaibol synthetase activity comprising or consisting of SEQ ID NO: 2.

In another aspect, the peptaibol synthetase gene comprises a polynucleotide comprising or consisting of a nucleotide sequence having at least 60%, *e.g.*, at least 65%, at least 70%, at least 75%, at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to SEQ ID NO: 1 or the cDNA sequence thereof. In another aspect, the peptaibol synthetase gene comprises a polynucleotide

comprising or consisting of SEQ ID NO: 1.

In another aspect, the peptaibol synthetase gene comprises a polynucleotide that hybridizes under very low stringency conditions, low stringency conditions, medium stringency conditions, medium-high stringency conditions, high stringency conditions, or very high stringency conditions with SEQ ID NO: 1 or the cDNA sequence thereof; or the full-length complement thereof.

In another aspect, the paracelsin synthetase gene encodes a polypeptide having paracelsin synthetase activity comprising or consisting of an amino acid sequence having at least 60%, *e.g.*, at least 65%, at least 70%, at least 75%, at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to SEQ ID NO: 4. In another aspect, the paracelsin synthetase gene encodes a polypeptide having paracelsin synthetase activity comprising or consisting of SEQ ID NO: 4.

In another aspect, the paracelsin synthetase gene comprises a polynucleotide comprising or consisting of a nucleotide sequence having at least 60%, *e.g.*, *e.g.*, at least 65%, at least 70%, at least 75%, at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to SEQ ID NO: 3. In another aspect, the paracelsin synthetase gene comprises a polynucleotide comprising or consisting of SEQ ID NO: 3.

In another aspect, the paracelsin synthetase gene comprises a polynucleotide that hybridizes under very low stringency conditions, low stringency conditions, medium stringency conditions, medium-high stringency conditions, high stringency conditions, or very high stringency conditions with SEQ ID NO: 3 or the cDNA sequence thereof; or the full-length complement thereof.

In another aspect, the first terpene cyclase gene encodes a polypeptide having terpene cyclase activity comprising or consisting of an amino acid sequence having at least 60%, *e.g.*, at least 65%, at least 70%, at least 75%, at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to SEQ ID NO: 6. In another aspect, the first terpene cyclase gene encodes a polypeptide having terpene cyclase activity comprising or consisting of SEQ ID NO: 6.

In another aspect, the first terpene cyclase gene comprises a polynucleotide comprising or consisting of a nucleotide sequence having at least 60%, *e.g.*, at least 65%, at

least 70%, at least 75%, at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to SEQ ID NO: 5 or the genomic DNA sequence thereof. In another aspect, the first terpene cyclase gene comprises a polynucleotide comprising or consisting of SEQ ID NO: 5.

In another aspect, the first terpene cyclase gene comprises a polynucleotide that hybridizes under very low stringency conditions, low stringency conditions, medium stringency conditions, medium-high stringency conditions, high stringency conditions, or very high stringency conditions with SEQ ID NO: 5 or the genomic DNA sequence thereof; or the full-length complement thereof.

In another aspect, the second terpene cyclase gene encodes a polypeptide having terpene cyclase activity comprising or consisting of an amino acid sequence having at least 60%, e.g., at least 65%, at least 70%, at least 75%, at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to SEQ ID NO: 8. In another aspect, the second terpene cyclase gene encodes a polypeptide having terpene cyclase activity comprising or consisting of SEQ ID NO: 8.

In another aspect, the second terpene cyclase gene comprises a polynucleotide comprising or consisting of a nucleotide sequence having at least 60%, e.g., at least 65%, at least 70%, at least 75%, at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to SEQ ID NO: 7 or the genomic DNA sequence thereof. In another aspect, the second terpene cyclase gene comprises a polynucleotide comprising or consisting of SEQ ID NO: 7.

In another aspect, the second terpene cyclase gene comprises a polynucleotide that hybridizes under very low stringency conditions, low stringency conditions, medium stringency conditions, medium-high stringency conditions, high stringency conditions, or very high stringency conditions with SEQ ID NO: 7 or the genomic DNA sequence; or the full-length complement thereof.

In another aspect, the third terpene cyclase gene encodes a polypeptide having terpene cyclase activity comprising or consisting of an amino acid sequence having at least 60%, e.g., at least 65%, at least 70%, at least 75%, at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at

least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to SEQ ID NO: 10. In another aspect, the third terpene cyclase gene encodes a polypeptide having terpene cyclase activity comprising or consisting of SEQ ID NO: 10.

In another aspect, the third terpene cyclase gene comprises a polynucleotide comprising or consisting of a nucleotide sequence having at least 60%, *e.g.*, at least 65%, at least 70%, at least 75%, at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to SEQ ID NO: 9 or the genomic DNA sequence thereof. In another aspect, the third terpene cyclase gene comprises a polynucleotide comprising or consisting of SEQ ID NO: 9.

In another aspect, the third terpene cyclase gene comprises a polynucleotide that hybridizes under very low stringency conditions, low stringency conditions, medium stringency conditions, medium-high stringency conditions, high stringency conditions, or very high stringency conditions with SEQ ID NO: 9 or the genomic DNA sequence thereof; or the full-length complement thereof.

The nucleotide sequences disclosed herein or subsequences thereof, as well as the amino acid sequences thereof or fragments thereof, may be used to design nucleic acid probes to identify and clone homologous DNA of the genes described above from strains of different genera or species according to methods well known in the art. In particular, such probes can be used for hybridization with the genomic DNA or cDNA of the genus or species of interest, following standard Southern blotting procedures, in order to identify and isolate the corresponding gene therein. Such probes can be considerably shorter than the entire sequence, but should be at least 14, *e.g.*, at least 25, at least 35, or at least 70 nucleotides in length. Preferably, the nucleic acid probe is at least 100 nucleotides in length, *e.g.*, at least 200 nucleotides, at least 300 nucleotides, at least 400 nucleotides, at least 500 nucleotides, at least 600 nucleotides, at least 700 nucleotides, at least 800 nucleotides, or at least 900 nucleotides in length. Both DNA and RNA probes can be used. The probes are typically labeled for detecting the corresponding gene (for example, with ³²P, ³H, ³⁵S, biotin, or avidin).

Thus, a genomic DNA or cDNA library prepared from such other organisms may be screened for DNA that hybridizes with the probes described above. Genomic or other DNA from such other organisms may be separated by agarose or polyacrylamide gel electrophoresis, or other separation techniques. DNA from the libraries or the separated DNA may be transferred to and immobilized on nitrocellulose or other suitable carrier material. In order to identify a clone or DNA that is homologous with the nucleotide sequences disclosed herein or subsequences thereof, the carrier material is used in a

Southern blot. For purposes of the present invention, hybridization indicates that the nucleic acid sequence hybridizes to a labeled nucleic acid probe corresponding to the nucleotide sequences disclosed herein, its complementary strand, or a subsequence thereof, under very low to very high stringency conditions. Molecules to which the nucleic acid probe hybridizes under these conditions are detected using X-ray film.

For short probes of about 15 nucleotides to about 70 nucleotides in length, stringency conditions are defined as prehybridization and hybridization at about 5°C to about 10°C below the calculated T_m using the calculation according to Bolton and McCarthy (1962, *Proc. Natl. Acad. Sci. USA* 48:1390) in 0.9 M NaCl, 0.09 M Tris-HCl pH 7.6, 6 mM EDTA, 0.5% NP-40, 1X Denhardt's solution, 1 mM sodium pyrophosphate, 1 mM sodium monobasic phosphate, 0.1 mM ATP, and 0.2 mg of yeast RNA per ml following standard Southern blotting procedures for 12 to 24 hours optimally. The carrier material is finally washed once in 6X SCC plus 0.1% SDS for 15 minutes and twice each for 15 minutes using 6X SSC at 5°C to 10°C below the calculated T_m .

A nucleotide sequence homologous or complementary to a gene described herein may be used from other microbial sources to modify the corresponding gene in a *Trichoderma* strain of choice.

In another aspect, the modification of a gene in the *Trichoderma* mutant strain is unmarked with a selectable marker. Removal of the selectable marker gene may be accomplished by culturing the mutants on a counter-selection medium. Where the selectable marker gene contains repeats flanking its 5' and 3' ends, the repeats will facilitate the looping out of the selectable marker gene by homologous recombination when the mutant strain is submitted to counter-selection. The selectable marker gene may also be removed by homologous recombination by introducing into the mutant strain a nucleic acid fragment comprising 5' and 3' regions of the defective gene, but lacking the selectable marker gene, followed by selecting on a counter-selection medium. By homologous recombination, the defective gene containing the selectable marker gene is replaced with the nucleic acid fragment lacking the selectable marker gene. Other methods known in the art may also be used.

It will be understood that the methods of the present invention are not limited to a particular order for obtaining the *Trichoderma* mutant strain. The modification of a gene may be introduced into the parent strain at any step in the construction of the strain for the production of a polypeptide of interest. It is preferred that the *Trichoderma* mutant strain has already been made peptaibol, paracelsin, and/or terpene-deficient prior to such a construction.

In a further aspect of the present invention, the mutants of *Trichoderma* strains may contain additional modifications, e.g., deletions or disruptions, of other genes, which may

encode substances detrimental to the production, recovery, or application of a polypeptide of interest.

In one aspect, the *Trichoderma* strain further comprises a modification, e.g., disruption or deletion, of one or more (e.g., several) genes encoding a proteolytic activity selected from the group consisting of an aminopeptidase, dipeptidylaminopeptidase, tripeptidylaminopeptidase, carboxypeptidase, metalloprotease, cysteine protease, and vacuolar protease.

In a preferred aspect, the *Trichoderma* strain further comprises a modification, e.g., disruption or deletion, of one or more (e.g., several) genes selected from the group consisting of a first subtilisin-like serine protease gene, a first aspartic protease gene, a trypsin-like serine protease gene, a second subtilisin-like serine protease gene, and a second aspartic protease gene, as described in WO 2011/075677, which is incorporated herein by reference in its entirety.

In another aspect, the *Trichoderma* strain further comprises a modification, e.g., disruption or deletion, of one or more (e.g., several) additional genes encoding enzymes selected from the group consisting of an oxidoreductase, a transferase, a hydrolase, a lyase, an isomerase, or a ligase.

In another aspect, the *Trichoderma* strain further comprises a modification, e.g., disruption or deletion, of one or more (e.g., several) additional genes encoding enzymes selected from the group consisting of an acetylmannan esterase, acetylxylan esterase, aminopeptidase, alpha-amylase, arabinanase, arabinofuranosidase, carbohydrase, carboxypeptidase, catalase, cellobiohydrolase, cellulase, cellulose inducing protein, chitinase, coumaric acid esterase, cyclodextrin glycosyltransferase, cutinase, cyclodextrin glycosyltransferase, deoxyribonuclease, endoglucanase, esterase, expansin, feruloyl esterase, AA9 (GH61) polypeptide, alpha-galactosidase, beta-galactosidase, glucocerebrosidase, glucose oxidase, alpha-glucosidase, beta-glucosidase, glucuronidase, glucuronoyl esterase, haloperoxidase, hemicellulase, invertase, isomerase, laccase, ligase, lipase, mannanase, mannosidase, mutanase, oxidase, pectinolytic enzyme, peroxidase, phospholipase, phytase, phenoloxidase, polyphenoloxidase, proteolytic enzyme, ribonuclease, swollenin, alpha-1,6-transglucosidase, transglutaminase, urokinase, xylanase, or beta-xylosidase.

In another aspect, the *Trichoderma* strain further comprises a modification, e.g., disruption or deletion, of one or more (e.g., several) additional genes encoding enzymes selected from the group consisting of an endoglucanase, a cellobiohydrolase, and a beta-glucosidase,

In the methods of the present invention, the *Trichoderma* mutant strain preferably produces at least the same amount of a heterologous polypeptide of interest as the

corresponding parent *Trichoderma* strain when cultured under identical production conditions. In another aspect, the mutant strain produces at least 5% more, *e.g.*, at least 10% more, at least 25% more, at least 50% more, at least 75% more, and at least 100% more of the heterologous polypeptide than the corresponding parent *Trichoderma* strain when cultured under identical production conditions.

The *Trichoderma* mutant strains are cultivated in a nutrient medium for production of the heterologous polypeptide of interest using methods known in the art. For example, the strain may be cultivated by shake flask cultivation or small-scale or large-scale fermentation (including continuous, batch, fed-batch, or solid state fermentations) in laboratory or industrial fermentors performed in a suitable medium and under conditions allowing the polypeptide to be expressed and/or isolated. The cultivation takes place in a suitable nutrient medium comprising carbon and nitrogen sources and inorganic salts, using procedures known in the art. Suitable media are available from commercial suppliers or may be prepared according to published compositions (*e.g.*, in catalogues of the American Type Culture Collection). The secreted polypeptide can be recovered directly from the medium. If the polypeptide is not secreted, it may be obtained from cell lysates. A whole broth comprising a heterologous polypeptide of interest can also be recovered.

The polypeptide of interest may be detected using methods known in the art that are specific for the polypeptide. These detection methods may include use of specific antibodies, high performance liquid chromatography, capillary chromatography, formation of an enzyme product, disappearance of an enzyme substrate, or SDS-PAGE. For example, an enzyme assay may be used to determine the activity of an enzyme. Procedures for determining enzyme activity are known in the art for many enzymes (see, for example, D. Schomburg and M. Salzmann (eds.), *Enzyme Handbook*, Springer-Verlag, New York, 1990).

The resulting polypeptide may be isolated by methods known in the art. For example, a polypeptide of interest may be isolated from the cultivation medium by conventional procedures including, but not limited to, centrifugation, filtration, extraction, spray-drying, evaporation, or precipitation. The isolated polypeptide may then be further purified by a variety of procedures known in the art including, but not limited to, chromatography (*e.g.*, ion exchange, affinity, hydrophobic, chromatofocusing, and size exclusion), electrophoretic procedures (*e.g.*, preparative isoelectric focusing (IEF), differential solubility (*e.g.*, ammonium sulfate precipitation), or extraction (see, *e.g.*, *Protein Purification*, J.-C. Janson and Lars Ryden, editors, VCH Publishers, New York, 1989).

The heterologous polypeptide of interest may be any polypeptide foreign to the *Trichoderma* strain. The polypeptide may be encoded by a single gene or two or more genes. The term "polynucleotide encoding the polypeptide" will be understood to encompass one or more (*e.g.*, several) genes involved in the production of the polypeptide. The term

"heterologous polypeptide" is defined herein as a polypeptide that is not native to the host strain; a native polypeptide in which structural modifications have been made to alter the native polypeptide, e.g., the protein sequence of a native polypeptide; or a native polypeptide whose expression is quantitatively altered as a result of a manipulation of the polynucleotide or host strain by recombinant DNA techniques, e.g., a stronger promoter, multiple copies of a DNA encoding the polypeptide. Thus, the present invention also encompasses, within the scope of the term "heterologous polypeptides," such recombinant production of native polypeptides, to the extent that such expression involves the use of genetic elements not native to the *Trichoderma* strain, or use of native elements that have been manipulated to function in a manner that do not normally occur in the host strain.

The heterologous polypeptide may be any polypeptide having a biological activity of interest. The term "polypeptide" is not meant herein to refer to a specific length of the encoded product and, therefore, encompasses peptides, oligopeptides, and proteins. The term "polypeptide" also includes naturally occurring allelic and engineered variations of a polypeptide. The term "polypeptide" further encompasses hybrid and fusion polypeptides.

A hybrid polypeptide comprises a combination of partial polypeptide sequences obtained from at least two different polypeptides wherein one or more (e.g., several) may be heterologous to the *Trichoderma* strain.

A fusion polypeptide or cleavable fusion polypeptide comprises a polypeptide fused at the N-terminus or the C-terminus of another polypeptide. A fusion polypeptide is produced by fusing a polynucleotide encoding one polypeptide to a polynucleotide encoding another polypeptide. Techniques for producing fusion polypeptides are known in the art, and include ligating the coding sequences encoding the polypeptides so that they are in frame and that expression of the fusion polypeptide is under control of the same promoter(s) and terminator. Fusion polypeptides may also be constructed using intein technology in which fusion polypeptides are created post-translationally (Cooper *et al.*, 1993, *EMBO J.* 12: 2575-2583; Dawson *et al.*, 1994, *Science* 266: 776-779).

A fusion polypeptide can further comprise a cleavage site between the two polypeptides. Upon secretion of the fusion protein, the site is cleaved releasing the two polypeptides. Examples of cleavage sites include, but are not limited to, the sites disclosed in Martin *et al.*, 2003, *J. Ind. Microbiol. Biotechnol.* 3: 568-576; Svetina *et al.*, 2000, *J. Biotechnol.* 76: 245-251; Rasmussen-Wilson *et al.*, 1997, *Appl. Environ. Microbiol.* 63: 3488-3493; Ward *et al.*, 1995, *Biotechnology* 13: 498-503; and Contreras *et al.*, 1991, *Biotechnology* 9: 378-381; Eaton *et al.*, 1986, *Biochemistry* 25: 505-512; Collins-Racie *et al.*, 1995, *Biotechnology* 13: 982-987; Carter *et al.*, 1989, *Proteins: Structure, Function, and Genetics* 6: 240-248; and Stevens, 2003, *Drug Discovery World* 4: 35-48.

In one aspect, the polypeptide is an antibody, an antigen, an antimicrobial peptide,

an enzyme, a growth factor, a hormone, an immunodilator, a neurotransmitter, a receptor, a reporter protein, a structural protein, or a transcription factor.

In another aspect, the polypeptide is an oxidoreductase, a transferase, a hydrolase, a lyase, an isomerase, or a ligase. In another aspect, the polypeptide is an acetylmannan
 5 esterase, acetylxylan esterase, aminopeptidase, alpha-amylase, arabinanase, arabinofuranosidase, carbohydrase, carboxypeptidase, catalase, cellobiohydrolase, cellulase, cellulose inducing protein, chitinase, coumaric acid esterase, cyclodextrin glycosyltransferase, cutinase, cyclodextrin glycosyltransferase, deoxyribonuclease, endoglucanase, esterase, expansin, feruloyl esterase, AA9 (GH61) polypeptide, alpha-
 10 galactosidase, beta-galactosidase, glucocerebrosidase, glucose oxidase, alpha-glucosidase, beta-glucosidase, glucuronidase, glucuronoyl esterase, haloperoxidase, hemicellulase, invertase, isomerase, laccase, ligase, lipase, mannanase, mannosidase, mutanase, oxidase, pectinolytic enzyme, peroxidase, phospholipase, phytase, phenoloxidase, polyphenoloxidase, proteolytic enzyme, ribonuclease, swollenin, alpha-1,6-transglucosidase,
 15 transglutaminase, urokinase, xylanase, or beta-xylosidase.

In another aspect, the polypeptide is an albumin, a collagen, a tropoelastin, an elastin, or a gelatin.

In another aspect, the polypeptide is an endoglucanase. In another aspect, the polypeptide is a cellobiohydrolase. In another aspect, the polypeptide is a beta-glucosidase.
 20 In another aspect, the polypeptide is an AA9 (GH61) polypeptide. In another aspect, the polypeptide is a xylanase. In another aspect, the polypeptide is a beta-xylosidase. In another aspect, the polypeptide is an acetylxylan esterase. In another aspect, the polypeptide is a feruloyl esterase. In another aspect, the polypeptide is an arabinofuranosidase. In another aspect, the polypeptide is a glucuronidase. In another aspect, the polypeptide is an
 25 acetylmannan esterase. In another aspect, the polypeptide is an arabinanase. In another aspect, the polypeptide is a coumaric acid esterase. In another aspect, the polypeptide is a galactosidase. In another aspect, the polypeptide is a glucuronoyl esterase. In another aspect, the polypeptide is a mannanase. In another aspect, the polypeptide is a mannosidase. In another aspect, the polypeptide is a cellulose inducing protein. In another
 30 aspect, the polypeptide is an expansin. In another aspect, the polypeptide is a Swollenin.

In the methods of the present invention, the mutant of the *Trichoderma* strain is a recombinant strain, comprising a polynucleotide encoding a heterologous polypeptide, which is advantageously used in the recombinant production of the polypeptide. The strain is preferably transformed with a vector comprising the polynucleotide encoding the
 35 heterologous polypeptide followed by integration of the vector into the chromosome. "Transformation" means introducing a vector comprising the polynucleotide into a host strain so that the vector is maintained as a chromosomal integrant or as a self-replicating extra-

chromosomal vector. Integration is generally considered to be an advantage as the polynucleotide is more likely to be stably maintained in the strain. Integration of the vector into the chromosome can occur by homologous recombination, non-homologous recombination, or transposition.

5 The polynucleotide encoding a heterologous polypeptide may be obtained from any prokaryotic, eukaryotic, or other source, e.g., archaeabacteria. For purposes of the present invention, the term "obtained from" as used herein in connection with a given source shall mean that the polypeptide is produced by the source or by a strain in which a gene from the source has been inserted.

10 In the methods of the present invention, a mutant *Trichoderma* strain of the present invention may also be used for the recombinant production of a polypeptide that is native to the *Trichoderma* strain. The native polypeptide may be produced by recombinant means by, for example, placing a gene encoding the polypeptide under the control of a different promoter to enhance expression of the substance, expediting its export outside the strain by use of, for example, a signal sequence, or increasing the copy number of a gene encoding the polypeptide normally produced by the *Trichoderma* strain.

15 The techniques used to isolate or clone a polynucleotide encoding a polypeptide of interest are known in the art and include isolation from genomic DNA, preparation from cDNA, or a combination thereof. The cloning of such a polynucleotide from such genomic DNA can be effected, e.g., by using the well-known polymerase chain reaction (PCR). See, 20 for example, Innis *et al.*, 1990, *PCR Protocols: A Guide to Methods and Application*, Academic Press, New York. The cloning procedures may involve excision and isolation of a desired nucleic acid fragment comprising the polynucleotide encoding the polypeptide, insertion of the fragment into a vector molecule, and incorporation of the recombinant vector 25 into a mutant *Trichoderma* strain of the present invention where multiple copies or clones of the polynucleotide will be replicated. The polynucleotide may be of genomic, cDNA, RNA, semisynthetic, synthetic origin, or any combinations thereof.

30 An isolated polynucleotide encoding a heterologous polypeptide may be manipulated in a variety of ways to provide for expression of the polypeptide in a mutant *Trichoderma* strain of the present invention. Manipulation of the polynucleotide's sequence prior to its insertion into a vector may be desirable or necessary depending on the expression vector. The techniques for modifying polynucleotide sequences utilizing recombinant DNA methods are well known in the art.

35 A nucleic acid construct comprising a polynucleotide encoding a polypeptide may be operably linked to one or more (e.g., several) control sequences capable of directing expression of the coding sequence in a mutant *Trichoderma* strain of the present invention under conditions compatible with the control sequences.

The control sequence may be an appropriate promoter, a nucleotide sequence that is recognized by a mutant *Trichoderma* strain of the present invention for expression of the polynucleotide encoding the polypeptide. The promoter contains transcriptional control sequences that mediate expression of the polypeptide. The promoter may be any nucleotide sequence that shows transcriptional activity in the mutant *Trichoderma* strain, including mutant, truncated, and hybrid promoters, and may be obtained from genes encoding extracellular or intracellular polypeptides either native or heterologous (foreign) to the mutant *Trichoderma* strain.

Examples of suitable promoters for directing the transcription of the nucleic acid constructs in the methods of the present invention are promoters obtained from the genes for *Aspergillus nidulans* acetamidase, *Aspergillus niger* neutral alpha-amylase, *Aspergillus niger* acid stable alpha-amylase, *Aspergillus niger* or *Aspergillus awamori* glucoamylase (*glaA*), *Aspergillus oryzae* TAKA amylase, *Aspergillus oryzae* alkaline protease, *Aspergillus oryzae* triose phosphate isomerase, *Fusarium oxysporum* trypsin-like protease (WO 96/00787), *Fusarium venenatum* amyloglucosidase (WO 00/56900), *Fusarium venenatum* Daria (WO 00/56900), *Fusarium venenatum* Quinn (WO 00/56900), *Rhizomucor miehei* lipase, *Rhizomucor miehei* aspartic proteinase, *Trichoderma reesei* beta-glucosidase, *Trichoderma reesei* cellobiohydrolase I, *Trichoderma reesei* cellobiohydrolase II, *Trichoderma reesei* endoglucanase I, *Trichoderma reesei* endoglucanase II, *Trichoderma reesei* endoglucanase III, *Trichoderma reesei* endoglucanase V, *Trichoderma reesei* xylanase I, *Trichoderma reesei* xylanase II, *Trichoderma reesei* xylanase III, *Trichoderma reesei* beta-xylosidase, *Trichoderma reesei* glyceraldehyde-6 phosphate dehydrogenase, and *Trichoderma reesei* translation elongation factor, as well as the NA2-tpi promoter (a modified promoter from an *Aspergillus* neutral alpha-amylase gene in which the untranslated leader has been replaced by an untranslated leader from an *Aspergillus* triose phosphate isomerase gene; non-limiting examples include modified promoters from an *Aspergillus niger* neutral alpha-amylase gene in which the untranslated leader has been replaced by an untranslated leader from an *Aspergillus nidulans* or *Aspergillus oryzae* triose phosphate isomerase gene); and mutant, truncated, and hybrid promoters thereof. Other promoters are described in U.S. Patent No. 6,011,147.

The control sequence may also be a transcription terminator, which is recognized by a host cell to terminate transcription. The terminator is operably linked to the 3'-terminus of the polynucleotide encoding the polypeptide. Any terminator that is functional in a *Trichoderma* strain may be used in the present invention.

Preferred terminators are obtained from the genes for *Aspergillus nidulans* acetamidase, *Aspergillus nidulans* anthranilate synthase, *Aspergillus niger* glucoamylase, *Aspergillus niger* alpha-glucosidase, *Aspergillus oryzae* TAKA amylase, *Fusarium*

oxysporum trypsin-like protease, *Trichoderma reesei* beta-glucosidase, *Trichoderma reesei* cellobiohydrolase I, *Trichoderma reesei* cellobiohydrolase II, *Trichoderma reesei* endoglucanase I, *Trichoderma reesei* endoglucanase II, *Trichoderma reesei* endoglucanase III, *Trichoderma reesei* endoglucanase V, *Trichoderma reesei* xylanase I, *Trichoderma reesei* xylanase II, *Trichoderma reesei* xylanase III, *Trichoderma reesei* beta-xylosidase, and *Trichoderma reesei* translation elongation factor.

The control sequence may also be a leader, a nontranslated region of an mRNA that is important for translation by a mutant *Trichoderma* strain of the present invention. The leader is operably linked to the 5'-terminus of the polynucleotide encoding the polypeptide. Any leader that is functional in the mutant *Trichoderma* strain may be used in the present invention.

Preferred leaders for filamentous fungal host cells are obtained from the genes for *Aspergillus oryzae* TAKA amylase and *Aspergillus nidulans* triose phosphate isomerase.

The control sequence may also be a polyadenylation sequence, a sequence operably linked to the 3' terminus of the nucleotide sequence and, when transcribed, is recognized by the mutant *Trichoderma* strain as a signal to add polyadenosine residues to transcribed mRNA. Any polyadenylation sequence that is functional in the mutant *Trichoderma* strain may be used in the present invention.

Preferred polyadenylation sequences for filamentous fungal host cells are obtained from the genes for *Aspergillus oryzae* TAKA amylase, *Aspergillus niger* glucoamylase, *Aspergillus nidulans* anthranilate synthetase, *Fusarium oxysporum* trypsin-like protease, and *Aspergillus niger* alpha-glucosidase.

The control sequence may also be a signal peptide coding region that encodes a signal peptide linked to the N-terminus of a polypeptide and directs the polypeptide into the cell's secretory pathway. The 5'-end of the coding sequence of the polynucleotide may inherently contain a signal peptide coding sequence naturally linked in translation reading frame with the segment of the coding sequence that encodes the polypeptide. Alternatively, the 5'-end of the coding sequence may contain a signal peptide coding sequence that is foreign to the coding sequence. A foreign signal peptide coding sequence may be required where the coding sequence does not naturally contain a signal peptide coding sequence. Alternatively, a foreign signal peptide coding sequence may simply replace the natural signal peptide coding sequence in order to enhance secretion of the polypeptide. However, any signal peptide coding sequence that directs the expressed polypeptide into the secretory pathway of the mutant *Trichoderma* strain, *i.e.*, secreted into a culture medium, may be used in the present invention.

Effective signal peptide coding regions for the mutant *Trichoderma* strains are the signal peptide coding regions obtained from the genes for *Aspergillus niger* neutral amylase,

Aspergillus niger glucoamylase, *Aspergillus oryzae* TAKA amylase, *Humicola insolens* cellulase, *Humicola insolens* endoglucanase V, *Humicola lanuginosa* lipase, and *Rhizomucor miehei* aspartic proteinase.

The control sequence may also be a propeptide coding sequence that encodes a propeptide positioned at the N-terminus of a polypeptide. The resultant polypeptide is known as a proenzyme or propolypeptide (or a zymogen in some cases). A propolypeptide is generally inactive and can be converted to an active polypeptide by catalytic or autocatalytic cleavage of the propeptide from the propolypeptide. The propeptide coding region may be obtained from genes for *Saccharomyces cerevisiae* alpha-factor, *Rhizomucor miehei* aspartic proteinase, and *Myceliophthora thermophila* laccase (WO 95/33836).

Where both signal peptide and propeptide sequences are present, the propeptide sequence is positioned next to the N-terminus of a polypeptide and the signal peptide sequence is positioned next to the N-terminus of the propeptide sequence.

It may also be desirable to add regulatory sequences that regulate expression of the polypeptide relative to the growth of the mutant *Trichoderma* strain. Examples of regulatory sequences are those that cause expression of the gene to be turned on or off in response to a chemical or physical stimulus, including the presence of a regulatory compound. Regulatory systems in filamentous fungi such as the *Aspergillus niger* glucoamylase promoter, *Aspergillus oryzae* TAKA alpha-amylase promoter, and *Aspergillus oryzae* glucoamylase promoter, *Trichoderma reesei* cellobiohydrolase I promoter, and *Trichoderma reesei* cellobiohydrolase II promoter may be used. Other examples of regulatory sequences are those that allow for gene amplification. In eukaryotic systems, these regulatory sequences include the dihydrofolate reductase gene that is amplified in the presence of methotrexate, and the metallothionein genes that are amplified with heavy metals. In these cases, the polynucleotide encoding the polypeptide would be operably linked to the regulatory sequence.

In the methods of the present invention, a recombinant expression vector comprising a polynucleotide encoding a polypeptide of interest, a promoter, and transcriptional and translational stop signals may be used for the recombinant production of the polypeptide of interest. The various nucleotide and control sequences may be joined together to produce a recombinant expression vector that may include one or more convenient restriction sites to allow for insertion or substitution of the polynucleotide encoding the polypeptide at such sites. Alternatively, the polynucleotide may be expressed by inserting the polynucleotide or a nucleic acid construct comprising the polynucleotide into an appropriate vector for expression. In creating the expression vector, the coding sequence is located in the vector so that the coding sequence is operably linked with the appropriate control sequences for expression.

The recombinant expression vector may be any vector (e.g., a plasmid or virus) that can be conveniently subjected to recombinant DNA procedures and can bring about expression of the nucleotide sequence. The choice of the vector will typically depend on its compatibility with the mutant *Trichoderma* strain into which the vector is to be introduced.

5 The vector may be a linear or closed circular plasmid.

The recombinant expression vector may be any vector (e.g., a plasmid or virus) that can be conveniently subjected to recombinant DNA procedures and can bring about expression of the polynucleotide. The choice of the vector will typically depend on the compatibility of the vector with the host cell into which the vector is to be introduced. The
10 vector may be a linear or closed circular plasmid.

The vector may be an autonomously replicating vector, i.e., a vector that exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication, e.g., a plasmid, an extrachromosomal element, a minichromosome, or an artificial chromosome. The vector may contain any means for assuring self-replication. Alternatively,
15 the vector may be one that, when introduced into the host cell, is integrated into the genome and replicated together with the chromosome(s) into which it has been integrated. Furthermore, a single vector or plasmid or two or more vectors or plasmids that together contain the total DNA to be introduced into the genome of the host cell, or a transposon, may be used.

20 The vector preferably contains one or more selectable markers that permit easy selection of transformed, transfected, transduced, or the like cells. A selectable marker is a gene the product of which provides for biocide or viral resistance, resistance to heavy metals, prototrophy to auxotrophs, and the like.

Selectable markers for use in a filamentous fungal host cell include, but are not
25 limited to, *adeA* (phosphoribosylaminoimidazole-succinocarboxamide synthase), *adeB* (phosphoribosyl-aminoimidazole synthase), *amdS* (acetamidase), *argB* (ornithine carbamoyltransferase), *bar* (phosphinothricin acetyltransferase), *hph* (hygromycin phosphotransferase), *niaD* (nitrate reductase), *pyrG* (orotidine-5'-phosphate decarboxylase), *sC* (sulfate adenylyltransferase), and *trpC* (anthranilate synthase), as well as equivalents thereof. Preferred for use in a *Trichoderma* cell are *adeA*, *adeB*, *amdS*, *hph*, and *pyrG*
30 genes.

The selectable marker may be a dual selectable marker system as described in WO 2010/039889. In one aspect, the dual selectable marker is an *hph-tk* dual selectable marker system.

35 The vector preferably contains an element(s) that permits integration of the vector into the host cell's genome or autonomous replication of the vector in the cell independent of the genome.

For integration into the host cell genome, the vector may rely on the polynucleotide's sequence encoding the polypeptide or any other element of the vector for integration into the genome by homologous or non-homologous recombination. Alternatively, the vector may contain additional polynucleotides for directing integration by homologous recombination into the genome of the host cell at a precise location(s) in the chromosome(s). To increase the likelihood of integration at a precise location, the integrational elements should contain a sufficient number of nucleic acids, such as 100 to 10,000 base pairs, 400 to 10,000 base pairs, and 800 to 10,000 base pairs, which have a high degree of sequence identity to the corresponding target sequence to enhance the probability of homologous recombination. The integrational elements may be any sequence that is homologous with the target sequence in the genome of the host cell. Furthermore, the integrational elements may be non-encoding or encoding polynucleotides. On the other hand, the vector may be integrated into the genome of the host cell by non-homologous recombination.

For autonomous replication, the vector may further comprise an origin of replication enabling the vector to replicate autonomously in the host cell in question. The origin of replication may be any plasmid replicator mediating autonomous replication that functions in a cell. The term "origin of replication" or "plasmid replicator" means a polynucleotide that enables a plasmid or vector to replicate *in vivo*.

Examples of origins of replication useful in a filamentous fungal cell are AMA1 and ANS1 (Gems *et al.*, 1991, *Gene* 98: 61-67; Cullen *et al.*, 1987, *Nucleic Acids Res.* 15: 9163-9175; WO 00/24883). Isolation of the AMA1 gene and construction of plasmids or vectors comprising the gene can be accomplished according to the methods disclosed in WO 00/24883.

More than one copy of a polynucleotide may be inserted into a host cell to increase production of a polypeptide. An increase in the copy number of the polynucleotide can be obtained by integrating at least one additional copy of the sequence into the host cell genome or by including an amplifiable selectable marker gene with the polynucleotide where cells containing amplified copies of the selectable marker gene, and thereby additional copies of the polynucleotide, can be selected for by cultivating the cells in the presence of the appropriate selectable agent.

The procedures used to ligate the elements described above to construct the recombinant expression vectors of the present invention are well known to one skilled in the art (see, e.g., Sambrook *et al.*, 1989, *Molecular Cloning, A Laboratory Manual*, 2d edition, Cold Spring Harbor, New York).

A vector comprising the nucleotide sequence can be introduced, e.g., by transformation, into the mutant *Trichoderma* strain so that the vector is maintained as a chromosomal integrant or as a self-replicating extra-chromosomal vector. Integration is

generally considered to be an advantage as the nucleotide sequence is more likely to be stably maintained in the strain. Integration of the vector into the chromosome occurs by homologous recombination, non-homologous recombination, or transposition.

The introduction of an expression vector into the mutant *Trichoderma* strain may involve a process consisting of protoplast formation, transformation of the protoplasts, and regeneration of the strain wall in a manner known *per se*. Suitable procedures for transformation of *Trichoderma* strains are described in Malardier *et al.*, 1989, *Gene* 78: 147-156, and WO 96/00787.

The present invention is further described by the following examples that should not be construed as limiting the scope of the invention.

Examples

Strains

Trichoderma reesei strain 981-O-8 (D4) is a mutagenized strain of *Trichoderma reesei* RutC30 (Montenecourt and Eveleigh, 1979, *Adv. Chem. Ser.* 181: 289-301. *Trichoderma reesei* strain AgJg115-104-7B1 is a ku70 disrupted strain of *Trichoderma reesei* RutC30.

Media and Solutions

Overlay PDA medium was composed of 39 g of Potato Dextrose Agar (Difco) and deionized water to 1 liter supplemented with 100 µg of hygromycin B per ml.

PDA plates were composed of 39 g of Potato Dextrose Agar (Difco) and deionized water to 1 liter.

PEG buffer was composed of 500 g of PEG 4000, 10 mM CaCl₂, 10 mM Tris-HCl pH 7.5, and deionized water to 1 liter; filter sterilized.

SOC medium was composed of 20 g of tryptone, 5 g of yeast extract, 0.5 g of NaCl, 10 ml of 20 mM KCl, and deionized water to 1 liter.

STC was composed of 0.8 M or 1 M sorbitol, 10 mM or 25 mM CaCl₂, and 10 mM or 25 mM Tris-HCl, pH 7.5 or pH 8; filter sterilized.

TAE buffer was composed of 4.82 g of Tris Base, 1.14 ml of Glacial acetic acid, 2 ml of 0.5 M EDTA pH 8.0, and deionized water to 1 liter.

TBE buffer was composed of 10.8 g of Tris base, 5.5 g of boric acid, 4 ml of 0.5 M EDTA pH 8.0, and deionized water to 1 liter.

TE buffer was composed of 10 mM Tris-0.1 mM EDTA pH 8.0.

2XYT plus ampicillin plates were composed of 16 g of tryptone, 10 g of yeast extract,

5 g of NaCl, 15 g of Bacto agar, 1 ml of 100 mg/ml ampicillin stock, and deionized water to 1 liter.

YEG medium was composed of 5 g of yeast extract, 20 g of glucose, and deionized water to 1 liter.

YP medium was composed of 10 g of yeast extract, 20 g of Bacto peptone, and deionized water to 1 liter.

Example 1: *Trichoderma reesei* strain 981-O-8 genomic DNA extraction

Trichoderma reesei strain 981-O-8 was grown in 50 ml of YEG medium in a baffled shake flask at 28°C for 2 days with agitation at 200 rpm. Mycelia were harvested by filtration using MIRACLOTH® (Calbiochem, La Jolla, CA, USA), washed twice in deionized water, and frozen under liquid nitrogen. Frozen mycelia were ground, by mortar and pestle, to a fine powder, and total DNA was isolated using a DNEASY® Plant Maxi Kit (QIAGEN Inc., Valencia, CA, USA).

Example 2: *Trichoderma reesei* protoplast generation and transformation

Protoplast preparation and transformation were performed using a modification of the protocol described by Penttilä *et al.*, 1987, *Gene* 61: 155-164. Briefly, *Trichoderma reesei* strain AgJg115-104-7B1 was cultivated in 25 ml of YP medium supplemented with 2% (w/v) glucose at 28°C for 17 hours with gentle agitation at 90 rpm. Mycelia were collected by filtration using a Millipore Vacuum Driven Disposable Filtration System (Millipore, Bedford, MA, USA) and washed twice with deionized water and twice with 1.2 M sorbitol. Protoplasts were generated by suspending the washed mycelia in 30 ml of 1.2 M sorbitol containing 15 mg of GLUCANEX® 200 G (Novozymes A/S, Bagsvaerd, Denmark) per ml and 0.36 units of chitinase (Sigma Chemical Co., St. Louis, MO, USA) per ml for 15-25 minutes at 34°C with gentle shaking at 90 rpm. Protoplasts were collected by centrifugation at 400 x g for 7 minutes and washed twice with cold 1.2 M sorbitol. The protoplasts were counted using a hemacytometer and re-suspended to a final concentration of 1x10⁸ protoplasts per ml in STC. Excess protoplasts were stored in a Cryo 1°C Freezing Container (Nalgene, Rochester, NY, USA) at -80°C.

Approximately 2 µg of each of the deletion cassettes described in the following Examples were digested with *Pme* I. Each digestion reaction was purified by 0.8% agarose gel electrophoresis using TBE buffer where a DNA band was excised from the gel and extracted using a NUCLEOSPIN® Gel and PCR Cleanup kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany). The resulting purified DNA was added to 100 µl of the protoplast solution and mixed gently. PEG buffer (250 µl) was added and mixed, and then the mixture

was incubated at 34°C for 30 minutes. STC (3 ml) was then added and mixed, and the mixture was spread onto PDA plates supplemented with 1 M sucrose. After incubation at 28°C for 16 hours, 15 ml of overlay PDA medium supplemented with 100 µg of hygromycin B per ml were added to each plate. The plates were incubated at 28°C for 4-6 days.

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Example 3: Construction of a peptaibol synthetase gene deletion vector

The 5' flanking sequence of the *Trichoderma reesei* strain 981-O-8 peptaibol synthetase gene (Tirre2:123786; SEQ ID NO: 1 for the genomic DNA sequence and SEQ ID NO: 2 for the deduced amino acid sequence) was amplified from genomic DNA of *T. reesei* strain 981-O-8 using the gene-specific forward and reverse primers shown below.

10

Forward primer (1201181):

5'-*tcacatggtttaaacg*gcgcgccGACCCGAAAGAACGCAAAAGTCCAT-3' (SEQ ID NO: 11)

Reverse primer (1201182):

5'-*agccttg*ttttgtcGTGTCAAGAACTTGGATCTCCTAGGAG-3' (SEQ ID NO: 12)

15

The underlined portion is an *Asc* I site introduced for cloning and the region in italics represents an introduced extension corresponding to a homologous region of the site of vector insertion necessary for cloning.

The amplification reaction was composed of 137 ng of *T. reesei* 981-O-8 genomic DNA, 200 µm dNTP's, 0.4 µM primers, 3% DMSO, 5X PHUSION® HF Buffer with 1.5 mM MgCl₂ (New England Biolabs, Inc., Ipswich, MA, USA), and 2 units of PHUSION® Hot Start High-Fidelity DNA Polymerase (New England Biolabs, Inc., Ipswich, MA, USA) in a final volume of 50 µl. The amplification reaction was performed in a ROBOCYCLER® (Agilent Technologies, Santa Clara, CA, USA) programmed for 1 cycle at 98°C for 3 minutes; 35 cycles each at 98°C for 10 seconds, 55°C for 30 seconds, and 72°C for 1.5 minutes; and 1 cycle at 72°C for 10 minutes. PCR products were separated by 0.8% agarose gel electrophoresis using TBE buffer where a 2.4 kb product was observed. The 2.4 kb PCR product was excised from the gel and purified using a MINELUTE® Gel Extraction Kit (QIAGEN Inc., Valencia, CA, USA) according to the manufacturer's protocol.

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The 2.4 kb PCR product was inserted into *Asc* I-digested pJfyS1579-41-11 (WO 2011/075677) using an IN-FUSION® HD ADVANTAGE® PCR Cloning Kit (Clontech, Palo Alto, CA, USA) according to the manufacturer's protocol. The reaction was composed of 100 ng of pJfyS1579-41-11, 60 ng of the PCR product, and 2 µl of IN-FUSION® HD Enzyme Premix (Clontech, Palo Alto, CA, USA) in a 10 µl reaction volume. The reaction was incubated for 15 minutes at 50°C. Two and a half µl were used to transform SOLOPACK® Gold Supercompetent (Agilent, Santa Clara, CA, USA) cells. The DNA was added to the cells and incubated for 30 minutes on ice followed by a heat-shock at 42°C for 30 seconds. Then SOC medium (250 µl) was added and incubated at 37°C for 1 hour. The reaction was

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spread onto 2XYT plus ampicillin plates and incubated overnight at 37°C. Plasmid DNA was purified from several transformants using a BIOROBOT® 9600 (QIAGEN Inc., Valencia, CA, USA) and analyzed by DNA sequencing using a 3130XL Genetic Analyzer (Life Technologies, Carlsbad, CA, USA) to identify a plasmid containing the desired insert. One clone containing the insert was identified and designated plasmid pJyHi002A. Plasmid pJyHi002A was used to insert the 3' flank of the peptaibol synthetase gene.

The 3' peptaibol synthetase gene flanking sequence was amplified from *T. reesei* 981-O-8 genomic DNA using PHUSION® Hot Start High-Fidelity DNA Polymerase (New England Biolabs, Inc., Ipswich, MA, USA) and the gene-specific forward and reverse primers shown below.

Forward Primer (1201183):

5'-cctagttggagtattcctgcaggTCCTCATCTGTGGCTCATATTAGGT-3' (SEQ ID NO: 13)

Reverse primer (1201524):

5'-tggccatatttaaatcctgcagg**gtttaac**CAAGGCGGGATAGTGTCTCGGTTCTT-3' (SEQ ID NO: 14)

The underlined portion is a *Sbf* I site introduced for cloning, the region in italics represents an introduced extension corresponding to a homologous region of the site of vector insertion necessary for cloning, and the bold portion is an introduced *Pme* I site for later removal of the bacterial propagation portion of the plasmid.

The amplification reaction was composed of 137 ng of *T. reesei* 981-O-8 genomic DNA, 200 µM dNTP's, 0.4 µM primers, 3% DMSO, 5X PHUSION® HF Buffer with 1.5 mM MgCl₂, and 2 units of PHUSION® Hot Start High-Fidelity DNA Polymerase in a final volume of 50 µl. The amplification reaction was performed in an ROBOCYCLER® programmed for 1 cycle at 98°C for 3 minutes; 35 cycles each at 98°C for 10 seconds, 55°C for 30 seconds, and 72°C for 1 minute; and 1 cycle at 72°C for 10 minutes. PCR products were separated by 0.8% agarose gel electrophoresis using TBE buffer where a 2.0 kb fragment was excised from the gel and agarose was extracted using a NUCLEOSPIN® Extract II Kit (Clontech, Palo Alto, CA, USA) according to the manufacturer's protocol.

The 2.0 kb PCR product was inserted into *Sbf* I-digested pJyHi002A using an IN-FUSION® HD ADVANTAGE® PCR Cloning Kit according to the manufacturer's protocol. The reaction was composed of 100 ng of pJyHi002A, 50 ng of the 2.0 kb PCR product, and 2 µl of IN-FUSION® Enzyme (Clontech, Palo Alto, CA, USA) in a 10 µl reaction volume. The reaction was incubated for 15 minutes at 50°C. Forty µl of TE buffer were added to the reaction and 2.5 µl were used to transform SOLOPACK® Gold Supercompetent cells according to the manufacturer's protocol. The DNA was added to the cells and incubated for 30 minutes on ice followed by a heat-shock at 42°C for 30 seconds. Then SOC medium (250 µl) was added and incubated at 37°C for 1 hour. The reaction was spread onto 2XYT plus

ampicillin plates and incubated overnight at 37°C. Plasmid DNA was purified from several transformants using a BIOROBOT® 9600 and analyzed by DNA sequencing using a 3130XL Genetic Analyzer to identify a plasmid containing the desired insert. One clone containing the insert was identified and designated plasmid pJyHi002 (Figure 1). Plasmid pJyHi002 was used to delete the peptaibol synthetase gene.

Example 4: Generation of peptaibol synthetase gene deleted *Trichoderma reesei* strain

Trichoderma reesei strain AgJg115-104-7B1 was transformed as described in Example 2 with plasmid pJyHi002. Transformants were transferred from PDA plates supplemented with 1 M sucrose with sterile inoculation loops to new PDA plates and grown at 28°C for 7 days.

Transformants of *Trichoderma reesei* strain AgJg115-104-7B1 containing the pJyHi002 deletion vector in the peptaibol synthetase locus, thereby deleting the peptaibol synthetase gene, were screened by fungal spore PCR using a PHIRE® Plant Direct PCR Kit (Thermo Fisher Scientific, Waltham, MA, USA) and the primers shown below.

Forward Primer (1202690):

5'-TGCCCCACGATATCTCTCCTTCTCC-3' (SEQ ID NO: 15)

Reverse Primer (067947):

5'-CTACATCGAAGCTGAAAGCACGAGA-3' (SEQ ID NO: 16)

Spores from several transformants were collected using a 1 µl loop and transferred to 15 µl of dilution buffer provided by the Kit. The spore samples were incubated at room temperature for 3 minutes and centrifuged at 2000 x g for one minute. One microliter of each spore sample was used as template. The reaction was composed of 1 µl of a spore suspension, 10 µl of 2X PHIRE® Plant PCR Buffer (Thermo Scientific, Waltham, MA, USA), 0.5 µM primer 1202690, 0.5 µM primer 067947, 0.4 µl of PHIRE® Hot Start DNA Polymerase (Thermo Scientific, Waltham, MA, USA), and 8.2 µl of water. The reaction was performed in an EPPENDORF® MASTERCYCLER® (Eppendorf AG, Hamburg, Germany) programmed for 1 cycle at 95°C for 10 minutes; 35 cycles each at 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 5 minutes 45 seconds; 1 cycle at 72°C for 15 minutes; and a 4°C hold. Since primer 1202690 is located upstream of the 5' flanking region and primer 067947 is located in the *hpt* marker, only transformants with the deletion cassette in the correct locus would yield a PCR product. PCR products were separated by 0.8% agarose gel electrophoresis using TBE buffer where a 3.7 kb fragment was observed indicating the deletion cassette was in the correct locus.

The transformants were then analyzed by Southern analysis according to the following procedure. Genomic DNA from each of the transformants was extracted using a

MASTERPURE™ Yeast DNA Purification Kit (Epicentre, Madison, WI, USA). Each transformant was cultivated in 25 ml of YP medium supplemented with 2% glucose in shake flasks for 72 hours with agitation at 200 rpm. The mycelia were collected from the cultures by filtration using Whatman 1 filter paper (Fisher Scientific, Pittsburgh, PA, USA). The filter paper was inserted in a ceramic funnel placed into a sidearm flask. Under vacuum, the culture broth was filtered and rinsed with water and filtered again. The mycelia were transferred to 2 ml tubes and then dried overnight using a SPEEDVAC® concentrator (Thermo Fisher Scientific, Waltham, MA, USA). The dried mycelia were crushed with a metal tool in each tube and approximately 50 µl of the dried mycelia were transferred to new tubes. Three hundred microliters of Yeast Cell Lysis Solution (Epicentre, Madison, WI, USA) were added to each tube and vortexed. The samples were incubated at 65°C for 20 minutes and then placed on ice for 5 minutes. One hundred fifty microliters of MPC Protein Precipitation Reagent (Epicentre, Madison, WI, USA) were added to each tube and then the reactions were vortexed. The tubes were centrifuged for 10 minutes at 9300 x g. The supernatants were transferred to microcentrifuge tubes followed by 0.5 ml of isopropanol, and then the tubes were centrifuged at 9300 x g for 10 minutes. The supernatants were discarded and each of the pellets was washed with 0.5 ml of 70% ethanol. The ethanol was removed and discarded and the pellets were briefly dried using a SPEEDVAC® concentrator. Then the pellets were resuspended in 60 µl of TE buffer. The samples were incubated at 65°C for about an hour to dissolve the pellet and then 0.3 µl of 100 mg/ml RNase A (QIAGEN Inc., Valencia, CA, USA) was added and the samples were incubated at 37°C for an hour.

Approximately two micrograms of each genomic DNA sample were digested with 30 units of *Bcl* I and 10 units of *Swa* I for 16 hours. The digestions were subjected to 0.8% agarose gel electrophoresis using TAE buffer and blotted onto NYTRAN® SuperCharge blotting membrane (Schleicher & Schuell BioScience, Keene, NH, USA) using a TURBOBLOTTER® (Schleicher & Schuell BioScience, Keene, NH, USA) for approximately 12-16 hours following the manufacturer's recommendations. A PCR probe, hybridizing to the 5' flanking sequence of the peptaibol synthetase gene, was synthesized by incorporation of digoxigenin-11-dUTP by PCR using a PCR DIG Probe Synthesis Kit (Roche Molecular Biochemicals, Indianapolis, IN, USA) according to the manufacturer's protocol and the following forward and reverse primers:

Forward primer (1201259):

5'-TAGCTAGCTGTCTTGGATGAATCGAGGTTG-3' (SEQ ID NO: 17)

Reverse primer (1202008):

5'-TCGTCTTCATGAGCATGTTGTTGGG-3' (SEQ ID NO: 18)

The amplification reaction (50 µl) was composed of 200 µM dNTP's, 0.5 µM primers, 3% DMSO, 5X PHUSION® HF Buffer with 1.5 mM MgCl₂, and 2 units of PHUSION® Hot

Start High-Fidelity DNA Polymerase in a final volume of 50 µl. The amplification reaction was performed in a ROBOCYCLER® programmed for 1 cycle at 98°C for 3 minutes; 35 cycles each at 98°C for 10 seconds, 55°C for 15 seconds, and 72°C for 1.5 minutes; and 1 cycle at 72°C for 10 minutes. PCR products were separated by 0.8% agarose gel electrophoresis using TBE buffer where a 0.5 kb fragment was excised from the gel and agarose was extracted using a MINELUTE® Gel Extraction Kit according to the manufacturer's protocol. The purified fragment was used as template for labelling with digoxigenin using a PCR DIG Probe Synthesis Kit. The reaction consisted of 5 µl of PCR buffer with MgCl₂ (Roche, Indianapolis, IN, USA), 5 µl of PCR DIG probe synthesis mix which includes 200 µM dNTP (Roche Molecular Biochemicals, Indianapolis, IN, USA), 1 µM forward primer 1201259, 1 µM reverse primer 1202008, 2.6 units of Enzyme mix, EXPAND® High Fidelity (Roche, Indianapolis, IN, USA), and 100 pg of template DNA up to a volume of 50 µl. The amplification reaction was performed in a ROBOCYCLER® programmed for 1 cycle at 95°C for 2 minutes; 30 cycles each at 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 40 seconds; and 1 cycle at 72°C for 10 minutes.

The incorporation of digoxigenin was confirmed by a molecular weight shift of the labelled probe which ran at approximately 0.5 kb. Hybridization was performed in DIG Easy Hyb buffer (Roche Molecular Biochemicals, Indianapolis, IN, USA) at 42°C for 15-17 hours. The membrane was then washed in 2X SSC plus 0.1% SDS for 5 minutes at room temperature followed by two washes in 0.5X SSC plus 0.1% SDS for 15 minutes each at 65°C. The probe-target hybrids were detected by a chemiluminescent assay (Roche Molecular Biochemicals, Indianapolis, IN, USA) following the manufacturer's instructions

Southern analysis indicated that several of the transformants harbored the deletion cassette in a single copy. One transformant containing the peptaibol synthase gene deletion was designated *Trichoderma reesei* JyHi002-26A.

Example 5: Construction of a paracelsin synthetase gene deletion vector

The 5' flanking sequence of the *Trichoderma reesei* strain 981-O-8 paracelsin synthetase gene (Trirre2:23171; SEQ ID NO: 3 for the genomic DNA sequence and SEQ ID NO: 4 for the deduced amino acid sequence) was amplified from genomic DNA of *T. reesei* strain 981-O-8 using the gene-specific forward and reverse primers shown below.

Forward primer (1201177):

5'-*tcacatggtttaacggcgccgc*TACTACCTAGTACAGTGCTTATTTA-3' (SEQ ID NO: 19)

Reverse primer (1201178):

5'-*agccttggtttgtc*GTTTTTCTCCAAATTTGTACAGAATTATCT-3' (SEQ ID NO: 20)

The underlined portion is an Asc I site introduced for cloning and the region in italics represents an introduced extension corresponding to a homologous region of the site of

vector insertion necessary for cloning.

The amplification reaction was composed of 137 ng of *T. reesei* 981-O-8 genomic DNA, 200 µm dNTP's, 0.4 µM primers, 3% DMSO, 5X PHUSION® HF Buffer with 1.5 mM MgCl₂, and 2 units of PHUSION® Hot Start High-Fidelity DNA Polymerase in a final volume of 50 µl. The amplification reaction was performed in an ROBOCYCLER® programmed for 1 cycle at 98°C for 3 minutes; 35 cycles each at 98°C for 10 seconds, 55°C for 30 seconds, and 72°C for 1 minute; and 1 cycle at 72°C for 10 minutes. PCR products were separated by 0.8% agarose gel electrophoresis using TBE buffer where a 2.0 kb PCR product was observed. The 2.0 kb PCR product was excised from the gel and purified using a MINELUTE® Gel Extraction Kit according to the manufacturer's protocol.

The 2.0 kb PCR product was inserted into Asc I-digested pJfyS1579-41-11 (WO 2011/075677) using an IN-FUSION® HD ADVANTAGE® PCR Cloning Kit according to the manufacturer's protocol. The reaction was composed of 200 ng of pJfyS1579-41-11, 100 ng of the PCR product, and 2 µl of IN-FUSION® HD Enzyme Premix in a 10 µl reaction volume. The reaction was incubated for 15 minutes at 50°C. Two and a half µl were used to transform SOLOPACK® Gold Supercompetent cells. The DNA was added to the cells and incubated for 30 minutes on ice followed by a heat-shock at 42°C for 30 seconds. Then SOC medium (250 µl) was added and incubated at 37°C for 1 hour. The reaction was spread onto 2XYT plus ampicillin plates and incubated overnight at 37°C. Plasmid DNA was purified from several transformants using a BIOROBOT® 9600 and analyzed by DNA sequencing using a 3130XL Genetic Analyzer to identify a plasmid containing the desired insert. One clone containing the insert was identified and designated plasmid pJyHi001A. Plasmid pJyHi001A was used to insert the 3' flank of the paracelsin synthetase gene.

The 3' paracelsin synthetase gene flanking sequence was amplified from *T. reesei* 981-O-8 genomic DNA using PHUSION® Hot Start High-Fidelity DNA Polymerase and the gene-specific forward and reverse primers shown below.

Forward Primer (1201179):

5'-*cctagttggagtattcctgcagg*AGGAATTGTGCCTGGCTGTTGAGTT-3' (SEQ ID NO: 21)

Reverse primer (1201523):

5'-*tggccatatttaaattcctgcagggtttaaac*GCTTATCGATCCGGCATATCGCTCT-3' (SEQ ID NO: 22)

The underlined portion is a *Sbf* I site introduced for cloning, the region in italics represents an introduced extension corresponding to a homologous region of the site of vector insertion necessary for cloning, and the bold portion is an introduced *Pme* I site for later removal of the bacterial propagation portion of the plasmid.

The amplification reaction was composed of 137 ng of *T. reesei* 981-O-8 genomic DNA, 200 µm dNTP's, 0.4 µM primers, 3% DMSO, 5X PHUSION® HF Buffer with 1.5 mM

MgCl₂, and 2 units of PHUSION® Hot Start High-Fidelity DNA Polymerase in a final volume of 50 µl. The amplification reaction was performed in an ROBOCYCLER® programmed for 1 cycle at 98°C for 3 minutes; 35 cycles each at 98°C for 10 seconds, 55°C for 30 seconds, and 72°C for 1 minute; and 1 cycle at 72°C for 10 minutes. PCR products were separated by 0.8% agarose gel electrophoresis using TBE buffer where a 2.0 kb fragment was excised from the gel and agarose was extracted using a NUCLEOSPIN® Extract II Kit according to the manufacturer's protocol.

The 2.0 kb PCR product was inserted into *Sbf* I-digested pJyHi001A using an IN-FUSION® HD ADVANTAGE® PCR Cloning Kit according to the manufacturer's protocol. The reaction was composed of 200 ng of pJyHi001A, 50 ng of the 2.0 kb PCR product, and 2 µl of IN-FUSION® Enzyme in a 10 µl reaction volume. The reaction was incubated for 15 minutes at 50°C. Forty µl of TE buffer were added to the reaction and 2.5 µl were used to transform SOLOPACK® Gold Supercompetent cells. The DNA was added to the cells and incubated for 30 minutes on ice followed by a heat-shock at 42°C for 30 seconds. Then SOC medium (250 µl) was added and incubated at 37°C for 1 hour. The reaction was spread onto 2XYT plus ampicillin plates and incubated overnight at 37°C. Plasmid DNA was purified from several transformants using a BIOROBOT® 9600 and analyzed by DNA sequencing using a 3130XL Genetic Analyzer to identify a plasmid containing the desired insert. One clone containing the insert was identified and designated plasmid pJyHi001 (Figure 2). Plasmid pJyHi001 was used to delete the paracelsin synthetase gene.

Example 6: Generation of paracelsin synthetase gene deleted *Trichoderma reesei* strain

Trichoderma reesei strain AgJg115-104-7B1 was transformed as described in Example 2 with plasmid pJyHi001. Transformants were transferred from PDA plates supplemented with 1 M sucrose with sterile inoculation loops to new PDA plates and grown at 28°C for 7 days.

Transformants of *Trichoderma reesei* strain AgJg115-104-7B1 containing the pJyHi001 deletion vector in the paracelsin synthetase locus, thereby deleting the paracelsin synthetase gene, were screened by fungal spore PCR using a PHIRE® Plant Direct PCR Kit and the primers shown below.

Forward Primer (1202687):

5'-TACCTTACAGGCCCTCCGCGAGCTA-3' (SEQ ID NO: 23)

Reverse Primer (067947):

5'-CTACATCGAAGCTGAAAGCACGAGA-3' (SEQ ID NO: 24)

Spores from several transformants were collected using a 1 µl loop and transferred to 15 µl of dilution buffer provided by the Kit. The spore samples were incubated at room

temperature for 3 minutes and centrifuged at 2000 x *g* for one minute. One microliter of each spore sample was used as template. The reaction was composed of 1 µl of a spore suspension, 10 µl of 2X PHIRE® Plant PCR Buffer, 0.5 µM primer 1202687, 0.5 µM primer 067947, 0.4 µl of PHIRE® Hot Start DNA Polymerase, and 8.2 µl of water. The reaction was performed in an EPPENDORF® MASTERCYCLER® programmed for 1 cycle at 98°C for 5 minutes; 40 cycles each at 98°C for 5 seconds and 72°C for 1 minute 30 seconds; 1 cycle at 72°C for 1 minute; and a 4°C hold. Since primer 1202687 is located upstream of the 5' flanking region and primer 067947 is located in the *hpt* marker, only transformants with the deletion cassette in the correct locus would yield a PCR product. PCR products were separated by 0.8% agarose gel electrophoresis using TBE buffer where a 3.2 kb fragment was observed indicating the deletion cassette was in the correct locus.

The transformants were then analyzed by Southern analysis according to the following procedure. Genomic DNA from each of the transformants was extracted using a MASTERPURE™ Yeast DNA Purification Kit. Each transformant was cultivated in 25 ml of YP medium supplemented with 2% glucose in shake flasks for 72 hours with agitation at 200 rpm. The mycelia were collected from the cultures by filtration using Whatman 1 filter paper. The filter paper was inserted in a ceramic funnel placed into a sidearm flask. Under vacuum, the culture broth was filtered and rinsed with water and filtered again. The mycelia were transferred to 2 ml tubes and then dried overnight using a SPEEDVAC® concentrator. The dried mycelia were crushed with a metal tool in each tube and approximately 50 µl of the dried mycelia was transferred to new tubes. Three hundred microliters of Yeast Cell Lysis Solution were added to each tube and vortexed. The samples were incubated at 65°C for 20 minutes and then placed on ice for 5 minutes. One hundred fifty microliters of MPC Protein Precipitation Reagent were added to each tube and then the reactions were vortexed. The tubes were centrifuged for 10 minutes at 9300 x *g*. The supernatants were transferred to microcentrifuge tubes followed by 0.5 ml of isopropanol, and then the tubes were centrifuged at 9300 x *g* for 10 minutes. The supernatants were discarded and each of the pellets was washed with 0.5 ml of 70% ethanol. The ethanol was removed and discarded and the pellets were briefly dried using a SPEEDVAC® concentrator. Then the pellets were resuspended in 60 µl of TE buffer. The samples were incubated at 65°C for about an hour to dissolve the pellet and then 0.3 µl of 100 mg/ml RNase A was added and the samples were incubated at 37°C for an hour.

Approximately two micrograms of each genomic DNA sample were digested with 20 units of *BsiW* I for 6 hours. The digestions were subjected to 0.8% agarose gel electrophoresis using TAE buffer and blotted onto NYTRAN® SuperCharge blotting membrane using a TURBOBLOTTER® for approximately 12-16 hours following the manufacturer's recommendations. A PCR probe, hybridizing to the 5' flanking sequence of

the paracelsin synthetase gene, was synthesized by incorporation of digoxigenin-11-dUTP by PCR using a PCR DIG Probe Synthesis Kit according to the manufacturer's protocol and the following forward and reverse primers:

Forward primer (1201253):

5'-ATGTTGGAGCCTTGCCTCCAGAGTCCTCAC-3' (SEQ ID NO: 25)

Reverse primer (1202005):

5'-GGGTTTCAGTCCAGAAGCAGAACCAGGATCA-3' (SEQ ID NO: 26)

The amplification reaction (50 µl) was composed of 200 µM dNTP's, 0.5 µM primers, 3% DMSO, 5X PHUSION® HF Buffer with 1.5 mM MgCl₂, and 2 units of PHUSION® Hot Start High-Fidelity DNA Polymerase in a final volume of 50 µl. The amplification reaction was performed in a ROBOCYCLER® programmed for 1 cycle at 98°C for 1 minute; 35 cycles each at 98°C for 10 seconds, 55°C for 30 seconds, and 72°C for 15 seconds; and 1 cycle at 72°C for 10 minutes. PCR products were separated by 0.8% agarose gel electrophoresis using TBE buffer where a 0.5 kb fragment was excised from the gel and agarose was extracted using a MINELUTE® Gel Extraction Kit. The purified fragment was used as template for labelling with digoxigenin using a PCR DIG Probe Synthesis Kit. The reaction consisted of 5 µl of PCR buffer with MgCl₂, 5 µl of PCR DIG probe synthesis mix which includes 200 µM dNTP, 1 µM forward primer 1201253, 1 µM reverse primer 1202005, 2.6 units of Enzyme mix, EXPAND® High Fidelity, and 100 pg of template DNA up to a volume of 50 µl. The amplification reaction was performed in a ROBOCYCLER® programmed for 1 cycle at 95°C for 2 minutes; 30 cycles each at 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 40 seconds; and 1 cycle at 72°C for 10 minutes.

The incorporation of digoxigenin was confirmed by a molecular weight shift of the labelled probe which ran at approximately 0.5 kb. Hybridization was performed in DIG Easy Hyb buffer at 42°C for 15-17 hours. The membrane was then washed in 2X SSC plus 0.1% SDS for 5 minutes at room temperature followed by two washes in 0.5X SSC plus 0.1% SDS for 15 minutes each at 65°C. The probe-target hybrids were detected by a chemiluminescent assay (Roche Molecular Biochemicals, Indianapolis, IN, USA) following the manufacturer's instructions.

The transformants were also analyzed by PCR using PHUSION® Hot Start High-Fidelity DNA Polymerase and the primers shown below.

Forward Primer (1202689):

5'-CAGATGAGCCCTACATGACGCCAGC-3' (SEQ ID NO: 27)

Reverse Primer (1200592):

5'-GGCTCCATACCGACGATATGC-3' (SEQ ID NO: 28)

The amplification reaction was composed of 150 ng of genomic DNA, 200 µM dNTP's, 0.4 µM primers, 3% DMSO, 5X PHUSION® HF Buffer with 1.5 mM MgCl₂, and 2

units of PHUSION® Hot Start High-Fidelity DNA Polymerase in a final volume of 50 µl. The amplification reaction was performed in an ROBOCYCLER® programmed for 1 cycle at 98°C for 30 seconds; 35 cycles each at 98°C for 10 seconds, 70°C for 30 seconds, and 72°C for 2 minutes 18 seconds; and 1 cycle at 72°C for 10 minutes. PCR products were separated by 0.8% agarose gel electrophoresis using TBE buffer where a 4.6 kb PCR product was expected if the paracelsin synthetase deletion cassette was integrated.

Both Southern and PCR analysis indicated that several of the transformants harbored the deletion cassette in a single copy. One transformant containing the paracelsin synthetase gene deletion was designated *Trichoderma reesei* JyHi001-4B.

The present invention is further described by the following numbered paragraphs:

[1] A mutant of a parent *Trichoderma* strain, comprising a polynucleotide encoding a heterologous polypeptide and one or more genes selected from the group consisting of a peptaibol synthetase gene, a paracelsin synthetase gene, a first terpene cyclase gene, a second terpene cyclase gene, and a third terpene cyclase gene, wherein one or more of the genes are modified rendering the mutant strain deficient in the production of one or more enzymes selected from the group consisting of a peptaibol synthetase, a paracelsin synthetase, a first terpene cyclase, a second terpene cyclase, and a third terpene cyclase compared to the parent *Trichoderma* strain when cultivated under identical conditions.

[2] The mutant of paragraph 1, which comprises a modification of the peptaibol synthetase gene.

[3] The mutant of paragraph 1 or 2, which comprises a modification of the paracelsin synthetase gene.

[4] The mutant of any of paragraphs 1-3, which comprises a modification of the first terpene cyclase gene.

[5] The mutant of any of paragraphs 1-4, which comprises a modification of the second terpene cyclase gene.

[6] The mutant of any of paragraphs 1-5, which comprises a modification of the third terpene cyclase gene.

[7] The mutant of paragraph 1-6, which comprises a modification of the peptaibol synthetase gene, the paracelsin synthetase gene, the first terpene cyclase gene, the second terpene cyclase gene, and the third terpene cyclase gene.

[8] The mutant of any of paragraphs 1-7, wherein the *Trichoderma* strain is selected from the group consisting of *Trichoderma harzianum*, *Trichoderma koningii*, *Trichoderma longibrachiatum*, *Trichoderma reesei*, and *Trichoderma viride*.

[9] The mutant of any of paragraphs 1-8, wherein the *Trichoderma* strain is *Trichoderma reesei*.

[10] The mutant of any of paragraphs 1-9, which produces at least 25% less of one or more of the enzymes selected from the group consisting of the peptaibol synthetase, the paracelsin synthetase, the first terpene cyclase, the second terpene cyclase, and the third terpene cyclase compared to the parent *Trichoderma* strain when cultivated under identical conditions.

[11] The mutant of any of paragraphs 1-10, which is completely deficient in one or more of the enzymes selected from the group consisting of the peptaibol synthetase, the paracelsin synthetase, the first terpene cyclase, the second terpene cyclase, and the third terpene cyclase compared to the parent *Trichoderma* strain when cultivated under identical conditions.

[12] The mutant of any of paragraphs 1-11, wherein the peptaibol synthetase gene encodes a polypeptide having peptaibol synthetase activity selected from the group consisting of: (a) a polypeptide comprising an amino acid sequence having at least 60%, e.g., at least 65%, at least 70%, at least 75%, at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to SEQ ID NO: 2; (b) a polypeptide encoded by a polynucleotide that hybridizes under at least low stringency conditions, at least medium stringency conditions, at least medium-high stringency conditions, at least high stringency conditions, or at least very high stringency conditions with SEQ ID NO: 1 or the cDNA sequence thereof; or the full-length complement thereof; and (c) a polypeptide encoded by a polynucleotide comprising a nucleotide sequence having at least 60%, e.g., at least 65%, at least 70%, at least 75%, at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to SEQ ID NO: 1 or the cDNA sequence thereof.

[13] The mutant of any of paragraphs 1-12, wherein the peptaibol synthetase gene encodes a polypeptide having peptaibol synthetase activity comprising or consisting of SEQ ID NO: 2.

[14] The mutant of any of paragraphs 1-13, wherein the paracelsin synthetase gene encodes a polypeptide having paracelsin synthetase activity selected from the group consisting of: (a) polypeptide comprising or consisting of an amino acid sequence having at least 60%, e.g., at least 65%, at least 70%, at least 75%, at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to SEQ ID

NO: 4; (b) a polypeptide encoded by a polynucleotide that hybridizes under at least low stringency conditions, at least medium stringency conditions, at least medium-high stringency conditions, at least high stringency conditions, or at least very high stringency conditions with SEQ ID NO: 3 or the cDNA sequence thereof; or the full-length complement thereof; and (c) a polypeptide encoded by a polynucleotide comprising or consisting of a nucleotide sequence having at least 60%, *e.g.*, at least 65%, at least 70%, at least 75%, at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to SEQ ID NO: 3 or the cDNA sequence thereof.

[15] The mutant of any of paragraphs 1-14, wherein the paracelsin synthetase gene encodes a polypeptide having paracelsin synthetase activity comprising or consisting of SEQ ID NO: 4.

[16] The mutant of any of paragraphs 1-15, wherein the first terpene cyclase gene encodes a polypeptide having terpene cyclase activity selected from the group consisting of: (a) a polypeptide comprising an amino acid sequence having at least 60%, *e.g.*, at least 65%, at least 70%, at least 75%, at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to SEQ ID NO: 6; (b) a polypeptide encoded by a polynucleotide that hybridizes under at least low stringency conditions, at least medium stringency conditions, at least medium-high stringency conditions, at least high stringency conditions, or at least very high stringency conditions with SEQ ID NO: 5 or the genomic DNA sequence thereof; or the full-length complement thereof; and (c) a polypeptide encoded by a polynucleotide comprising a nucleotide sequence having at least 60%, *e.g.*, at least 65%, at least 70%, at least 75%, at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to SEQ ID NO: 5 or the genomic DNA sequence thereof.

[17] The mutant of any of paragraphs 1-16, wherein the first terpene cyclase gene encodes a polypeptide having terpene cyclase activity comprising or consisting of SEQ ID NO: 6.

[18] The mutant of any of paragraphs 1-17, wherein the second terpene cyclase gene encodes a polypeptide having terpene cyclase activity selected from the group consisting of: (a) a polypeptide comprising or consisting of an amino acid sequence having at least 60%, *e.g.*, at least 65%, at least 70%, at least 75%, at least 80%, at least 81%, at least 82%, at

least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to SEQ ID NO: 8; (b) a polypeptide encoded by a polynucleotide that hybridizes under at least low stringency conditions, at least medium stringency conditions, at least medium-high stringency conditions, at least high stringency conditions, or at least very high stringency conditions with SEQ ID NO: 7 or the genomic DNA sequence thereof; or the full-length complement thereof; and (c) a polypeptide encoded by a polynucleotide comprising or consisting of a nucleotide sequence having at least 60%, *e.g.*, at least 65%, at least 70%, at least 75%, at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to SEQ ID NO: 7 or the genomic DNA sequence thereof.

[19] The mutant of any of paragraphs 1-18, wherein the second terpene cyclase gene encodes a polypeptide having terpene cyclase activity comprising or consisting of SEQ ID NO: 8.

[20] The mutant of any of paragraphs 1-19, wherein the third terpene cyclase gene encodes a polypeptide having terpene cyclase activity selected from the group consisting of: (a) a polypeptide comprising an amino acid sequence having at least 60%, *e.g.*, at least 65%, at least 70%, at least 75%, at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to SEQ ID NO: 10; (b) a polypeptide encoded by a polynucleotide that hybridizes under at least low stringency conditions, at least medium stringency conditions, at least medium-high stringency conditions, at least high stringency conditions, or at least very high stringency conditions with SEQ ID NO: 9 or the genomic DNA sequence thereof; or the full-length complement thereof; and (c) a polypeptide encoded by a polynucleotide comprising a nucleotide sequence having at least 60%, *e.g.*, at least 65%, at least 70%, at least 75%, at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to SEQ ID NO: 9 or the genomic DNA sequence thereof.

[21] The mutant of any of paragraphs 1-20, wherein the third terpene cyclase gene encodes a polypeptide having terpene cyclase activity comprising or consisting of SEQ ID NO: 10.

[22] A method of producing a heterologous polypeptide, comprising: cultivating a

mutant of a parent *Trichoderma* strain in a medium for the production of the heterologous polypeptide, wherein the mutant strain comprises a polynucleotide encoding the heterologous polypeptide and one or more genes selected from the group consisting of a peptaibol synthetase gene, a paracelsin synthetase gene, a first terpene cyclase gene, a second terpene cyclase gene, and a third terpene cyclase gene, wherein one or more of the genes are modified rendering the mutant strain deficient in the production of one or more enzymes selected from the group consisting of a peptaibol synthetase, a paracelsin synthetase, a first terpene cyclase, a second terpene cyclase, and a third terpene cyclase compared to the parent *Trichoderma* strain when cultivated under identical conditions.

[23] The method of paragraph 22, further comprising recovering the heterologous polypeptide from the cultivation medium.

[24] The method of paragraph 22 or 23, wherein the mutant comprises a modification of the peptaibol synthetase gene.

[25] The method of any of paragraphs 22-24, wherein the mutant comprises a modification of the paracelsin synthetase gene.

[26] The method of any of paragraphs 22-25, wherein the mutant comprises a modification of the first terpene cyclase gene.

[27] The method of any of paragraphs 22-26, wherein the mutant comprises a modification of the second terpene cyclase gene.

[28] The method of any of paragraphs 22-27, wherein the mutant comprises a modification of the third terpene cyclase gene.

[29] The method of any of paragraphs 22-28, wherein the mutant comprises a modification of the peptaibol synthetase gene, the paracelsin synthetase gene, the first terpene cyclase gene, the second terpene cyclase gene, and the third terpene cyclase gene.

[30] The method of any of paragraphs 22-29, wherein the *Trichoderma* strain is selected from the group consisting of *Trichoderma harzianum*, *Trichoderma koningii*, *Trichoderma longibrachiatum*, *Trichoderma reesei*, and *Trichoderma viride*.

[31] The method of any of paragraphs 22-30, wherein the *Trichoderma* strain is *Trichoderma reesei*.

[32] The method of any of paragraphs 22-31, wherein the mutant strain produces at least 25% less of one or more of the enzymes selected from the group consisting of the peptaibol synthetase, the paracelsin synthetase, the first terpene cyclase, the second terpene cyclase, and the third terpene cyclase compared to the parent *Trichoderma* strain when cultivated under identical conditions.

[33] The method of any of paragraphs 22-32, wherein the mutant strain is completely deficient in one or more of the enzymes selected from the group consisting of the peptaibol synthetase, the paracelsin synthetase, the first terpene cyclase, the second terpene cyclase,

and the third terpene cyclase compared to the parent *Trichoderma* strain when cultivated under identical conditions.

[34] The method of any of paragraphs 22-33, wherein the peptaibol synthetase gene encodes a polypeptide having peptaibol synthetase activity selected from the group consisting of: (a) a polypeptide comprising an amino acid sequence having at least 60%, e.g., at least 65%, at least 70%, at least 75%, at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to SEQ ID NO: 2; (b) a polypeptide encoded by a polynucleotide that hybridizes under at least low stringency conditions, at least medium stringency conditions, at least medium-high stringency conditions, at least high stringency conditions, or at least very high stringency conditions with SEQ ID NO: 1 or the cDNA sequence thereof; or the full-length complement thereof; and (c) a polypeptide encoded by a polynucleotide comprising a nucleotide sequence having at least 60%, e.g., at least 65%, at least 70%, at least 75%, at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to SEQ ID NO: 1 or the cDNA sequence thereof.

[35] The method of any of paragraphs 22-34, wherein the peptaibol synthetase gene encodes a polypeptide having peptaibol synthetase activity comprising or consisting of SEQ ID NO: 2.

[36] The method of any of paragraphs 22-35, wherein the paracelsin synthetase gene encodes a polypeptide having paracelsin synthetase activity selected from the group consisting of: (a) a polypeptide comprising or consisting of an amino acid sequence having at least 60%, e.g., at least 65%, at least 70%, at least 75%, at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to SEQ ID NO: 4; (b) a polypeptide encoded by a polynucleotide that hybridizes under at least low stringency conditions, at least medium stringency conditions, at least medium-high stringency conditions, high stringency conditions, or very high stringency conditions with SEQ ID NO: 3 or the cDNA sequence thereof; or the full-length complement thereof; and (c) a polypeptide encoded by a polynucleotide comprising or consisting of a nucleotide sequence having at least 60%, e.g., at least 65%, at least 70%, at least 75%, at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at

least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to SEQ ID NO: 3 or the cDNA sequence thereof.

[37] The method of any of paragraphs 22-36, wherein the paracelsin synthetase gene encodes a polypeptide having paracelsin synthetase activity comprising or consisting of SEQ ID NO: 4.

[38] The method of any of paragraphs 22-37, wherein the first terpene cyclase gene encodes a polypeptide having terpene cyclase activity selected from the group consisting of: (a) a polypeptide comprising an amino acid sequence having at least 60%, *e.g.*, at least 65%, at least 70%, at least 75%, at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to SEQ ID NO: 6; (b) a polypeptide encoded by a polynucleotide that hybridizes under at least low stringency conditions, at least medium stringency conditions, at least medium-high stringency conditions, at least high stringency conditions, or at least very high stringency conditions with SEQ ID NO: 5 or the genomic DNA sequence thereof; or the full-length complement thereof; and (c) a polypeptide encoded by a polynucleotide comprising a nucleotide sequence having at least 60%, *e.g.*, at least 65%, at least 70%, at least 75%, at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to SEQ ID NO: 5 or the genomic DNA sequence thereof.

[39] The method of any of paragraphs 22-38, wherein the first terpene cyclase gene encodes a polypeptide having terpene cyclase activity comprising or consisting of SEQ ID NO: 6.

[40] The method of any of paragraphs 22-39, wherein the second terpene cyclase gene encodes a polypeptide having terpene cyclase activity selected from the group consisting of: (a) a polypeptide comprising or consisting of an amino acid sequence having at least 60%, *e.g.*, at least 65%, at least 70%, at least 75%, at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to SEQ ID NO: 8; (b) a polypeptide encoded by a polynucleotide that hybridizes under at least low stringency conditions, at least medium stringency conditions, at least medium-high stringency conditions, at least high stringency conditions, or at least very high stringency conditions with SEQ ID NO: 7 or the genomic DNA sequence thereof; or the full-length complement thereof; and (c) a polypeptide encoded by a polynucleotide comprising or

consisting of a nucleotide sequence having at least 60%, *e.g.*, at least 65%, at least 70%, at least 75%, at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to SEQ ID NO: 7 or the genomic DNA sequence thereof.

[41] The method of any of paragraphs 22-40, wherein the second terpene cyclase gene encodes a polypeptide having terpene cyclase activity comprising or consisting of SEQ ID NO: 8.

[42] The method of any of paragraphs 22-41, wherein the third terpene cyclase gene encodes a polypeptide having terpene cyclase activity selected from the group consisting of: (a) a polypeptide comprising an amino acid sequence having at least 60%, *e.g.*, at least 65%, at least 70%, at least 75%, at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to SEQ ID NO: 10; (b) a polypeptide encoded by a polynucleotide that hybridizes under at least low stringency conditions, at least medium stringency conditions, at least medium-high stringency conditions, at least high stringency conditions, or at least very high stringency conditions with SEQ ID NO: 9 or the genomic DNA sequence thereof; or the full-length complement thereof; and (c) a polypeptide encoded by a polynucleotide comprising a nucleotide sequence having at least 60%, *e.g.*, at least 65%, at least 70%, at least 75%, at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to SEQ ID NO: 9 or the genomic DNA sequence thereof.

[43] The method of any of paragraphs 22-42, wherein the third terpene cyclase gene encodes a polypeptide having terpene cyclase activity comprising or consisting of SEQ ID NO: 10.

[44] A method for obtaining a mutant of a parent *Trichoderma* strain, comprising: modifying one or more genes selected from the group consisting of a peptaibol synthetase gene, a paracelsin synthetase gene, a first terpene cyclase gene, a second terpene cyclase gene, and a third terpene cyclase gene; and identifying a mutant strain from step (a) wherein one or more of the genes are modified rendering the mutant strain deficient in the production of one or more of the enzymes selected from the group consisting of a peptaibol synthetase, a paracelsin synthetase, a first terpene cyclase, a second terpene cyclase, and a third terpene cyclase compared to the parent *Trichoderma* strain when cultivated under identical

conditions.

[45] The method of paragraph 44, wherein the mutant comprises a modification of the peptaibol synthetase gene.

[46] The method of paragraph 44 or 45, wherein the mutant comprises a modification
5 of the paracelsin synthetase gene.

[47] The method of any of paragraphs 44-46, wherein the mutant comprises a modification of the first terpene cyclase gene.

[48] The method of any of paragraphs 44-47, wherein the mutant comprises a modification of the second terpene cyclase gene.

[49] The method of any of paragraphs 44-48, wherein the mutant comprises a
10 modification of the third terpene cyclase gene.

[50] The method of any of paragraphs 44-49, wherein the mutant comprises a modification of the peptaibol synthetase gene, the paracelsin synthetase gene, the first terpene cyclase gene, the second terpene cyclase gene, and the third terpene cyclase gene.

[51] The method of any of paragraphs 44-50, wherein the *Trichoderma* strain is
15 selected from the group consisting of *Trichoderma harzianum*, *Trichoderma koningii*, *Trichoderma longibrachiatum*, *Trichoderma reesei*, and *Trichoderma viride*.

[52] The method of any of paragraphs 44-51, wherein the *Trichoderma* strain is
Trichoderma reesei.

[53] The method of any of paragraphs 44-52, wherein the mutant strain produces at
20 least 25% less of one or more of the enzymes selected from the group consisting of the peptaibol synthetase, the paracelsin synthetase, the first terpene cyclase, the second terpene cyclase, and the third terpene cyclase compared to the parent *Trichoderma* strain when cultivated under identical conditions.

[54] The method of any of paragraphs 44-53, wherein the mutant strain is completely
25 deficient in one or more of the enzymes selected from the group consisting of the peptaibol synthetase, the paracelsin synthetase, the first terpene cyclase, the second terpene cyclase, and the third terpene cyclase compared to the parent *Trichoderma* strain when cultivated under identical conditions.

[55] The method of any of paragraphs 44-54, wherein the peptaibol synthetase gene
30 encodes a polypeptide having peptaibol synthetase activity selected from the group consisting of: (a) a polypeptide comprising an amino acid sequence having at least 60%, e.g., at least 65%, at least 70%, at least 75%, at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%,
35 at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to SEQ ID NO: 2; (b) a polypeptide encoded by a polynucleotide that hybridizes under at least low stringency

conditions, at least medium stringency conditions, at least medium-high stringency conditions, at least high stringency conditions, or at least very high stringency conditions with SEQ ID NO: 1 or the cDNA sequence thereof; or the full-length complement thereof; and (c) a polypeptide encoded by a polynucleotide comprising a nucleotide sequence having at least 60%, e.g., at least 65%, at least 70%, at least 75%, at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to SEQ ID NO: 1 or the cDNA sequence thereof.

[56] The method of any of paragraphs 44-55, wherein the peptaibol synthetase gene encodes a polypeptide having peptaibol synthetase activity comprising or consisting of SEQ ID NO: 2.

[57] The method of any of paragraphs 44-56, wherein the paracelsin synthetase gene encodes a polypeptide having paracelsin synthetase activity selected from the group consisting of: (a) a polypeptide comprising or consisting of an amino acid sequence having at least 60%, e.g., at least 65%, at least 70%, at least 75%, at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to SEQ ID NO: 4; (b) a polypeptide encoded by a polynucleotide that hybridizes under at least low stringency conditions, at least medium stringency conditions, at least medium-high stringency conditions, at least high stringency conditions, or at least very high stringency conditions with SEQ ID NO: 3 or the cDNA sequence thereof; or the full-length complement thereof; and (c) a polypeptide encoded by a polynucleotide comprising or consisting of a nucleotide sequence having at least 60%, e.g., at least 65%, at least 70%, at least 75%, at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to SEQ ID NO: 3 or the cDNA sequence thereof.

[58] The method of any of paragraphs 44-57, wherein the paracelsin synthetase gene encodes a polypeptide having paracelsin synthetase activity comprising or consisting of SEQ ID NO: 4.

[59] The method of any of paragraphs 44-58, wherein the first terpene cyclase gene encodes a polypeptide having terpene cyclase activity selected from the group consisting of: (a) a polypeptide comprising an amino acid sequence having at least 60%, e.g., at least 65%, at least 70%, at least 75%, at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%,

at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to SEQ ID NO: 6; (b) a polypeptide encoded by a polynucleotide that hybridizes under at least low stringency conditions, at least medium stringency conditions, at least medium-high stringency conditions, high stringency conditions, or very high stringency conditions with SEQ ID NO: 5 or the genomic DNA sequence thereof; or the full-length complement thereof; and (c) a polypeptide encoded by a polynucleotide comprising a nucleotide sequence having at least 60%, e.g., at least 65%, at least 70%, at least 75%, at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to SEQ ID NO: 5 or the genomic DNA sequence thereof.

[60] The method of any of paragraphs 44-59, wherein the first terpene cyclase gene encodes a polypeptide having terpene cyclase activity comprising or consisting of SEQ ID NO: 6.

[61] The method of any of paragraphs 44-60, wherein the second terpene cyclase gene encodes a polypeptide having terpene cyclase activity selected from the group consisting of: (a) a polypeptide comprising or consisting of an amino acid sequence having at least 60%, e.g., at least 65%, at least 70%, at least 75%, at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to SEQ ID NO: 8; (b) a polypeptide encoded by a polynucleotide that hybridizes under at least low stringency conditions, at least medium stringency conditions, at least medium-high stringency conditions, at least high stringency conditions, or at least very high stringency conditions with SEQ ID NO: 7 or the genomic DNA sequence thereof; or the full-length complement thereof; and (c) a polypeptide encoded by a polynucleotide comprising or consisting of a nucleotide sequence having at least 60%, e.g., at least 65%, at least 70%, at least 75%, at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to SEQ ID NO: 7 or the genomic DNA sequence thereof.

[62] The method of any of paragraphs 44-61, wherein the second terpene cyclase gene encodes a polypeptide having terpene cyclase activity comprising or consisting of SEQ ID NO: 8.

[63] The method of any of paragraphs 44-62, wherein the third terpene cyclase gene

encodes a polypeptide having terpene cyclase activity selected from the group consisting of:
(a) a polypeptide comprising an amino acid sequence having at least 60%, *e.g.*, at least 65%, at least 70%, at least 75%, at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%,
5 at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to SEQ ID NO: 10; (b) a polypeptide encoded by a polynucleotide that hybridizes under at least low stringency conditions, at least medium stringency conditions, at least medium-high stringency conditions, at least high stringency conditions, or at least very high stringency conditions with
10 SEQ ID NO: 9 or the genomic DNA sequence thereof; or the full-length complement thereof; and (c) a polypeptide encoded by a polynucleotide comprising a nucleotide sequence having at least 60%, *e.g.*, at least 65%, at least 70%, at least 75%, at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%,
15 at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to SEQ ID NO: 9 or the genomic DNA sequence thereof.

[64] The method of any of paragraphs 44-63, wherein the third terpene cyclase gene encodes a polypeptide having terpene cyclase activity comprising or consisting of SEQ ID NO: 10.

20 The invention described and claimed herein is not to be limited in scope by the specific aspects herein disclosed, since these aspects are intended as illustrations of several aspects of the invention. Any equivalent aspects are intended to be within the scope of this invention. Indeed, various modifications of the invention in addition to those shown and
25 described herein will become apparent to those skilled in the art from the foregoing description. Such modifications are also intended to fall within the scope of the appended claims. In the case of conflict, the present disclosure including definitions will control. Various references are cited herein, the disclosures of which are incorporated by reference in their entireties.

Claims

What is claimed is:

- 5 1. A mutant of a parent *Trichoderma* strain, comprising a polynucleotide encoding a heterologous polypeptide and one or more genes selected from the group consisting of a peptaibol synthetase gene, a paracelsin synthetase gene, a first terpene cyclase gene, a second terpene cyclase gene, and a third terpene cyclase gene, wherein one or more of the genes are modified rendering the mutant strain deficient in the production of one or more
10 enzymes selected from the group consisting of a peptaibol synthetase, a paracelsin synthetase, a first terpene cyclase, a second terpene cyclase, and a third terpene cyclase compared to the parent *Trichoderma* strain when cultivated under identical conditions.
- 15 2. The mutant of claim 1, wherein the peptaibol synthetase gene encodes a polypeptide having peptaibol synthetase activity selected from the group consisting of: (a) a polypeptide comprising an amino acid sequence having at least 60% sequence identity to SEQ ID NO: 2; (b) a polypeptide encoded by a polynucleotide that hybridizes under at least low stringency conditions with SEQ ID NO: 1 or the cDNA sequence thereof; or the full-length complement thereof; (c) a polypeptide encoded by a polynucleotide comprising a nucleotide sequence
20 having at least 60% sequence identity to SEQ ID NO: 1 or the cDNA sequence thereof; and (d) a polypeptide comprising or consisting of SEQ ID NO: 2.
- 25 3. The mutant of claim 1 or 2, wherein the paracelsin synthetase gene encodes a polypeptide having paracelsin synthetase activity selected from the group consisting of: (a) polypeptide comprising or consisting of an amino acid sequence having at least 60% sequence identity to SEQ ID NO: 4; (b) a polypeptide encoded by a polynucleotide that hybridizes under at least low stringency conditions with SEQ ID NO: 3 or the cDNA sequence thereof; or the full-length complement thereof; (c) a polypeptide encoded by a polynucleotide comprising or consisting of a nucleotide sequence having at least 60%
30 sequence identity to SEQ ID NO: 3 or the cDNA sequence thereof; and (d) a polypeptide comprising or consisting of SEQ ID NO: 4.
- 35 4. The mutant of any of claims 1-3, wherein the first terpene cyclase gene encodes a polypeptide having terpene cyclase activity selected from the group consisting of: (a) a polypeptide comprising an amino acid sequence having at least 60% sequence identity to SEQ ID NO: 6; (b) a polypeptide encoded by a polynucleotide that hybridizes under at least low stringency conditions with SEQ ID NO: 5 or the genomic DNA sequence thereof; or the

full-length complement thereof; (c) a polypeptide encoded by a polynucleotide comprising a nucleotide sequence having at least 60% sequence identity to SEQ ID NO: 5 or the genomic DNA sequence thereof; and (d) a polypeptide comprising or consisting of SEQ ID NO: 6.

5 5. The mutant of any of claims 1-4, wherein the second terpene cyclase gene encodes a polypeptide having terpene cyclase activity selected from the group consisting of: (a) a polypeptide comprising or consisting of an amino acid sequence having at least 60% sequence identity to SEQ ID NO: 8; (b) a polypeptide encoded by a polynucleotide that hybridizes under at least low stringency conditions with SEQ ID NO: 7 or the genomic DNA
10 sequence thereof; or the full-length complement thereof; (c) a polypeptide encoded by a polynucleotide comprising or consisting of a nucleotide sequence having at least 60% sequence identity to SEQ ID NO: 7 or the genomic DNA sequence thereof; and (d) a polypeptide comprising or consisting of SEQ ID NO: 8.

15 6. The mutant of any of claims 1-5, wherein the third terpene cyclase gene encodes a polypeptide having terpene cyclase activity selected from the group consisting of: (a) a polypeptide comprising an amino acid sequence having at least 60% sequence identity to SEQ ID NO: 10; (b) a polypeptide encoded by a polynucleotide that hybridizes under at least low stringency conditions with SEQ ID NO: 9 or the genomic DNA sequence thereof; or the
20 full-length complement thereof; (c) a polypeptide encoded by a polynucleotide comprising a nucleotide sequence having at least 60% sequence identity to SEQ ID NO: 9 or the genomic DNA sequence thereof; and (d) a polypeptide comprising or consisting of SEQ ID NO: 10.

7. A method of producing a heterologous polypeptide, comprising: cultivating a mutant
25 of a parent *Trichoderma* strain in a medium for the production of the heterologous polypeptide, wherein the mutant strain comprises a polynucleotide encoding the heterologous polypeptide and one or more genes selected from the group consisting of a peptaibol synthetase gene, a paracelsin synthetase gene, a first terpene cyclase gene, a second terpene cyclase gene, and a third terpene cyclase gene, wherein one or more of the
30 genes are modified rendering the mutant strain deficient in the production of one or more enzymes selected from the group consisting of a peptaibol synthetase, a paracelsin synthetase, a first terpene cyclase, a second terpene cyclase, and a third terpene cyclase compared to the parent *Trichoderma* strain when cultivated under identical conditions.

35 8. The method of claim 7, further comprising recovering the heterologous polypeptide from the cultivation medium.

9. The method of claim 7 or 8, wherein the peptaibol synthetase gene encodes a polypeptide having peptaibol synthetase activity selected from the group consisting of: (a) a polypeptide comprising an amino acid sequence having at least 60% sequence identity to SEQ ID NO: 2; (b) a polypeptide encoded by a polynucleotide that hybridizes under at least low stringency conditions with SEQ ID NO: 1 or the cDNA sequence thereof; or the full-length complement thereof; (c) a polypeptide encoded by a polynucleotide comprising a nucleotide sequence having at least 60% sequence identity to SEQ ID NO: 1 or the cDNA sequence thereof; and (d) a polypeptide comprising or consisting of SEQ ID NO: 2.

10. The method of any of claims 7-9, wherein the paracelsin synthetase gene encodes a polypeptide having paracelsin synthetase activity selected from the group consisting of: (a) polypeptide comprising or consisting of an amino acid sequence having at least 60% sequence identity to SEQ ID NO: 4; (b) a polypeptide encoded by a polynucleotide that hybridizes under at least low stringency conditions with SEQ ID NO: 3 or the cDNA sequence thereof; or the full-length complement thereof; (c) a polypeptide encoded by a polynucleotide comprising or consisting of a nucleotide sequence having at least 60% sequence identity to SEQ ID NO: 3 or the cDNA sequence thereof; and (d) a polypeptide comprising or consisting of SEQ ID NO: 4.

11. The method of any of claims 7-10, wherein the first terpene cyclase gene encodes a polypeptide having terpene cyclase activity selected from the group consisting of: (a) a polypeptide comprising an amino acid sequence having at least 60% sequence identity to SEQ ID NO: 6; (b) a polypeptide encoded by a polynucleotide that hybridizes under at least low stringency conditions with SEQ ID NO: 5 or the genomic DNA sequence thereof; or the full-length complement thereof; (c) a polypeptide encoded by a polynucleotide comprising a nucleotide sequence having at least 60% sequence identity to SEQ ID NO: 5 or the genomic DNA sequence thereof; and (d) a polypeptide comprising or consisting of SEQ ID NO: 6.

12. The method of any of claims 7-11, wherein the second terpene cyclase gene encodes a polypeptide having terpene cyclase activity selected from the group consisting of: (a) a polypeptide comprising or consisting of an amino acid sequence having at least 60% sequence identity to SEQ ID NO: 8; (b) a polypeptide encoded by a polynucleotide that hybridizes under at least low stringency conditions with SEQ ID NO: 7 or the genomic DNA sequence thereof; or the full-length complement thereof; (c) a polypeptide encoded by a polynucleotide comprising or consisting of a nucleotide sequence having at least 60% sequence identity to SEQ ID NO: 7 or the genomic DNA sequence thereof; and (d) a polypeptide comprising or consisting of SEQ ID NO: 8.

13. The method of any of claims 7-12, wherein the third terpene cyclase gene encodes a polypeptide having terpene cyclase activity selected from the group consisting of: (a) a polypeptide comprising an amino acid sequence having at least 60% sequence identity to SEQ ID NO: 10; (b) a polypeptide encoded by a polynucleotide that hybridizes under at least low stringency conditions with SEQ ID NO: 9 or the genomic DNA sequence thereof; or the full-length complement thereof; (c) a polypeptide encoded by a polynucleotide comprising a nucleotide sequence having at least 60% sequence identity to SEQ ID NO: 9 or the genomic DNA sequence thereof; and (d) a polypeptide comprising or consisting of SEQ ID NO: 10.

14. A method for obtaining a mutant of a parent *Trichoderma* strain, comprising: modifying one or more genes selected from the group consisting of a peptaibol synthetase gene, a paracelsin synthetase gene, a first terpene cyclase gene, a second terpene cyclase gene, and a third terpene cyclase gene; and identifying a mutant strain from step (a) wherein one or more of the genes are modified rendering the mutant strain deficient in the production of one or more of the enzymes selected from the group consisting of a peptaibol synthetase, a paracelsin synthetase, a first terpene cyclase, a second terpene cyclase, and a third terpene cyclase compared to the parent *Trichoderma* strain when cultivated under identical conditions.

15. The method of claim 14, wherein the peptaibol synthetase gene encodes a polypeptide having peptaibol synthetase activity selected from the group consisting of: (a) a polypeptide comprising an amino acid sequence having at least 60% sequence identity to SEQ ID NO: 2; (b) a polypeptide encoded by a polynucleotide that hybridizes under at least low stringency conditions with SEQ ID NO: 1 or the cDNA sequence thereof; or the full-length complement thereof; (c) a polypeptide encoded by a polynucleotide comprising a nucleotide sequence having at least 60% sequence identity to SEQ ID NO: 1 or the cDNA sequence thereof; and (d) a polypeptide comprising or consisting of SEQ ID NO: 2.

16. The method of claim 14 or 15, wherein the paracelsin synthetase gene encodes a polypeptide having paracelsin synthetase activity selected from the group consisting of: (a) a polypeptide comprising or consisting of an amino acid sequence having at least 60% sequence identity to SEQ ID NO: 4; (b) a polypeptide encoded by a polynucleotide that hybridizes under at least low stringency conditions with SEQ ID NO: 3 or the cDNA sequence thereof; or the full-length complement thereof; (c) a polypeptide encoded by a polynucleotide comprising or consisting of a nucleotide sequence having at least 60% sequence identity to SEQ ID NO: 3 or the cDNA sequence thereof; and (d) a polypeptide

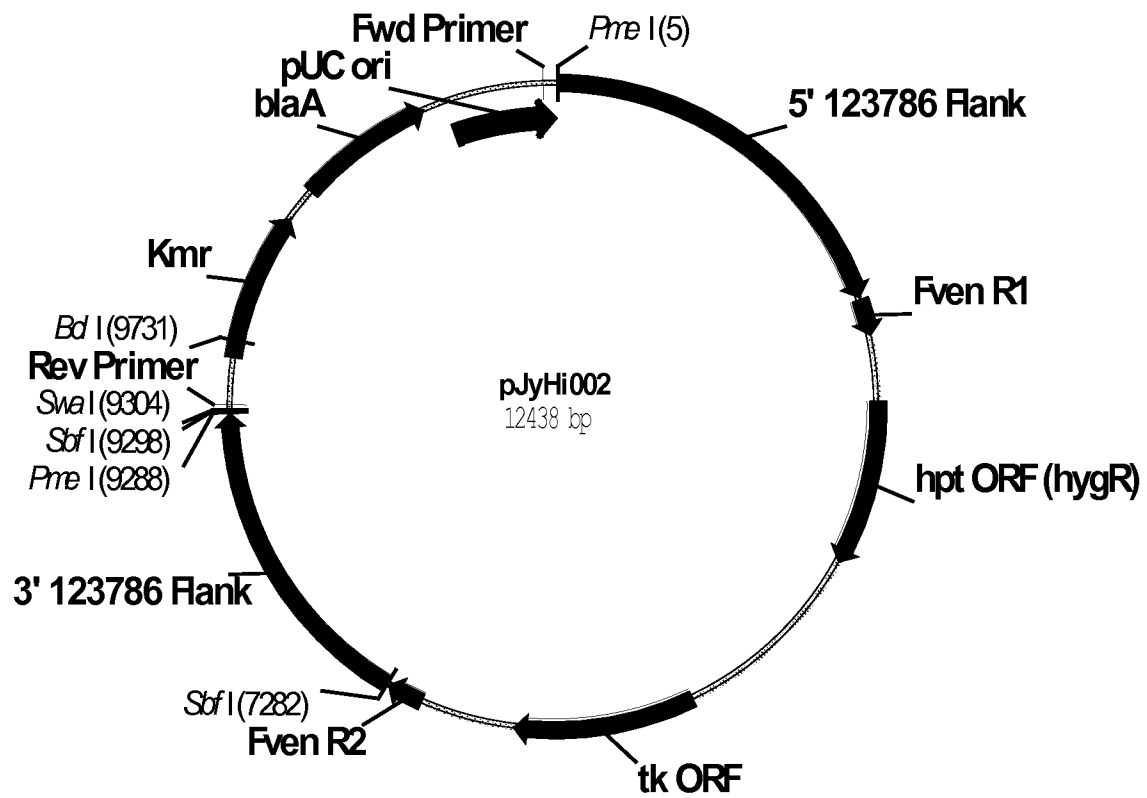
comprising or consisting of SEQ ID NO: 4.

17. The method of any of claims 14-16, wherein the first terpene cyclase gene encodes a polypeptide having terpene cyclase activity selected from the group consisting of: (a) a polypeptide comprising an amino acid sequence having at least 60% sequence identity to SEQ ID NO: 6; (b) a polypeptide encoded by a polynucleotide that hybridizes under at least low stringency conditions with SEQ ID NO: 5 or the genomic DNA sequence thereof; or the full-length complement thereof; (c) a polypeptide encoded by a polynucleotide comprising a nucleotide sequence having at least 60% sequence identity to SEQ ID NO: 5 or the genomic DNA sequence thereof; and (d) a polypeptide comprising or consisting of SEQ ID NO: 6.

18. The method of any of claims 14-17, wherein the second terpene cyclase gene encodes a polypeptide having terpene cyclase activity selected from the group consisting of: (a) a polypeptide comprising or consisting of an amino acid sequence having at least 60% sequence identity to SEQ ID NO: 8; (b) a polypeptide encoded by a polynucleotide that hybridizes under at least low stringency conditions with SEQ ID NO: 7 or the genomic DNA sequence thereof; or the full-length complement thereof; (c) a polypeptide encoded by a polynucleotide comprising or consisting of a nucleotide sequence having at least 60% sequence identity to SEQ ID NO: 7 or the genomic DNA sequence thereof; and (d) a polypeptide comprising or consisting of SEQ ID NO: 8.

19. The method of any of claims 14-18, wherein the third terpene cyclase gene encodes a polypeptide having terpene cyclase activity selected from the group consisting of: (a) a polypeptide comprising an amino acid sequence having at least 60% sequence identity to SEQ ID NO: 10; (b) a polypeptide encoded by a polynucleotide that hybridizes under at least low stringency conditions with SEQ ID NO: 9 or the genomic DNA sequence thereof; or the full-length complement thereof; (c) a polypeptide encoded by a polynucleotide comprising a nucleotide sequence having at least 60% sequence identity to SEQ ID NO: 9 or the genomic DNA sequence thereof; and (d) a polypeptide comprising or consisting of SEQ ID NO: 10.

1/2

**Fig. 1**

2/2

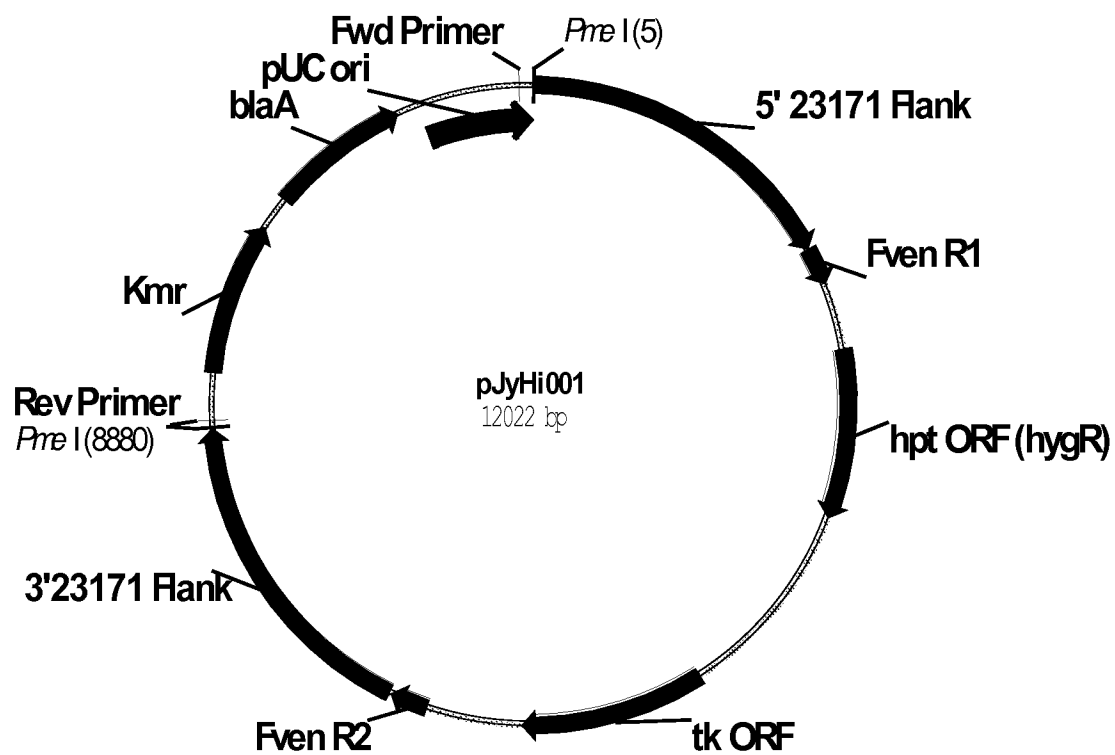


Fig. 2

INTERNATIONAL SEARCH REPORT

International application No

PCT/US2013/076977

A. CLASSIFICATION OF SUBJECT MATTER

INV. C12P21/02 C12N15/52 C12N15/60 C12N9/00 C12N9/88
ADD.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C12P C12N

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

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

EPO-Internal, BIOSIS, Sequence Search, FSTA, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2012/001169 A1 (DSM IP ASSETS BV [NL]; PEIJ VAN NOEL NICOLAAS MARIA ELISABETH [NL]; HA) 5 January 2012 (2012-01-05)	1-3,7-10
A	the whole document	14
	claims 1,10,18,19,26,37	

A	WO 2005/056772 A1 (NOVOZYMES INC [US]; NOVOZYMES AS [DK]) 23 June 2005 (2005-06-23)	1,7,14
	cited in the application	
	the whole document	
	page 13, lines 17,18	
	claims 1,6,12,17,23,28	

	-/-	

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

☒ See patent family annex.

* Special categories of cited documents :

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

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

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

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search

26 February 2014

Date of mailing of the international search report

15/05/2014

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2013/076977

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

2, 3, 9, 10, 15, 16(completely); 1, 7, 8, 14(partially)

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No

PCT/US2013/076977

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	P. K. MUKHERJEE ET AL: "Two Classes of New Peptaibols Are Synthesized by a Single Non-ribosomal Peptide Synthetase of <i>Trichoderma virens</i> ", JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 286, no. 6, 11 February 2011 (2011-02-11), pages 4544-4554, XP055101937, ISSN: 0021-9258, DOI: 10.1074/jbc.M110.159723 cited in the application the whole document	1-3,7,9, 10,14-16
X	----- A. WIEST: "Identification of Peptaibols from <i>Trichoderma virens</i> and Cloning of a Peptaibol Synthetase", JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 277, no. 23, 21 March 2002 (2002-03-21), pages 20862-20868, XP055102121, ISSN: 0021-9258, DOI: 10.1074/jbc.M201654200 the whole document	1-3,7,9, 10,14-16
X	----- WEI XIANYING ET AL: "Multiple non-ribosomal peptide synthetase genes determine peptaibol synthesis in <i>Trichoderma virens</i> ", CANADIAN JOURNAL OF MICROBIOLOGY, vol. 51, no. 5, 20 July 2005 (2005-07-20), pages 423-429, XP009173578, ISSN: 0008-4166 the whole document	1-3,7,9, 10,14-16
X	----- P. K. MUKHERJEE ET AL: "Secondary metabolism in <i>Trichoderma</i> - a genomic perspective", MICROBIOLOGY, vol. 158, no. 1, 13 October 2011 (2011-10-13), pages 35-45, XP055085056, ISSN: 1350-0872, DOI: 10.1099/mic.0.053629-0 cited in the application the whole document ----- -/-	1-3,7,9, 10,14-16

INTERNATIONAL SEARCH REPORT

International application No

PCT/US2013/076977

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	MALA MUKHERJEE ET AL: "A secondary metabolite biosynthesis cluster in <i>Trichoderma virens</i>: evidence from analysis of genes underexpressed in a mutant defective in morphogenesis and antibiotic production", CURRENT GENETICS, vol. 50, no. 3, 28 June 2006 (2006-06-28), pages 193-202, XP019423623, ISSN: 1432-0983, DOI: 10.1007/S00294-006-0075-0 the whole document -----	1,7,14
A	OWEN P. WARD: "Production of recombinant proteins by filamentous fungi", BIOTECHNOLOGY ADVANCES, vol. 30, no. 5, 24 September 2011 (2011-09-24), pages 1119-1139, XP055088348, ISSN: 0734-9750, DOI: 10.1016/j.biotechadv.2011.09.012 the whole document -----	1,7,14
A	SU XIAOYUN ET AL: "Heterologous gene expression in filamentous fungi.", ADVANCES IN APPLIED MICROBIOLOGY, vol. 81, 2012, pages 1-61, XP000816743, ISSN: 0065-2164 the whole document -----	1,7,14
A	CHRISTIAN P KUBICEK ET AL: "Comparative genome sequence analysis underscores mycoparasitism as the ancestral life style of <i>Trichoderma</i>", GENOME BIOLOGY, vol. 12, no. 4, 18 April 2011 (2011-04-18) , page R40, XP021102327, ISSN: 1465-6906, DOI: 10.1186/GB-2011-12-4-R40 cited in the application the whole document -----	1,7,14
A	J A Vizcaíno ET AL: "Detection of peptaibols and partial cloning of a putative peptaibol synthetase gene from <i>T. harzianum</i> CECT 2413", Folia microbiologica, 2006, pages 114-120, XP055102127, Czech Republic DOI: 10.1007/BF02932165 Retrieved from the Internet: URL: http://hal.archives-ouvertes.fr/hal-00148358/en/ the whole document ----- -/--	1,7,14

INTERNATIONAL SEARCH REPORT

International application No

PCT/US2013/076977

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	THOMAS DEGENKOLB ET AL: "The Production of Multiple Small Peptaibol Families by Single 14-Module Peptide Synthetases in Trichoderma/Hypocrea", CHEMISTRY & BIODIVERSITY, vol. 9, no. 3, 1 March 2012 (2012-03-01), pages 499-535, XP055101946, ISSN: 1612-1872, DOI: 10.1002/cbdv.201100212 the whole document	1,7,14
E	----- WO 2014/025605 A1 (DANISCO US INC [US]) 13 February 2014 (2014-02-13) the whole document -----	1-3, 7-10, 14-16

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/US2013/076977

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2012001169	A1	05-01-2012	
		AU 2011273351 A1	17-01-2013
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WO 2014025605	A1	13-02-2014	NONE

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 2, 3, 9, 10, 15, 16(completely); 1, 7, 8, 14(partially)

A mutant of a parent *Trichoderma* strain, comprising a polynucleotide encoding a heterologous polypeptide and one or more genes selected from the group consisting of a peptaibol synthetase gene and a paracelsin synthetase gene, wherein one or more of the genes are modified rendering the mutant strain deficient in the production of one or more enzymes selected from the group consisting of a peptaibol synthetase and a paracelsin synthetase compared to the parent *Trichoderma* strain when cultivated under identical conditions. A method of producing a heterologous polypeptide, comprising: cultivating a mutant of a parent *Trichoderma* strain in a medium for the production of the heterologous polypeptide, wherein the mutant strain comprises a polynucleotide encoding the heterologous polypeptide and one or more genes selected from the group consisting of a peptaibol synthetase gene and a paracelsin synthetase gene, wherein one or more of the genes are modified rendering the mutant strain deficient in the production of one or more enzymes selected from the group consisting of a peptaibol synthetase and a paracelsin synthetase compared to the parent *Trichoderma* strain when cultivated under identical conditions. A method for obtaining a mutant of a parent *Trichoderma* strain, comprising: modifying one or more genes selected from the group consisting of a peptaibol synthetase gene and a paracelsin synthetase gene; and identifying a mutant strain from step (a) wherein one or more of the genes are modified rendering the mutant strain deficient in the production of one or more of the enzymes selected from the group consisting of a peptaibol synthetase and a paracelsin synthetase compared to the parent *Trichoderma* strain when cultivated under identical conditions.

2. claims: 4-6, 11-13, 17-19(completely); 1, 7, 8, 14(partially)

A mutant of a parent *Trichoderma* strain, comprising a polynucleotide encoding a heterologous polypeptide and one or more genes selected from the group consisting of a first terpene cyclase gene, a second terpene cyclase gene, and a third terpene cyclase gene, wherein one or more of the genes are modified rendering the mutant strain deficient in the production of one or more enzymes selected from the group consisting of a first terpene cyclase, a second terpene cyclase, and a third terpene cyclase compared to the parent *Trichoderma* strain when cultivated under identical conditions. A method of producing a heterologous polypeptide, comprising: cultivating a mutant of a parent *Trichoderma* strain in a medium for the production of the heterologous polypeptide, wherein the mutant strain

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

comprises a polynucleotide encoding the heterologous polypeptide and one or more genes selected from the group consisting of a first terpene cyclase gene, a second terpene cyclase gene, and a third terpene cyclase gene, wherein one or more of the genes are modified rendering the mutant strain deficient in the production of one or more enzymes selected from the group consisting of a first terpene cyclase, a second terpene cyclase, and a third terpene cyclase compared to the parent *Trichoderma* strain when cultivated under identical conditions. A method for obtaining a mutant of a parent *Trichoderma* strain, comprising: modifying one or more genes selected from the group consisting of a first terpene cyclase gene, a second terpene cyclase gene, and a third terpene cyclase gene; and identifying a mutant strain from step (a) wherein one or more of the genes are modified rendering the mutant strain deficient in the production of one or more of the enzymes selected from the group consisting of a first terpene cyclase, a second terpene cyclase, and a third terpene cyclase compared to the parent *Trichoderma* strain when cultivated under identical conditions.
