(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
27 September 2018 (27.09.2018)

WIPO | PCT

(10) International Publication Number
WO 2018/172155 A1

(51) International Patent Classification:
C12P 21/00 (2006.01) *C12N 9/10* (2006.01)
C12N 1/14 (2006.01)

(21) International Application Number:
PCT/EP2018/056377

(22) International Filing Date:
14 March 2018 (14.03.2018)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
17162530.4 23 March 2017 (23.03.2017) EP

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report (Art. 21(3))*
— *with sequence listing part of description (Rule 5.2(a))*

WO 2018/172155 A1

(54) Title: IMPROVED FILAMENTOUS FUNGAL HOST CELLS

(57) Abstract: The present invention relates to improved ChsB-inactivated filamentous fungal cells producing a heterologous polypeptide of interest, methods of producing a heterologous polypeptide of interest in said cells as well as methods of producing said cells.

Improved Filamentous Fungal Host Cells

Reference to sequence listing

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

FIELD OF THE INVENTION

The present invention relates to improved ChsB-inactivated filamentous fungal cells producing a heterologous polypeptide of interest, methods of producing a heterologous polypeptide of interest in said cells as well as methods of producing said cells.

BACKGROUND OF THE INVENTION

The *Aspergillus nidulans* chitin synthase ChsB encoded by the *chsB* gene is necessary for normal hyphal growth and development, its inactivation by site-specifically inserting the native *A. nidulans argB* gene encoding ornithine transcarbamylase into the *chsB* coding sequence was disclosed (Borgia PT. *et al.* 1996. Fungal Genet Biol. 1996 Sep; 20(3): 193-203). The inactivation of *chsB* by *pyrG* disruption in *Aspergillus oryzae* has also been disclosed (Müller C. *et al.* 2003. Biotechnol Bioeng. 2003 Mar 5; 81(5): 525-34).

A reporter system for the identification of antifungal compounds in filamentous fungi through cell wall stress-induced promoters to drive expression of a reporter protein has been disclosed; one such induced promoter was the *Aspergillus niger agsA* promoter; the cloning of the full-length *agsA* gene using degenerate primers as well as the isolation of *ags*-homologues from other fungi was provided (WO 03/020922). The inactivation of *agsA* in *A. niger* has also been described (Damveld R. *et al.* 2004. Fungal Genetics and Biology 42 (2005) 165–177).

SUMMARY OF THE INVENTION

The present invention is directed to improved genetically modified filamentous fungal host cells producing a heterologous polypeptide, in which host cells the *chsB* gene has been inactivated. Inactivation of the *chsB* gene may be done by any suitable gene inactivation method known in the art. An example of a convenient way to eliminate or reduce *chsB* expression is based on techniques of gene replacement or gene interruption. The productivity or yield of the heterologous polypeptide is higher in the improved host cells of the invention, wherein the *chsB* gene is inactivated, as compared to *chsB* wild-type host cells.

Specifically, the inactivation of *chsB* in an *Aspergillus niger* filamentous fungal host cell resulted in increased yield of a heterologous phospholipase enzyme of interest produced by said cell.

Accordingly, in a first aspect, the invention relates to filamentous fungal host cells producing a heterologous polypeptide of interest and comprising an inactivated chitin synthase-encoding gene, where said chitin synthase-encoding gene in its active form:

i) encodes a chitin synthase polypeptide having at least 80% amino acid sequence identity with SEQ ID NO:3;

ii) comprises or consists of a genomic nucleotide sequence at least 80% identical to the genomic DNA sequence shown in SEQ ID NO:1; and/or

iii) comprises or consists of a genomic nucleotide sequence, the cDNA sequence of which is at least 80% identical to the cDNA sequence shown in SEQ ID NO:2.

The invention further provides methods for producing a heterologous polypeptide of interest by cultivating a filamentous fungal host cell of the invention under conditions conducive for expression of the heterologous polypeptide of interest and, optionally, recovering the heterologous polypeptide of interest.

Accordingly, in a second aspect, the invention relates to methods of producing a heterologous polypeptide of interest, said method comprising the steps of:

a) cultivating a filamentous fungal host cell producing the heterologous polypeptide of interest and comprising an inactivated chitin synthase-encoding gene under conditions conducive to the production of the heterologous polypeptide of interest, where said chitin synthase-encoding gene in its active form:

i) encodes a chitin synthase polypeptide having at least 80% amino acid sequence identity with SEQ ID NO:3;

ii) comprises or consists of a genomic nucleotide sequence at least 80% identical to the genomic DNA sequence shown in SEQ ID NO:1; and/or

iii) comprises or consists of a genomic nucleotide sequence, the cDNA sequence of which is at least 80% identical to the cDNA sequence shown in SEQ ID NO:2;

and, optionally,

b) recovering the heterologous polypeptide of interest.

In a final aspect, the invention relates to method of producing a filamentous fungal host cell having an improved productivity of a heterologous polypeptide of interest, said method comprising the following steps in no particular order:

a) transforming a filamentous fungal host cell with a polynucleotide encoding the heterologous polypeptide of interest; and

b) inactivating a chitin synthase-encoding gene in the filamentous fungal host cell, wherein said chitin synthase-encoding gene in its active form:

i) encodes a chitin synthase polypeptide having at least 80% amino acid sequence identity with SEQ ID NO:3;

ii) comprises or consists of a genomic nucleotide sequence at least 80% identical to the genomic DNA sequence shown in SEQ ID NO:1; and/or

iii) comprises or consists of a genomic nucleotide sequence, the cDNA sequence of which is at least 80% identical to the cDNA sequence shown in SEQ ID NO:2.

DEFINITIONS

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 or prokaryotic 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 mature polypeptide of the present invention. 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.

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.

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 of the present invention. 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).

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. It is known in the art that a host cell may produce a mixture of two of more different mature polypeptides (*i.e.*, with a different C-terminal and/or N-terminal amino acid) expressed by the same polynucleotide. It is also known in the art that different host cells process polypeptides differently, and thus, one host cell expressing a polynucleotide may produce a different mature polypeptide (*e.g.*, having a different C-terminal and/or N-terminal amino acid) as compared to another host cell expressing the same polynucleotide.

Mature polypeptide coding sequence: The term "mature polypeptide coding sequence" means a polynucleotide that encodes a mature polypeptide

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.

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 gap open penalty of 10, 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:

(Identical Residues x 100)/(Length of Alignment – 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 gap open penalty of 10, 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:

(Identical Deoxyribonucleotides x 100)/(Length of Alignment – Total Number of Gaps in Alignment)

DETAILED DESCRIPTION OF THE INVENTION

Host Cells

The present invention relates to recombinant host cells comprising a polynucleotide of the present invention operably linked to one or more control sequences that direct the production of a heterologous polypeptide of interest.

A construct or vector comprising a polynucleotide is introduced into a host cell so that the construct or vector is maintained as a chromosomal integrant or as a self-replicating extra-chromosomal vector as described earlier. 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. The choice of a host cell will to a large extent depend upon the gene encoding the polypeptide and its source.

The host cell may be a fungal cell. "Fungi" as used herein includes the phyla Ascomycota, Basidiomycota, Chytridiomycota, and Zygomycota as well as the Oomycota and all mitosporic fungi (as defined by Hawksworth *et al.*, *In*, *Ainsworth and Bisby's Dictionary of The Fungi*, 8th edition, 1995, CAB International, University Press, Cambridge, UK).

The fungal host cell of the invention is a filamentous fungal cell. "Filamentous fungi" include all filamentous forms of the subdivision Eumycota and Oomycota (as defined by Hawksworth *et al.*, 1995, *supra*). The filamentous fungi are generally characterized by a mycelial wall composed of chitin, cellulose, glucan, chitosan, mannan, and other complex polysaccharides. Vegetative growth is by hyphal elongation and carbon catabolism is obligately aerobic.

The filamentous fungal host cell may be an *Acremonium*, *Aspergillus*, *Aureobasidium*, *Bjerkandera*, *Ceriporiopsis*, *Chrysosporium*, *Coprinus*, *Coriolus*, *Cryptococcus*, *Filibasidium*, *Fusarium*, *Humicola*, *Magnaporthe*, *Mucor*, *Myceliophthora*, *Neocallimastix*, *Neurospora*, *Paecilomyces*, *Penicillium*, *Phanerochaete*, *Phlebia*, *Piromyces*, *Pleurotus*, *Schizophyllum*, *Talaromyces*, *Thermoascus*, *Thielavia*, *Tolypocladium*, *Trametes*, or *Trichoderma* cell.

For example, the filamentous fungal host cell may be an *Aspergillus awamori*, *Aspergillus foetidus*, *Aspergillus fumigatus*, *Aspergillus japonicus*, *Aspergillus nidulans*, *Aspergillus niger*, *Aspergillus oryzae*, *Bjerkandera adusta*, *Ceriporiopsis aneirina*, *Ceriporiopsis caregiea*, *Ceriporiopsis gilvescens*, *Ceriporiopsis pannocinta*, *Ceriporiopsis rivulosa*, *Ceriporiopsis subrufa*, *Ceriporiopsis subvermispora*, *Chrysosporium inops*, *Chrysosporium keratinophilum*,

Chrysosporium lucknowense, *Chrysosporium merdarium*, *Chrysosporium pannicola*, *Chrysosporium queenslandicum*, *Chrysosporium tropicum*, *Chrysosporium zonatum*, *Coprinus cinereus*, *Coriolus hirsutus*, *Fusarium bactridioides*, *Fusarium cerealis*, *Fusarium crookwellense*, *Fusarium culmorum*, *Fusarium graminearum*, *Fusarium graminum*, *Fusarium heterosporum*, *Fusarium negundi*, *Fusarium oxysporum*, *Fusarium reticulatum*, *Fusarium roseum*, *Fusarium sambucinum*, *Fusarium sarcochroum*, *Fusarium sporotrichioides*, *Fusarium sulphureum*, *Fusarium torulosum*, *Fusarium trichothecioides*, *Fusarium venenatum*, *Humicola insolens*, *Humicola lanuginosa*, *Mucor miehei*, *Myceliophthora thermophila*, *Neurospora crassa*, *Penicillium purpurogenum*, *Phanerochaete chrysosporium*, *Phlebia radiata*, *Pleurotus eryngii*, *Thielavia terrestris*, *Trametes villosa*, *Trametes versicolor*, *Trichoderma harzianum*, *Trichoderma koningii*, *Trichoderma longibrachiatum*, *Trichoderma reesei*, or *Trichoderma viride* cell.

Fungal cells may be transformed by a process involving protoplast formation, transformation of the protoplasts, and regeneration of the cell wall in a manner known *per se*. Suitable procedures for transformation of *Aspergillus* and *Trichoderma* host cells are described in EP 238023, Yelton *et al.*, 1984, *Proc. Natl. Acad. Sci. USA* 81: 1470-1474, and Christensen *et al.*, 1988, *Bio/Technology* 6: 1419-1422. Suitable methods for transforming *Fusarium* species are described by Malardier *et al.*, 1989, *Gene* 78: 147-156, and WO 96/00787.

In one aspect, the invention relates to methods of producing a filamentous fungal host cell having an improved productivity of a heterologous polypeptide of interest, said method comprising the following steps in no particular order:

a) transforming a filamentous fungal host cell with a polynucleotide encoding the heterologous polypeptide of interest; and

b) inactivating a chitin synthase-encoding gene in the filamentous fungal host cell, wherein said chitin synthase-encoding gene in its active form:

i) encodes a chitin synthase polypeptide having at least 80% amino acid sequence identity with SEQ ID NO:3;

ii) comprises or consists of a genomic nucleotide sequence at least 80% identical to the genomic DNA sequence shown in SEQ ID NO:1; and/or

iii) comprises or consists of a genomic nucleotide sequence, the cDNA sequence of which is at least 80% identical to the cDNA sequence shown in SEQ ID NO:2

In another aspect, the invention relates to the resulting host cells; filamentous fungal host cell producing a heterologous polypeptide of interest and comprising an inactivated chitin synthase-encoding gene, where said chitin synthase-encoding gene in its active form:

i) encodes a chitin synthase polypeptide having at least 80% amino acid sequence identity with SEQ ID NO:3;

ii) comprises or consists of a genomic nucleotide sequence at least 80% identical to the genomic DNA sequence shown in SEQ ID NO:1; and/or

iii) comprises or consists of a genomic nucleotide sequence, the cDNA sequence of which is at least 80% identical to the cDNA sequence shown in SEQ ID NO:2.

In a preferred embodiment of the aspects of the invention, the filamentous fungal host cell is of a genus selected from the group consisting of *Acremonium*, *Aspergillus*, *Aureobasidium*, *Bjerkandera*, *Ceriporiopsis*, *Chrysosporium*, *Coprinus*, *Coriolus*, *Cryptococcus*, *Filibasidium*, *Fusarium*, *Humicola*, *Magnaporthe*, *Mucor*, *Myceliophthora*, *Neocallimastix*, *Neurospora*, *Paecilomyces*, *Penicillium*, *Phanerochaete*, *Phlebia*, *Piromyces*, *Pleurotus*, *Schizophyllum*, *Talaromyces*, *Thermoascus*, *Thielavia*, *Tolypocladium*, *Trametes* and *Trichoderma*; even more preferably the filamentous fungal host cell is an *Aspergillus* cell; preferably an *Aspergillus awamori*, *Aspergillus foetidus*, *Aspergillus fumigatus*, *Aspergillus japonicus*, *Aspergillus nidulans*, *Aspergillus niger* or an *Aspergillus oryzae* cell.

Preferably, the heterologous polypeptide of interest is an enzyme; preferably the enzyme is a hydrolase, isomerase, ligase, lyase, oxidoreductase, or transferase, *e.g.*, an aminopeptidase, amylase, carbohydrase, carboxypeptidase, catalase, cellobiohydrolase, cellulase, chitinase, cutinase, cyclodextrin glycosyltransferase, deoxyribonuclease, endoglucanase, esterase, alpha-galactosidase, beta-galactosidase, glucoamylase, alpha-glucosidase, beta-glucosidase, invertase, laccase, lipase, mannosidase, mutanase, oxidase, pectinolytic enzyme, peroxidase, phospholipase, phytase, polyphenoloxidase, proteolytic enzyme, ribonuclease, transglutaminase, xylanase, or beta-xylosidase. In a preferred embodiment, the heterologous enzyme is secreted.

In a preferred embodiment of the invention, the ChsB chitin synthase polypeptide comprises or consists of an amino acid sequence at least 80% identical to the amino acid sequence shown in SEQ ID NO:3; preferably at least 85%, 90%, 95%, 96%, 97%, 98% or most preferably at least 99% identical to the amino acid sequence shown in SEQ ID NO:3.

Preferably, the *chsB* gene or homologue thereof in its active form, *i.e.* before its inactivation, comprises or consists of a genomic nucleotide sequence at least 80% identical to the genomic DNA sequence shown in SEQ ID NO:1; preferably at least 85%, 90%, 95%, 96%, 97%, 98% or most preferably at least 99% identical to the genomic DNA sequence shown in SEQ ID NO:1. Alternatively, the *chsB* gene or homologue thereof in its active form comprises or consists of a genomic nucleotide sequence, the cDNA sequence of which is at least 80% identical to the cDNA sequence shown in SEQ ID NO:2; preferably at least 85%, 90%, 95%, 96%, 97%, 98% or most preferably at least 99% identical to the cDNA sequence shown in SEQ ID NO:2.

In a preferred embodiment of the invention, the filamentous fungal host cells further comprises an inactivated 1,3-alpha-D-glucan synthase-encoding *agsA* gene, wherein said *agsA* gene in its active form:

i) encodes an 1,3-alpha-D-glucan synthase AgsA polypeptide having at least 80% amino acid sequence identity with SEQ ID NO:6; preferably at least 85%, 90%, 95%, 96%, 97%, 98% or most preferably at least 99% identical to the amino acid sequence shown in SEQ ID NO:6;

ii) comprises or consists of a genomic nucleotide sequence at least 80% identical to the genomic DNA sequence shown in SEQ ID NO:4; preferably at least 85%, 90%, 95%, 96%, 97%, 98% or most preferably at least 99% identical to the genomic DNA sequence shown in SEQ ID NO:4; and/or

iii) comprises or consists of a genomic nucleotide sequence, the cDNA sequence of which is at least 80% identical to the cDNA sequence shown in SEQ ID NO:5; preferably at least 85%, 90%, 95%, 96%, 97%, 98% or most preferably at least 99% identical to the cDNA sequence shown in SEQ ID NO:5.

Nucleic Acid Constructs

The present invention also relates to nucleic acid constructs comprising a polynucleotide of the present invention operably linked to one or more control sequences that direct the expression of the coding sequence in a suitable host cell under conditions compatible with the control sequences.

The polynucleotide may be manipulated in a variety of ways to provide for expression of the polypeptide. Manipulation of the polynucleotide prior to its insertion into a vector may be desirable or necessary depending on the expression vector. The techniques for modifying polynucleotides utilizing recombinant DNA methods are well known in the art.

The control sequence may be a promoter, a polynucleotide that is recognized by a host cell for expression of a polynucleotide encoding a polypeptide of the present invention. The promoter contains transcriptional control sequences that mediate the expression of the polypeptide. The promoter may be any polynucleotide that shows transcriptional activity in the host cell including mutant, truncated, and hybrid promoters, and may be obtained from genes encoding extracellular or intracellular polypeptides either homologous or heterologous to the host cell.

Examples of suitable promoters for directing transcription of the nucleic acid constructs of the present invention in a filamentous fungal host cell 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, 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 the host cell may be used in the present invention.

Preferred terminators for filamentous fungal host cells 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 an mRNA stabilizer region downstream of a promoter and upstream of the coding sequence of a gene which increases expression of the gene.

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

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 polynucleotide and, when transcribed, is recognized by the host cell as a signal to add polyadenosine residues to transcribed mRNA. Any polyadenylation sequence that is functional in the host cell may be used.

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

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 a host cell may be used.

Effective signal peptide coding sequences for filamentous fungal host cells are the signal peptide coding sequences 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 sequence may be obtained from the genes for *Bacillus subtilis* alkaline protease (*aprE*), *Bacillus subtilis* neutral protease (*nprT*), *Myceliophthora thermophila* laccase (WO 95/33836), *Rhizomucor miehei* aspartic proteinase, and *Saccharomyces cerevisiae* alpha-factor.

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 host cell. 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. In filamentous fungi, 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.

Expression Vectors

The present invention also relates to recombinant expression vectors comprising a polynucleotide of the present invention, a promoter, and transcriptional and translational stop signals. 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 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 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, 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.

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 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 adenyltransferase), and *trpC* (anthranilate synthase), as well as equivalents thereof. Preferred for use in an *Aspergillus* cell are *Aspergillus nidulans* or *Aspergillus oryzae amdS* and *pyrG* genes and a *Streptomyces hygroscopicus bar* gene. Preferred for use in a *Trichoderma* cell are *adeA*, *adeB*, *amdS*, *hph*, and *pyrG* 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.

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 of the present invention 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, *supra*).

Removal or Reduction of ChsB Activity

The present invention also relates to methods of producing a mutant of a parent cell, which comprises inactivating, disrupting or deleting a polynucleotide, or a portion thereof, encoding a ChsB chitin synthase polypeptide of the present invention, which results in the mutant cell producing less of the ChsB chitin synthase polypeptide than the parent cell when cultivated under the same conditions. In a preferred embodiment, the mutant cells is completely deficient in producing a ChsB chitin synthase polypeptide of the invention.

The mutant cell may be constructed by reducing or eliminating expression of the *chsB* polynucleotide or a homologue thereof using methods well known in the art, for example, insertions, disruptions, replacements, or deletions. In a preferred aspect, the polynucleotide is inactivated. The polynucleotide to be modified or inactivated may be, for example, the coding region or a part thereof essential for activity, or a regulatory element required for expression of the coding region. An example of such a regulatory or control sequence may be a promoter sequence or a functional part thereof, *i.e.*, a part that is sufficient for affecting expression of the polynucleotide. Other control sequences for possible modification include, but are not limited to, a leader, polyadenylation sequence, propeptide sequence, signal peptide sequence, transcription terminator, and transcriptional activator.

Modification or inactivation of the polynucleotide may be performed by subjecting the parent cell to mutagenesis and selecting for mutant cells in which expression of the polynucleotide has been reduced or eliminated. The mutagenesis, which may be specific or random, may be performed, for example, by use of a suitable physical or chemical mutagenizing agent, by use of a suitable oligonucleotide, or by subjecting the DNA sequence to PCR generated mutagenesis. Furthermore, the mutagenesis may be performed by use of any combination of these mutagenizing agents.

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), O-methyl hydroxylamine, 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 cell to be mutagenized in the presence of the mutagenizing agent of choice under suitable conditions, and screening and/or selecting for mutant cells exhibiting reduced or no expression of the gene.

Modification or inactivation of the *chsB* polynucleotide or homologue thereof may be accomplished by insertion, substitution, or deletion of one or more nucleotides in the gene or a regulatory element required for transcription or translation thereof. For example, nucleotides may be inserted or removed so as to result in the introduction of a stop codon, the removal of the start codon, or a change in the open reading frame. Such modification or inactivation may be accomplished by site-directed mutagenesis or PCR generated mutagenesis in accordance with

methods known in the art. Although, in principle, the modification may be performed *in vivo*, *i.e.*, directly on the cell expressing the polynucleotide to be modified, it is preferred that the modification be performed *in vitro* as exemplified below.

Methods for deleting or disrupting a targeted gene are described, for example, by Miller, et al (1985. Mol. Cell. Biol. 5:1714-1721); WO 90/00192; May, G. (1992. Applied Molecular Genetics of Filamentous Fungi. J. R. Kinghorn and G. Turner, eds., Blackie Academic and Professional, pp. 1-25); and Turner, G. (1994. Vectors for Genetic Manipulation. S. D. Martinelli and J. R. Kinghorn, eds., Elsevier, pp. 641-665).

An example of a convenient way to eliminate or reduce expression of a polynucleotide is based on techniques of gene replacement, gene deletion, or gene disruption. For example, in the gene disruption method, a nucleic acid sequence corresponding to the endogenous polynucleotide is mutagenized *in vitro* to produce a defective nucleic acid sequence that is then transformed into the parent cell to produce a defective gene. By homologous recombination, the defective nucleic acid sequence replaces the endogenous polynucleotide. It may be desirable that the defective polynucleotide also encodes a marker that may be used for selection of transformants in which the polynucleotide has been modified or destroyed. In an aspect, the polynucleotide is disrupted with a selectable marker such as those described herein.

The present invention also relates to methods of inhibiting the expression of a polypeptide having ChsB activity in a cell, comprising administering to the cell or expressing in the cell a double-stranded RNA (dsRNA) molecule, wherein the dsRNA comprises a subsequence of a chsB polynucleotide or homologue thereof. In a preferred aspect, the dsRNA is about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25 or more duplex nucleotides in length.

The dsRNA is preferably a small interfering RNA (siRNA) or a micro RNA (miRNA). In a preferred aspect, the dsRNA is small interfering RNA for inhibiting transcription. In another preferred aspect, the dsRNA is micro RNA for inhibiting translation.

The present invention also relates to such double-stranded RNA (dsRNA) molecules, comprising a portion of the polypeptide coding sequence of SEQ ID NO: 1 for inhibiting expression of the polypeptide in a cell. While the present invention is not limited by any particular mechanism of action, the dsRNA can enter a cell and cause the degradation of a single-stranded RNA (ssRNA) of similar or identical sequences, including endogenous mRNAs. When a cell is exposed to dsRNA, mRNA from the homologous gene is selectively degraded by a process called RNA interference (RNAi); see, for example, U.S. Patent No. 5,190,931.

The dsRNAs of the present invention can be used in gene-silencing. In one aspect, the invention provides methods to selectively degrade RNA using a dsRNAi of the present invention. The process may be practiced *in vitro*, *ex vivo* or *in vivo*. In one aspect, the dsRNA molecules can be used to generate a loss-of-function mutation in a cell, an organ or an animal. Methods for making and using dsRNA molecules to selectively degrade RNA are well known in the art; see, for example, U.S. Patent Nos. 6,489,127; 6,506,559; 6,511,824 and 6,515,109.

The ChsB chitin synthase polypeptide-deficient mutant cells are particularly useful as host cells for expression of a heterologous polypeptide of interest; preferably a secreted heterologous polypeptide of interest; preferable embodiments thereof may be found herein.

The methods used for cultivation and purification of the heterologous polypeptide of interest may be performed by methods known in the art.

Methods of Production

The host cells are cultivated in a nutrient medium suitable for production of the polypeptide using methods known in the art. For example, the cells 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 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). If the polypeptide is secreted into the nutrient medium, the polypeptide can be recovered directly from the medium. If the polypeptide is not secreted, it can be recovered from cell lysates.

The polypeptide may be detected using methods known in the art that are specific for the polypeptides. These detection methods include, but are not limited to, use of specific antibodies, formation of an enzyme product, or disappearance of an enzyme substrate. For example, an enzyme assay may be used to determine the activity of the polypeptide.

The polypeptide may be recovered using methods known in the art. For example, the polypeptide may be recovered from the nutrient medium by conventional procedures including, but not limited to, collection, centrifugation, filtration, extraction, spray-drying, evaporation, or precipitation. In one aspect, a fermentation broth comprising the polypeptide is recovered.

The polypeptide may be 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), differential solubility (*e.g.*, ammonium sulfate precipitation), SDS-PAGE, or extraction (see, *e.g.*, *Protein Purification*, Janson and Ryden, editors, VCH Publishers, New York, 1989) to obtain substantially pure polypeptides.

In an alternative aspect, the polypeptide is not recovered, but rather a host cell of the present invention expressing the polypeptide is used as a source of the polypeptide.

One aspect of the invention relates to methods of producing a heterologous polypeptide of interest, said method comprising the steps of:

a) cultivating a filamentous fungal host cell producing the heterologous polypeptide of interest and comprising an inactivated chitin synthase-encoding gene under conditions

conducive to the production of the heterologous polypeptide of interest, where said chitin synthase-encoding gene in its active form:

i) encodes a chitin synthase polypeptide having at least 80% amino acid sequence identity with SEQ ID NO:3;

ii) comprises or consists of a genomic nucleotide sequence at least 80% identical to the genomic DNA sequence shown in SEQ ID NO:1; and/or

iii) comprises or consists of a genomic nucleotide sequence, the cDNA sequence of which is at least 80% identical to the cDNA sequence shown in SEQ ID NO:2;

and, optionally,

b) recovering the heterologous polypeptide of interest.

In a preferred embodiment, the filamentous fungal host cell is of a genus selected from the group consisting of *Acremonium*, *Aspergillus*, *Aureobasidium*, *Bjerkandera*, *Ceriporiopsis*, *Chrysosporium*, *Coprinus*, *Coriolus*, *Cryptococcus*, *Filibasidium*, *Fusarium*, *Humicola*, *Magnaporthe*, *Mucor*, *Myceliophthora*, *Neocallimastix*, *Neurospora*, *Paecilomyces*, *Penicillium*, *Phanerochaete*, *Phlebia*, *Piromyces*, *Pleurotus*, *Schizophyllum*, *Talaromyces*, *Thermoascus*, *Thielavia*, *Tolypocladium*, *Trametes* and *Trichoderma*; even more preferably the filamentous fungal host cell is an *Aspergillus* cell; preferably an *Aspergillus awamori*, *Aspergillus foetidus*, *Aspergillus fumigatus*, *Aspergillus japonicus*, *Aspergillus nidulans*, *Aspergillus niger* or an *Aspergillus oryzae* cell.

Preferably, the heterologous polypeptide of interest is an enzyme; preferably the enzyme is a hydrolase, isomerase, ligase, lyase, oxidoreductase, or transferase, *e.g.*, an aminopeptidase, amylase, carbohydrase, carboxypeptidase, catalase, cellobiohydrolase, cellulase, chitinase, cutinase, cyclodextrin glycosyltransferase, deoxyribonuclease, endoglucanase, esterase, alpha-galactosidase, beta-galactosidase, glucoamylase, alpha-glucosidase, beta-glucosidase, invertase, laccase, lipase, mannosidase, mutanase, oxidase, pectinolytic enzyme, peroxidase, phytase, polyphenoloxidase, proteolytic enzyme, ribonuclease, transglutaminase, xylanase, or beta-xylosidase. In a preferred embodiment, the heterologous enzyme is secreted.

In a preferred embodiment of the invention, the ChsB chitin synthase polypeptide comprises or consists of an amino acid sequence at least 80% identical to the amino acid sequence shown in SEQ ID NO:3; preferably at least 85%, 90%, 95%, 96%, 97%, 98% or most preferably at least 99% identical to the amino acid sequence shown in SEQ ID NO:3.

Preferably, the *chsB* gene or homologue thereof in its active form comprises or consists of a genomic nucleotide sequence at least 80% identical to the genomic DNA sequence shown in SEQ ID NO:1; preferably at least 85%, 90%, 95%, 96%, 97%, 98% or most preferably at least 99% identical to the genomic DNA sequence shown in SEQ ID NO:1. Alternatively, the *chsB* gene or homologue thereof in its active form comprises or consists of a genomic nucleotide sequence, the cDNA sequence of which is at least 80% identical to the cDNA sequence shown in SEQ ID

NO:2; preferably at least 85%, 90%, 95%, 96%, 97%, 98% or most preferably at least 99% identical to the cDNA sequence shown in SEQ ID NO:2.

EXAMPLES

Materials and methods

Molecular cloning techniques are described in Sambrook,J., Fritsch,E.F., Maniatis,T. (1989) Mo-lecular cloning: a laboratory manual (2nd edn.) Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Enzymes

Enzymes for DNA manipulations (e.g. restriction endonucleases, ligases etc.) are obtainable from New England Biolabs, Inc. and were used according to the manufacturer's instructions.

Media and Solutions

AMG trace metals solution was composed of 0.3 g of citric acid, 0.68 g of $ZnCl_2$, 0.25 g of $CuSO_4 \cdot 5H_2O$, 0.024 g of $NiCl_2 \cdot 6H_2O$, 1.39 g of $FeSO_4 \cdot 7H_2O$, 1.356 g of $MnSO_4 \cdot 5H_2O$, and deionized water to 1 liter.

COVE-N-gly plates were composed of 218 g of sorbitol, 10 g of glycerol, 2.02 g of KNO_3, 50 ml of COVE salt solution, 25 g of Noble agar, and deionized water to 1 liter.

COVE medium was composed of 342.3 g of sucrose, 20 ml of 50X COVE salts solution, 10 ml of 1 M acetamide, 10 ml of 1.5 M CsCl2, 25 g of Noble agar, and deionized water to 1 liter.

COVE2 medium was composed of 30 g of sucrose, 20 ml of 50X COVE salts solution, 10 ml of 1 M acetamide, 25 g of Noble agar, and deionized water to 1 liter.

COVE-N plates were composed of 342.3 g of sucrose, 20 ml of COVE salt solution, 3 g of $NaNO_3$, 30 g of Noble agar, and deionized water to 1 liter.

COVE-N top agarose was composed of 342.3 g of sucrose, 20 ml of COVE salt solution, 3 g of $NaNO_3$, 10 g of low melt agarose, and deionized water to 1 liter.

COVE-N JP plates were composed of 30 g of sucrose, 20 ml of COVE salt solution, 3 g of $NaNO_3$, 30 g of Noble agar, and deionized water to 1 liter.

COVE salt solution was composed of 26 g of KCl, 26 g of $MgSO_4 \cdot 7H_2O$, 76 g of KH_2PO_4, 50 ml of COVE trace metals solution, and deionized water to 1 liter.

COVE trace metals was composed of 0.04 g of $Na_2B_4O_7 \cdot 10H_2O$, 0.4 g of $CuSO_4 \cdot 5H_2O$, 1.2 g of $FeSO_4 \cdot 7H_2O$, 1.0 g of $MnSO_4 \cdot 5H_2O$, 0.8 g of $Na_2MoO_4 \cdot 2H_2O$, 10 g of $ZnSO_4 \cdot 7H_2O$, and deionized water to 1 liter.

LB medium was composed of 10 g of tryptone, 5 g of yeast extract, 5 g of sodium chloride, and deionized water to 1 liter.

LB plus ampicillin plates were composed of 10 g of tryptone, 5 g of yeast extract, 5 g of sodium chloride, 15 g of Bacto agar, ampicillin at 100 µg per ml, and deionized water to 1 liter.

MSS medium was composed of 70 g of sucrose, 100 g of soy bean powder, three drops of pluronic antifoam, and deionized water to 1 liter; pH adjusted to 6.0.

MU-1 glu medium without urea was composed of 260 g of glucose, 3 g of $MgSO_4 \cdot 7H_2O$, 6 g of K_2SO_4, 5 g of KH_2PO_4, 0.5 ml of AMG trace metals solution, a few drops of antifoam, and deionized water to 1 liter; pH adjusted to 4.5.

50% Urea was composed of 500 g of urea and deionized water to 1 liter.

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

STC was composed of 0.8 M sorbitol, 25 mM or 50 mM Tris pH 8, and 25 mM or 50 mM $CaCl_2$.

SPTC was composed of 40% polyethyleneglycol 4000 (PEG4000) in STC buffer.

SOC medium was composed of 20 g of tryptone, 5 g of yeast extract, 0.5 g of NaCl, 10 ml of 250 mM KCl, and deionized water to 1 liter.

TAE buffer was composed of 4.84 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.

Purchased materials (*E.coli*, plasmid and kits)

E.coli DH5-alpha (Toyobo) was used for plasmid construction and PCR amplification. The commercial plasmids pBluescript II SK- (Stratagene #212206) were used for cloning of PCR fragments. Amplified plasmids were recovered with Qiagen Plasmid Kit (Qiagen). Ligation was done with DNA ligation kit (Takara) or T4 DNA ligase (Boehringer Mannheim). Polymerase Chain Reaction (PCR) was carried out with Expand TM PCR system (Boehringer Mannheim). QIAquickTM Gel Extraction Kit (Qiagen) was used for the purification of PCR fragments and extraction of DNA fragments from agarose gel.

Strains

The expression host strain *Aspergillus niger* C3105 was isolated by Novozymes and is a derivative of *Aspergillus niger* NN049184 which was isolated from soil. C3105 is genetically modified to disrupt expression of amyloglycosidase activities and alpha-amylase activities followed by introducing *Aspergillus niger* cytosine deaminase gene (*fcy1*).

The expression host strain *Aspergillus niger* M1405-1435-16 was isolated by Novozymes and is an *agsA*-minus derivative of *Aspergillus niger* C3105 (its construction is disclosed in Example 3 of WO 2016/066690).

Plasmids

Plasmid pHUda801 is disclosed in Example 4 of WO 2012/160093.

Plasmid pRika147 for the expression of enzyme genes is also disclosed in Example 9 of WO 2012/160093.

Transformation of *Aspergillus niger*

Transformation of the parent *Aspergillus niger* host cell was achieved using the general methods known for transformation in filamentous fungi, as described in the Yelton et al., "Transformation of *Aspergillus nidulans* by using a trpC plasmid," Proc Natl Acad Sci U S A. 1984 Mar;81(5):1470-4, and as follows:

Aspergillus niger host strain was inoculated to 100 ml of YPG medium supplemented with 10 mM uridine in case the host strain is a pyrG deficient mutant, and incubated for 16 hrs at 32°C at 80 rpm. Pellets were collected and washed with 0.6 M KCl, and resuspended 20 ml 0.6 M KCl containing a commercial □-glucanase product (GLUCANEX™, Novozymes A/S, Bagsværd, Denmark) at a final concentration of 20 mg per ml. The suspension was incubated at 32 °C at 80 rpm until protoplasts were formed, and then washed twice with STC buffer. The protoplasts were counted with a hematometer and resuspended and adjusted in an 8:2:0.1 solution of STC:STPC:DMSO to a final concentration of $2.5x10^7$ protoplasts/ml. Approximately 4µg of plasmid DNA was added to 100 µl of the protoplast suspension, mixed gently, and incubated on ice for 30 minutes. One ml of SPTC was added and the protoplast suspension was incubated for 20 minutes at 37°C. After the addition of 10 ml of 50°C COVE-N top agarose, the mixture was poured onto the minimum medium and the plates were incubated at 30°C for 5days.

PCR amplification

5x PCR buffer (incl.MgCl2)	20 µl
2.5mM dNTP mix	10 µl
Forward primer (100 µM)	1 µl
Reverse primer (100 µM)	1 µl
Expand High Fidelity polymerase (Roche)	1 µl
Template DNA (50-100 ng/ µl)	1 µl
Distilled water to	100 µl

PCR conditions

94°C	2 min	1 cycle
92°C	1 min	
55°C	1min	30 cycles
72°C	1-2 min	
72°C	7 min	1 cycle

Shake flask cultivation for reporter enzyme production

Spores of the selected transformants were inoculated in 100 ml of MSS media and cultivated at 30 C for 3 days. 10 % of seed culture was transferred to MU-1 glu medium in lab-scale tanks with feeding the appropriate amounts of glucose and ammonium and cultivated at 30 C for 6 days. The supernatant was obtained by centrifugation. Culture supernatant after centrifugation was used for enzyme assay.

Southern hybridization

Mycelia of the selected transformants were harvested from overnight culture in 3 ml YPG medium, rinsed with distilled water. Ground mycelia were subject to genome DNA preparation using FastDNA SPIN Kit for Soil (MP Biomedicals) follows by manufacture's instruction. Non-radioactive probes were synthesized using a PCR DIG probe synthesis kit (Roche Applied Science, Indianapolis IN) followed by manufacture's instruction. DIG labeled probes were gel purified using a QIAquickTM Gel Extraction Kit (QIAGEN Inc., Valencia, CA) according to the manufacturer's instructions.

Five micrograms of genome DNA was digested with appropriate restriction enzymes completely for 16 hours (40 µl total volume, 4U enzyme/µl DNA) and run on a 0.8 % agarose gel. The DNA was fragmented in the gel by treating with 0.2 M HCl, denatured (0.5M NaOH, 1.5M NaCl) and neutralized (1M Tris, pH7.5; 1.5M NaCl) for subsequent transfer in 20X SSC to Hybond N+ membrane (Amersham). The DNA was UV cross-linked to the membrane and prehybridized for 1 hour at 42 oC in 20 ml DIG Easy Hyb (Roche Diagnostics Corporation, Mannheim, Germany). The denatured probe was added directly to the DIG Easy Hyb buffer and an overnight hybridization at 42 oC was done. Following the post hybridization washes (twice in 2X SSC, room temperature, 5 min and twice in 0.1X SSC, 68o C, 15 min. each), chemiluminescent detection using the DIG detection system and CPD-Star (Roche) was done followed by manufacture's protocol. The DIG-labeled DNA Molecular Weight Marker II (Roche) was used for the standard marker.

Bradford assay (whole protein determination)

The Bradford assay, a colorimetric protein assay, is based on an absorbance shift of the dye Coomassie Brilliant Blue G-250 in which under acidic conditions the red form of the dye is converted into its bluer form to bind to the protein being assayed. The binding of the dye to the protein stabilizes the blue anionic form. The increase of absorbance at 595 nm is proportional to the amount of bound dye, and thus to the amount (concentration) of protein present in the sample.

Enzyme samples were diluted by distilled water appropriately and were measured their protein amounts by use of Quick Start™ Bradford Protein Assay (Bio-Rad inc.) followed by manufactures' instruction.

Example 1: Construction of plasmid pHUda1435, a vector for targeted gene disruption of *Aspergillus niger chsB*

Plasmid pHUda1435 was constructed to contain 5' and 3' flanking regions for the *Aspergillus niger* chitin synthase (*chsB*) gene separated by the *A. nidulans* orotidine-5'-phosphate decarboxylase gene (*pyrG*) as a selectable marker with its terminator repeats, and the human *Herpes simplex* virus 1 (HSV-1) thymidine kinase gene. The HSV-1 thymidine kinase gene lies 3' of the 3' flanking region of the *chsB* gene, allowing for counter-selection of *Aspergillus niger* transformants that do not correctly target to the *chsB* gene locus. The plasmid was constructed in several steps as described below.

A PCR product containing the 5' flanking region of *A. niger chsB* was generated using the following primers:

Primer chsB1 (sense) (SEQ ID NO:7): ggtggcggccgcatttactacttgtttact

Primer chsB2 (antisense) (SEQ ID NO:8): ccactagtcgatcaaatcctaattattgtc

The desired fragment was amplified by PCR in a reaction composed of approximately 100 ng of genome DNA of *Aspergillus niger* C3105, 1 µl of Expand High Fidelity polymerase (Roche), 100 µM of primer chsB1, 100 µM of primer chsB2, 5x PCR buffer (incl.MgCl2), 20 µl 2.5mM dNTP mix (total volume; 100µl). The reaction was incubated in a Bio-Rad® C1000 Touch™ Thermal Cycler programmed for 1 cycle at 94^{O}C for 2 minutes; 30 cycles each at 94^{O}C for 30 seconds, 55^{O}C for 30 seconds, and 72^{O}C for 2 minutes; 1 cycle at 72^{O}C for 7 minutes; and a 4^{O}C hold. The resulting 2,136 bp PCR fragment was purified by 0.8% agarose gel electrophoresis using TAE buffer, excised from the gel, and extracted using a QIAQUICK® Gel Extraction Kit. The purified 2,136 bp PCR fragment was digested by *NotI* and *SpeI*.

Plasmid pHUda801 (Example 4 in WO 2012160093 A1) was digested with *Not* I and *SpeI*, and purified by 0.8% agarose gel electrophoresis using TAE buffer, where a 9,558 bp fragment was excised from the gel and extracted using a QIAQUICK® Gel Extraction Kit. The 9,558 bp fragment was ligated to the 2,136 bp PCR fragment in a reaction composed of 1 µl of the 9,558 bp fragment, 3 µl of the 2,136 bp fragment, 1 µl of 5X ligase Buffer, 5 µl of 2X Ligase Buffer and 1 µl of Ligase (Roche Rapid DNA Ligation Kit). The ligation reaction was incubated at room temperature for 10 minutes. Five µl of the ligation mixture were transformed into DH5α chemically competent *E. coli* cells. Transformants were spread onto LB plus ampicillin plates and incubated at 37°C overnight. Plasmid DNA was purified from several transformants using a QIA mini-prep kit. The plasmid DNA was screened for proper ligation by use of proper restriction enzymes followed by 0.8% agarose gel electrophoresis using TAE buffer. One plasmid was designated as pHUda801-5'*chsB*.

A PCR product containing the 3' flanking region of *A. niger chsB* was generated using the following primers:

Primer chsB3 (sense) (SEQ ID NO:9): cttctagagtgttgcctggtgctttctcag

Primer chsB4 (antisense) (SEQ ID NO:10): gagaattccattgcaccaccgcctgcccat

The desired fragment was amplified by PCR in a reaction composed of approximately 100 ng of genome DNA of *Aspergillus niger* C3105, 1 µl of Expand High Fidelity polymerase (Roche), 100 µM of primer chsB3, 100 µM of primer chsB4, 5x PCR buffer (incl.MgCl2), 20 µl 2.5mM dNTP mix (total volume; 100µl). The reaction was incubated in a Bio-Rad® C1000 Touch™ Thermal Cycler programmed for 1 cycle at 94^{O}C for 2 minutes; 30 cycles each at 94^{O}C for 30 seconds, 55^{O}C for 30 seconds, and 72^{O}C for 2 minutes; 1 cycle at 72^{O}C for 7 minutes; and a 4^{O}C hold. The resulting 1,976 bp PCR fragment was purified by 0.8% agarose gel electrophoresis using TAE buffer, excised from the gel, and extracted using a QIAQUICK® Gel Extraction Kit. The purified 1,976 bp PCR fragment was digested by *XbaI* and *EcoRI*.

Plasmid pHUda801-5'chsB was digested with *XbaI* and *EcoRI*, and purified by 0.8% agarose gel electrophoresis using TAE buffer, where a 9,638 bp fragment was excised from the gel and extracted using a QIAQUICK® Gel Extraction Kit. The 9,638 bp fragment was ligated to the 1,976 bp PCR fragment in a reaction composed of 1 µl of the 9,638 bp fragment, 3 µl of the 1,976 bp fragment, 1 µl of 5X ligase Buffer, 5 µl of 2X Ligase Buffer and 1 µl of Ligase (Roche Rapid DNA Ligation Kit). The ligation reaction was incubated at room temperature for 10 minutes. Five µl of the ligation mixture were transformed into DH5α chemically competent *E. coli* cells. Transformants were spread onto LB plus ampicillin plates and incubated at 37°C overnight. Plasmid DNA was purified from several transformants using a QIA mini-prep kit. The plasmid DNA was screened for proper ligation by use of proper restriction enzymes followed by 0.8% agarose gel electrophoresis using TAE buffer. One plasmid was designated as pHUda1435.

Example 2: The *Aspergillus niger chsB* gene disruption in C3105

The *pyrG* gene in C3105 was rescued as follows. The strain C3105 was inoculated on Cove-N JP media containing 10 mM uridine and 1 g/ L 5-fluoro-orotic acid (5-FOA) at 30°C for 5 days. Strains in which the *pyrG* gene has been deleted will grow in the presence of 5-FOA; those that retain the gene will convert 5-FOA to 5-fluoro-UMP, a toxic intermediate. The grown colonies were transferred with sterile toothpicks to COVE-N-gly plates supplemented with 10 mM uridine and were grown at 30°C for 7 days. The isolated strain was named M1405.

Protoplasts of *Aspergillus niger* strain M1405 were prepared by cultivating the strain in 100 ml of YPG medium supplemented with 10 mM uridine at 32°C for 16 hours with gentle agitation at 80 rpm. Pellets were collected and washed with 0.6 M KCl, and resuspended 20 ml 0.6 M KCl containing a commercial beta-glucanase product (GLUCANEX™, Novozymes A/S, Bagsværd, Denmark) at a final concentration of 20 mg per ml. The suspension was incubated at 32 °C at 80 rpm until protoplasts were formed. Protoplasts were filtered through a funnel lined with MIRACLOTH® into a 50 ml sterile plastic centrifuge tube and were washed with 0.6 M KCl to extract trapped protoplasts. The combined filtrate and supernatant were collected by

centrifugation at 2,000 rpm for 15 minutes. The supernatant was discarded and the pellet was washed with 10-25 ml of STC and centrifuged again at 2,000 rpm for 10 minutes and then washed twice with STC buffer. The protoplasts were counted with a hematometer and resuspended and adjusted in an 8:2:0.1 solution of STC:STPC:DMSO to a final concentration of $2.5x10^7$ protoplasts/ml.

Approximately 10 µg of pHUda1435 was added to 0.3 ml of the protoplast suspension, mixed gently, and incubated on ice for 30 minutes. Three ml of SPTC was added and the protoplast suspension was incubated for 20 minutes at 37°C. After the addition of 12 ml of 50°C COVE-N top agarose, the mixture was poured onto the COVE-N plates and the plates were incubated at 30°C for 7 days. The grown transformants were transferred with sterile toothpicks to Cove-N JP plates supplemented with 1.5 µM 5-Flouro-2-deoxyuridine (FdU), an agent which kills cells expressing the herpes simplex virus (HSV) thymidine kinase gene (TK) harboring in pHUda1435. Single spore isolates were transferred to COVE-N-gly plates.

Possible transformants of *Aspergillus niger* strain M1405 containing the pHUda1435 to disrupt *chsB* gene were screened by Southern analysis. Each of the spore purified transformants were cultivated in 3 ml of YPG medium and incubated at 30°C for 2 days with shaking at 200 rpm. Biomass was collected using a MIRACLOTH® lined funnel. Ground mycelia were subject to genome DNA preparation using FastDNA SPIN Kit for Soil (MP Biomedicals) follows by manufacture's instruction.

Southern blot analysis was performed to confirm the disruption of the *chsB* gene locus. Five µg of genomic DNA from each transformant were digested with *XhoI*. The genomic DNA digestion reactions were composed of 5 µg of genomic DNA, 1 µl of *XhoI*, 2 µl of 10X NEBuffer 4, and water to 20 µl. Genomic DNA digestions were incubated at 37°C for approximately 16 hours. The digestions were submitted to 0.8 % agarose gel electrophoresis using TAE buffer and blotted onto a hybond N+ (GE Healthcare Life Sciences, Manchester, NH, USA) using a TURBOBLOTTER® for approximately 1 hour following the manufacturer's recommendations. The membrane was hybridized with a 500 bp digoxigenin-labeled *Aspergillus niger chsB* probe, which was synthesized by incorporation of digoxigenin-11-dUTP by PCR using primers chsB5 (sense) and chsB6 (antisense) shown below:

Forward primer (chsB5) (SEQ ID NO:11): gtgttgcctggtgctttctc

Reverse primer (chsB6) (SEQ ID NO:12): aggtccataatgaccgatgttgta

The amplification reaction (100 µl) was composed of 200 µM PCR DIG Labeling Mix (vial 2) (Roche Applied Science, Palo Alto, CA, USA), 0.5 µM primers, EXPAND® High Fidelity Enzyme mix (vial 1) (Roche Applied Science, Palo Alto, USA), and 1 µl (100 pg/µl) of pHUda1435 as template in a final volume of 100 µl. The amplification reaction was incubated in a Bio-Rad® C1000 Touch™ Thermal Cycler programmed for 1 cycle at $94^{O}C$ for 2 minutes; 30 cycles each at $94^{O}C$ for 30 seconds, $55^{O}C$ for 30 seconds, and $72^{O}C$ for 30 seconds and a $4^{O}C$ hold. PCR

products were separated by 0.8 % agarose gel electrophoresis using TAE buffer where a 0.5 kb fragment was excised from the gel and extracted using a QIAQUICK® Gel Extraction Kit. The denatured probe was added directly to the DIG Easy Hyb buffer and an overnight hybridization at 42 oC was done. Following the post hybridization washes (twice in 2X SSC, room temperature, 5 min and twice in 0.1X SSC, 68o C, 15 min. each), chemiluminescent detection using the DIG detection system and CPD-Star (Roche) was done followed by manufacture's protocol. The DIG-labeled DNA Molecular Weight Marker II (Roche) was used for the standard marker. A strain, M1405-1435-9, giving the correct integration at the *chsB* loci (a hybridized band shifted from 9.7 kb to 4.8 kb) were selected for the subsequent experiments.

Example 3: The *Aspergillus niger chsB* gene disruption in M1405-1685-16

Protoplasts of *Aspergillus niger* strain M1405-1685-16 were prepared by cultivating the strain in 100 ml of YPG medium at 32°C for 16 hours with gentle agitation at 80 rpm. Pellets were collected and washed with 0.6 M KCl, and resuspended 20 ml 0.6 M KCl containing a commercial beta-glucanase product (GLUCANEX™, Novozymes A/S, Bagsværd, Denmark) at a final concentration of 20 mg per ml. The suspension was incubated at 32°C at 80 rpm until protoplasts were formed. Protoplasts were filtered through a funnel lined with MIRACLOTH® into a 50 ml sterile plastic centrifuge tube and were washed with 0.6 M KCl to extract trapped protoplasts. The combined filtrate and supernatant were collected by centrifugation at 2,000 rpm for 15 minutes. The supernatant was discarded and the pellet was washed with 10-25 ml of STC and centrifuged again at 2,000 rpm for 10 minutes and then washed twice with STC buffer. The protoplasts were counted with a hematometer and resuspended and adjusted in an 8:2:0.1 solution of STC:STPC:DMSO to a final concentration of $2.5x10^7$ protoplasts/ml.

Approximately 10 µg of pHUda1435 was added to 0.3 ml of the protoplast suspension, mixed gently, and incubated on ice for 30 minutes. Three ml of SPTC was added and the protoplast suspension was incubated for 20 minutes at 37°C. After the addition of 12 ml of 50°C COVE top agarose, the mixture was poured onto the COVE plates and the plates were incubated at 30°C for 7 days. The grown transformants were transferred with sterile toothpicks to Cove-2 plates supplemented with 1.5 µM 5-Flouro-2-deoxyuridine (FdU), an agent which kills cells expressing the herpes simplex virus (HSV) thymidine kinase gene (TK) harboring in pHUda1435. Single spore isolates were transferred to COVE-N-gly plates.

Possible transformants of *Aspergillus niger* strain M1405-1685-16 containing the pHUda1435 to disrupt *chsB* gene were screened by Southern analysis. Each of the spore purified transformants were cultivated in 3 ml of YPG medium and incubated at 30°C for 2 days with shaking at 200 rpm. Biomass was collected using a MIRACLOTH® lined funnel. Ground mycelia were subject to genome DNA preparation using FastDNA SPIN Kit for Soil (MP Biomedicals) follows by manufacture's instruction.

Southern blot analysis was performed to confirm the disruption of the *chsB* gene locus. Five µg of genomic DNA from each transformant were digested with *XhoI*. The genomic DNA digestion reactions were composed of 5 µg of genomic DNA, 1 µl of *XhoI*, 2 µl of 10X NEBuffer 4, and water to 20 µl. Genomic DNA digestions were incubated at 37°C for approximately 16 hours. The digestions were submitted to 0.8 % agarose gel electrophoresis using TAE buffer and blotted onto a hybond N+ (GE Healthcare Life Sciences, Manchester, NH, USA) using a TURBOBLOTTER® for approximately 1 hour following the manufacturer's recommendations. The membrane was hybridized with a 500 bp digoxigenin-labeled *Aspergillus niger chsB* probe, which was synthesized by incorporation of digoxigenin-11-dUTP by PCR using primers chsB5 (sense) and chsB6 (antisense) shown below:

Forward primer (chsB5) (SEQ ID NO:13): gtgttgcctggtgctttctc

Reverse primer (chsB6) (SEQ ID NO:14): aggtccataatgaccgatgttgta

The amplification reaction (100 µl) was composed of 200 µM PCR DIG Labeling Mix (vial 2) (Roche Applied Science, Palo Alto, CA, USA), 0.5 µM primers, EXPAND® High Fidelity Enzyme mix (vial 1) (Roche Applied Science, Palo Alto, USA), and 1 µl (100 pg/µl) of pHUda1435 as template in a final volume of 100 µl. The amplification reaction was incubated in a Bio-Rad® C1000 Touch™ Thermal Cycler programmed for 1 cycle at 94°C for 2 minutes; 30 cycles each at 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds and a 4°C hold. PCR products were separated by 0.8% agarose gel electrophoresis using TAE buffer where a 0.5 kb fragment was excised from the gel and extracted using a QIAQUICK® Gel Extraction Kit. The denatured probe was added directly to the DIG Easy Hyb buffer and an overnight hybridization at 42°C was done. Following the post hybridization washes (twice in 2X SSC, room temperature, 5 min and twice in 0.1X SSC, 68°C, 15 min. each), chemiluminescent detection using the DIG detection system and CPD-Star (Roche) was done followed by manufacture's protocol. The DIG-labeled DNA Molecular Weight Marker II (Roche) was used for the standard marker. A strain, M1405-1685-1435-24, giving the correct integration at the *chsB* loci (a hybridized band shifted from 9.7 kb to 4.8 kb) were selected for the subsequent experiments.

Example 4: Construction of *Kionochaeta* phospholipase C gene (*plc*) expression vector pHUda1556

Plasmid pHUda1556 was constructed to contain Kionochaeta phospholipase C gene (*plc*) driven by *Aspergillus niger* neutral amylase promoter II (*Pna2*) and glucoamylase terminator (*Tamg*), the *A. nidulans* acetamidase gene (*amdS*) as a selectable marker, and the yeast *Saccharamyces cerevisiae* FLP recombinase gene (*flp*) driven by *Aspergillus niger* acid stable amylase promoter (*PasaA*) and the *Aspergillus oryzae* nitrate reductase terminator (T*niad*).

Based on amino acid sequences of *Kionochaeta* phospholipase C, the codon optimized gene with no amino acid changes were synthesized and ordered from the commercial supplier GeneArt™.

A PCR product containing the synthetic *Kionochaeta* phospholipase C gene was generated using the following primers:

Primer KplcC-1 (sense) (SEQ ID NO:15): ggatccaccatgagagcatcgagcatcctg

Primer KplcC-2 (antisense) (SEQ ID NO:16): cacgtgctaaactgccattcggcgtttctc

The desired fragment was amplified by PCR in a reaction composed of approximately 100 ng of the plasmid DNA harboring synthetic *Kionochaeta* phospholipase C gene, 1 µl of Expand High Fidelity polymerase (Roche), 100 µM of primer KplcC -1, 100 µM of primer KplcC -2, 5x PCR buffer (incl.MgCl2), 20 µl 2.5mM dNTP mix (total volume; 100µl). The reaction was incubated in a Bio-Rad® C1000 Touch™ Thermal Cycler programmed for 1 cycle at 94^{O}C for 2 minutes; 30 cycles each at 94^{O}C for 30 seconds, 55^{O}C for 30 seconds, and 72^{O}C for 2 minute; 1 cycle at 72^{O}C for 7 minutes; and a 4^{O}C hold. The resulting 1,947 bp PCR fragment was purified by 0.8% agarose gel electrophoresis using TAE buffer, excised from the gel, and extracted using a QIAQUICK® Gel Extraction Kit. The purified 1,947 bp PCR fragment was digested by *BamHI* and *PmlI*.

Plasmid pRika147 (disclosed in Example9 of WO 2012/160093) was digested with *BamHI* and *PmlI*, and purified by 0.8% agarose gel electrophoresis using TAE buffer, where a 10,512 bp fragment was excised from the gel and extracted using a QIAQUICK® Gel Extraction Kit. The 10,512 bp fragment was ligated to the 1,947 bp PCR fragment in a reaction composed of 1 µl of the 10,512 bp fragment, 3 µl of the 1,947 bp fragment, 1 µl of 5X ligase Buffer, 5 µl of 2X Ligase Buffer and 1 µl of Ligase (Roche Rapid DNA Ligation Kit). The ligation reaction was incubated at room temperature for 10 minutes. Five µl of the ligation mixture were transformed into DH5α chemically competent *E. coli* cells. Transformants were spread onto LB plus ampicillin plates and incubated at 37°C overnight. Plasmid DNA was purified from several transformants using a QIA mini-prep kit. The plasmid DNA was screened for proper ligation by use of proper restriction enzymes followed by 0.8% agarose gel electrophoresis using TAE buffer. One plasmid was designated pHUda1556.

Example 5: Introduction of *Kionochaeta* phospholipase C gene (*plc*) expression vector pHUda1556 in *Aspergillus niger strains* C3105, M1405-1435-9 and M1405-1685-1435-24

The *plc* expression cassette of pHUda1556 should be introduced in each strain by site-specific FLP/*frt* recombination into four pre-determined chromosomal *loci*: neutral amylase I (*amyA*), neutral amylase II (*amyB*), acid stable amylase (*asaA*) and putative alkali sulfatase (*payA*).

Protoplasts of *Aspergillus niger* strains C3105, M1405-1435-9 and M1405-1685-1435-24 were prepared by cultivating the strains in 100 ml of YPG medium at 32°C for 16 hours with gentle agitation at 80 rpm. Pellets from each were collected and washed with 0.6 M KCl, and resuspended 20 ml 0.6 M KCl containing a commercial beta-glucanase product (GLUCANEX™, Novozymes A/S, Bagsværd, Denmark) at a final concentration of 20 mg per ml. The suspension was incubated at 32°C at 80 rpm until protoplasts were formed. Protoplasts were filtered through a funnel lined with MIRACLOTH® into a 50 ml sterile plastic centrifuge tube and were washed with 0.6 M KCl to extract trapped protoplasts. The combined filtrate and supernatant were collected by centrifugation at 2,000 rpm for 15 minutes. The supernatant was discarded and the pellet was washed with 10-25 ml of STC and centrifuged again at 2,000 rpm for 10 minutes and then washed twice with STC buffer. The protoplasts were counted with a hematometer and resuspended and adjusted in an 8:2:0.1 solution of STC:STPC:DMSO to a final concentration of $2.5x10^7$ protoplasts/ml.

Approximately 10 µg of pHUda1556 was added to 0.3 ml of the protoplast suspension, mixed gently, and incubated on ice for 30 minutes. Three ml of SPTC was added and the protoplast suspension was incubated for 20 minutes at 37°C. After the addition of 12 ml of 50°C COVE top agarose supplemented with 50 µg/ml of 5' fluorocytosine (5FC), an agent which kills cells expressing the *Aspergillus niger* cytosine deaminase (*fcy1*) gene harboured in strains C3105, M1405-1435-9 and M1405-1685-1435-24. The mixture was poured onto the COVE plates and the plates were incubated at 30°C for 10 days. The grown transformants were transferred with sterile toothpicks to Cove-2 plates supplemented with 10 µg/ml of 5' fluorocytosine (5FC). Single spore isolates were transferred to COVE-N-gly plates.

Possible pHUda1556 transformants of *Aspergillus niger* strain either C3105, M1405-1435-9 or M1405-1685-1435-24 were screened by Southern analysis. Each of the spore purified transformants were cultivated in 3 ml of YPG medium and incubated at 30°C for 2 days with shaking at 200 rpm. Biomass was collected using a MIRACLOTH® lined funnel. Ground mycelia were subject to genome DNA preparation using FastDNA SPIN Kit for Soil (MP Biomedicals) follows by manufacture's instruction.

Southern blot analysis was performed to confirm the introduction of the *plc* gene at the four pre-determined *loci*: *amyA*, *amyB*, *asaA* and *payA*. Five µg of genomic DNA from each transformant were digested with *HindIII*. The genomic DNA digestion reactions were composed of 5 µg of genomic DNA, 0.5 µl of SpeI and PmlI, 2 µl of 10X NEBuffer 4, and water to 20 µl. Genomic DNA digestions were incubated at 37°C for approximately 16 hours. The digestions were submitted to 0.5 % agarose gel electrophoresis using TAE buffer and blotted onto a hybond N+ (GE Healthcare Life Sciences, Manchester, NH, USA) using a TURBOBLOTTER® for approximately 1 hour following the manufacturer's recommendations. The membrane was hybridized with a 500 bp digoxigenin-labeled *plc* probe, which was synthesized by incorporation

of digoxigenin-11-dUTP by PCR using primers KplcC-3 (sense) and KplcC-4 (antisense) shown below:

Forward primer (KplcC-3) (SEQ ID NO:17): cgactatacttctgctagct

Reverse primer (KplcC-4) (SEQ ID NO:18): ggttcgtacaggtagaagtt

The amplification reaction (100 µl) was composed of 200 µM PCR DIG Labeling Mix (vial 2) (Roche Applied Science, Palo Alto, CA, USA), 0.5 µM primers, EXPAND® High Fidelity Enzyme mix (vial 1) (Roche Applied Science, Palo Alto, USA), and 1 µl (100 pg/µl) of pHUda1556 as template in a final volume of 100 µl. The amplification reaction was incubated in a Bio-Rad® C1000 Touch™ Thermal Cycler programmed for 1 cycle at 94^{O}C for 2 minutes; 30 cycles each at 94^{O}C for 30 seconds, 55^{O}C for 30 seconds, and 72^{O}C for 30 seconds and a 4^{O}C hold. PCR products were separated by 0.8% agarose gel electrophoresis using TAE buffer where a 0.5 kb fragment was excised from the gel and extracted using a QIAQUICK® Gel Extraction Kit. The denatured probe was added directly to the DIG Easy Hyb buffer and an overnight hybridization at 42^{O}C was done. Following the post hybridization washes (twice in 2X SSC, room temperature, 5 min and twice in 0.1X SSC, 68^{O}C, 15 min. each), chemiluminescent detection using the DIG detection system and CPD-Star (Roche) was done followed by manufacture's protocol. The DIG-labeled DNA Molecular Weight Marker II (Roche) was used for the standard marker. A strain from each of C3105-1556-1 &2, M1405-1435-3 & 4 and M1405-1685-1435-5 & 6, generated from C3105, M1405-1435-9 and M1405-1685-1435-24, respectively, giving the correct integration at the four *loci* (four hybridized bands at the size of 6.1 kb, 4.1 kb, 2.9 kb and 5.0 kb) was selected for the subsequent experiments.

Example 6: PLC expression evaluation in shake flask culture

Aspergillus niger C3105-1556-1 &2, M1405-1435-3 & 4 and M1405-1685-1435-5 & 6 were cultivated on COVE-N-gly plates at 30°C for about a week. A sterile transfer pipette was used to punch a piece of small plugs from each plate, which were each inoculated into 100 ml of MSS medium in 500 ml flasks. The flasks were incubated at 30°C for 3 days at 200 rpm. And then, 10 ml of culture broth was transferred to 100 ml of MU1 glu medium in 500 ml flasks. The flasks were incubated at 30°C for 6 days at 200 rpm. Each culture was centrifuged at 5,000 rpm for 10 minutes in a 10 ml test tubes and culture supernatant was recovered for determining phospholipaseC (PLC) productivities. The PLC productivity assay was performed using a Quick Start™ Bradford Protein Assay Kit (Bio-Rad inc.). Culture supernatants were diluted appropriately in distilled water. A bovine serum albumin (WAKO cat number 519-83921) was diluted using several steps starting with a 0.5 mg/ml concentration and ending with a 0.1 mg/ml concentration in the distilled water. Five µl of each dilution including standard were transferred to a 96-well flat bottom plate. 250 µl of 1x Dye Reagent solution were added to each well and then incubated at room temperature for 5 minutes. The endpoint of the reaction was measured at 595 nm. Whole

protein productivities were determined by extrapolation from the generated standard curve and compared to the *Aspergillus niger* strain C3105-1556-1 set at 100%.

The *chsB*-inactivated *A. niger* M1405-1435 strain showed a surprisingly improved phospholipase C (PLC) productivity of 10~15% in shake flasks compared with the non-inactivated parent *A. niger* C3105-1556 strain (table 1). The double *cshB*- and *agsA*-inactivated *A. niger* M1405-1685-1435 strain had an even more surprisingly improved PLC productivity of 60~65% compared to the non-inactivated parent (Table 1).

Table 1: PLC productivity in shake flask culture.

Strain	**Genotype**	**Relative PLC productivity (%)**
A. niger C3105-1556-1	Parent	100%
A. niger C3105-1556-2	Parent	95%
A. niger M1405-1435-3	*chsB*$^-$	112%
A. niger M1405-1435-4	*chsB*$^-$	115%
A. niger M1405-1685-1435-5	*chsB*$^-$, *agsA*$^-$	160%
A. niger M1405-1685-1435-6	*chsB*$^-$, *agsA*$^-$	165%

CLAIMS

1. A filamentous fungal host cell producing a heterologous polypeptide of interest and comprising an inactivated chitin synthase-encoding gene, where said chitin synthase-encoding gene in its active form:

i) encodes a chitin synthase polypeptide having at least 80% amino acid sequence identity with SEQ ID NO:3;

ii) comprises or consists of a genomic nucleotide sequence at least 80% identical to the genomic DNA sequence shown in SEQ ID NO:1; and/or

iii) comprises or consists of a genomic nucleotide sequence, the cDNA sequence of which is at least 80% identical to the cDNA sequence shown in SEQ ID NO:2.

2. The host cell of claim 1 which is of a genus selected from the group consisting of *Acremonium*, *Aspergillus*, *Aureobasidium*, *Bjerkandera*, *Ceriporiopsis*, *Chrysosporium*, *Coprinus*, *Coriolus*, *Cryptococcus*, *Filibasidium*, *Fusarium*, *Humicola*, *Magnaporthe*, *Mucor*, *Myceliophthora*, *Neocallimastix*, *Neurospora*, *Paecilomyces*, *Penicillium*, *Phanerochaete*, *Phlebia*, *Piromyces*, *Pleurotus*, *Schizophyllum*, *Talaromyces*, *Thermoascus*, *Thielavia*, *Tolypocladium*, *Trametes* and *Trichoderma*.

3. The host cell of claim 2 which is an *Aspergillus* cell; preferably an *Aspergillus awamori*, *Aspergillus foetidus*, *Aspergillus fumigatus*, *Aspergillus japonicus*, *Aspergillus nidulans*, *Aspergillus niger* or an *Aspergillus oryzae* cell.

4. The host cell of any preceding claim, wherein the heterologous polypeptide of interest is an enzyme; preferably the enzyme is a hydrolase, isomerase, ligase, lyase, oxidoreductase, or transferase, *e.g.*, an aminopeptidase, amylase, carbohydrase, carboxypeptidase, catalase, cellobiohydrolase, cellulase, chitinase, cutinase, cyclodextrin glycosyltransferase, deoxyribonuclease, endoglucanase, esterase, alpha-galactosidase, beta-galactosidase, glucoamylase, alpha-glucosidase, beta-glucosidase, invertase, laccase, lipase, mannosidase, mutanase, oxidase, pectinolytic enzyme, peroxidase, phytase, polyphenoloxidase, proteolytic enzyme, ribonuclease, transglutaminase, xylanase, or beta-xylosidase.

5. The host cell of any preceding claim, which further comprises an inactivated 1,3-alpha-D-glucan synthase-encoding *agsA* gene, where said *agsA* gene in its active form:

i) encodes an 1,3-alpha-D-glucan synthase AgsA polypeptide having at least 80% amino acid sequence identity with SEQ ID NO:6;

ii) comprises or consists of a genomic nucleotide sequence at least 80% identical to the genomic DNA sequence shown in SEQ ID NO:4; and/or

iii) comprises or consists of a genomic nucleotide sequence, the cDNA sequence of which is at least 80% identical to the cDNA sequence shown in SEQ ID NO:5.

6. A method of producing a heterologous polypeptide of interest, said method comprising the steps of:

c) cultivating a filamentous fungal host cell producing the heterologous polypeptide of interest and comprising an inactivated chitin synthase-encoding gene under conditions conducive to the production of the heterologous polypeptide of interest, where said chitin synthase-encoding gene in its active form:

i) encodes a chitin synthase polypeptide having at least 80% amino acid sequence identity with SEQ ID NO:3;

ii) comprises or consists of a genomic nucleotide sequence at least 80% identical to the genomic DNA sequence shown in SEQ ID NO:1; and/or

iii) comprises or consists of a genomic nucleotide sequence, the cDNA sequence of which is at least 80% identical to the cDNA sequence shown in SEQ ID NO:2;

and, optionally,

d) recovering the heterologous polypeptide of interest.

7. The method of claim 6, wherein the filamentous fungal host cell is of a genus selected from the group consisting of *Acremonium*, *Aspergillus*, *Aureobasidium*, *Bjerkandera*, *Ceriporiopsis*, *Chrysosporium*, *Coprinus*, *Coriolus*, *Cryptococcus*, *Filibasidium*, *Fusarium*, *Humicola*, *Magnaporthe*, *Mucor*, *Myceliophthora*, *Neocallimastix*, *Neurospora*, *Paecilomyces*, *Penicillium*, *Phanerochaete*, *Phlebia*, *Piromyces*, *Pleurotus*, *Schizophyllum*, *Talaromyces*, *Thermoascus*, *Thielavia*, *Tolypocladium*, *Trametes* and *Trichoderma*.

8. The method cell of claim 7, wherein the filamentous fungal host cell is an *Aspergillus* cell; preferably an *Aspergillus awamori*, *Aspergillus foetidus*, *Aspergillus fumigatus*, *Aspergillus japonicus*, *Aspergillus nidulans*, *Aspergillus niger* or an *Aspergillus oryzae* cell.

9. The method of any of claims 6-8, wherein the heterologous polypeptide of interest is an enzyme; preferably the enzyme is a hydrolase, isomerase, ligase, lyase, oxidoreductase, or transferase, *e.g.*, an aminopeptidase, amylase, carbohydrase, carboxypeptidase, catalase, cellobiohydrolase, cellulase, chitinase, cutinase, cyclodextrin glycosyltransferase, deoxyribonuclease, endoglucanase, esterase, alpha-galactosidase, beta-galactosidase, glucoamylase, alpha-glucosidase, beta-glucosidase, invertase, laccase, lipase, mannosidase, mutanase, oxidase, pectinolytic enzyme, peroxidase, phytase, polyphenoloxidase, proteolytic enzyme, ribonuclease, transglutaminase, xylanase, or beta-xylosidase.

10. The method of any of claims 6-8, wherein the filamentous fungal host cell further comprises an inactivated 1,3-alpha-D-glucan synthase-encoding *agsA* gene, wherein said *agsA* gene in its active form:

i) encodes an 1,3-alpha-D-glucan synthase AgsA polypeptide having at least 80% amino acid sequence identity with SEQ ID NO:6;

ii) comprises or consists of a genomic nucleotide sequence at least 80% identical to the genomic DNA sequence shown in SEQ ID NO:4; and/or

iii) comprises or consists of a genomic nucleotide sequence, the cDNA sequence of which is at least 80% identical to the cDNA sequence shown in SEQ ID NO:5.

11. A method of producing a filamentous fungal host cell having an improved productivity of a heterologous polypeptide of interest, said method comprising the following steps in no particular order:

c) transforming a filamentous fungal host cell with a polynucleotide encoding the heterologous polypeptide of interest; and

d) inactivating a chitin synthase-encoding gene in the filamentous fungal host cell, wherein said chitin synthase-encoding gene in its active form:

i) encodes a chitin synthase polypeptide having at least 80% amino acid sequence identity with SEQ ID NO:3;

ii) comprises or consists of a genomic nucleotide sequence at least 80% identical to the genomic DNA sequence shown in SEQ ID NO:1; and/or

iii) comprises or consists of a genomic nucleotide sequence, the cDNA sequence of which is at least 80% identical to the cDNA sequence shown in SEQ ID NO:2.

12. The method of claim 11, wherein the filamentous fungal host cell is of a genus selected from the group consisting of *Acremonium*, *Aspergillus*, *Aureobasidium*, *Bjerkandera*, *Ceriporiopsis*, *Chrysosporium*, *Coprinus*, *Coriolus*, *Cryptococcus*, *Filibasidium*, *Fusarium*, *Humicola*, *Magnaporthe*, *Mucor*, *Myceliophthora*, *Neocallimastix*, *Neurospora*, *Paecilomyces*, *Penicillium*, *Phanerochaete*, *Phlebia*, *Piromyces*, *Pleurotus*, *Schizophyllum*, *Talaromyces*, *Thermoascus*, *Thielavia*, *Tolypocladium*, *Trametes* and *Trichoderma*.

13. The method cell of claim 12, wherein the filamentous fungal host cell is an *Aspergillus* cell; preferably an *Aspergillus awamori*, *Aspergillus foetidus*, *Aspergillus fumigatus*, *Aspergillus japonicus*, *Aspergillus nidulans*, *Aspergillus niger* or an *Aspergillus oryzae* cell.

14. The method of any of claims 11-13, wherein the heterologous polypeptide of interest is an enzyme; preferably the enzyme is a hydrolase, isomerase, ligase, lyase, oxidoreductase, or transferase, *e.g.*, an aminopeptidase, amylase, carbohydrase, carboxypeptidase, catalase,

cellobiohydrolase, cellulase, chitinase, cutinase, cyclodextrin glycosyltransferase, deoxyribonuclease, endoglucanase, esterase, alpha-galactosidase, beta-galactosidase, glucoamylase, alpha-glucosidase, beta-glucosidase, invertase, laccase, lipase, mannosidase, mutanase, oxidase, pectinolytic enzyme, peroxidase, phospholipase, phytase, polyphenoloxidase, proteolytic enzyme, ribonuclease, transglutaminase, xylanase, or beta-xylosidase.

15. The method of any of claims 11-13, wherein the filamentous fungal host cell further comprises an inactivated 1,3-alpha-D-glucan synthase-encoding *agsA* gene, wherein said *agsA* gene in its active form:

i) encodes an 1,3-alpha-D-glucan synthase AgsA polypeptide having at least 80% amino acid sequence identity with SEQ ID NO:6;

ii) comprises or consists of a genomic nucleotide sequence at least 80% identical to the genomic DNA sequence shown in SEQ ID NO:4; and/or

iii) comprises or consists of a genomic nucleotide sequence, the cDNA sequence of which is at least 80% identical to the cDNA sequence shown in SEQ ID NO:5.

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2018/056377

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12P21/00 C12N1/14 C12N9/10
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, EMBASE, 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 Y	WO 98/11203 A1 (NOVO NORDISK BIOTECH INC [US]) 19 March 1998 (1998-03-19) Whole doc., in particular examples 3, 4, 8, 42	1-4,6-9, 11-14 5,10,15
X	CHRISTIAN MÜLLER ET AL: "Effect of deletion of chitin synthase genes on mycelial morphology and culture viscosity in Aspergillus oryzae : Chitin Synthesis, Morphology, and Viscosity", BIOTECHNOLOGY AND BIOENGINEERING, vol. 81, no. 5, 5 March 2003 (2003-03-05), pages 525-534, XP55471923, US ISSN: 0006-3592, DOI: 10.1002/bit.10491	1-4,6-9, 11-14
Y	Whole doc., in partic. abstract; p.525,col2; discussion	5,10,15

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

[X] See patent family annex.

* Special categories of cited documents :

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

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

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"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

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
3 May 2018	18/05/2018

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INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2018/056377

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 2016/066690 A1 (NOVOZYMES AS [DK]) 6 May 2016 (2016-05-06) Whole doc., in particular p.1, l.25-29; examples 3,8	5,10,15
A	RAST D M ET AL: "Cell wall-associated enzymes in fungi", PHYTOCHEMI, PERGAMON PRESS, GB, vol. 64, no. 2, 1 January 2004 (2004-01-01), pages 339-366, XP004448651, ISSN: 0031-9422, DOI: 10.1016/S0031-9422(03)00350-9 the whole document	1-15

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2018/056377

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