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(54) **Title:** METHOD FOR INCREASING GROWTH RATE

(57) **Abstract:** A method of producing a polypeptide of interest, comprising culturing the recombinant filamentous fungal host cell, wherein an NAD(H) kinase activity is increased compared to an otherwise identical wild type host cell cultured under identical conditions, and said recombinant filamentous fungal host cell further expresses a polypeptide of interest.

TITLE: METHOD FOR INCREASING GROWTH RATE**Reference to a Sequence Listing**

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

BACKGROUND OF THE INVENTION**FIELD OF THE INVENTION**

10 The present invention relates to recombinant host cells, to a method of producing a polypeptide of interest using the host cell of the invention as well as to a method of increasing the growth rate of a host cell.

DESCRIPTION OF THE RELATED ART

15 The use of recombinant host cells in the expression of heterologous proteins has in recent years greatly simplified the production of large quantities of commercially valuable proteins which otherwise are obtainable only by purification from their native sources. Currently, there is a varied selection of expression systems from which to choose for the production of any given protein, including bacterial and eukaryotic hosts. The selection of an appropriate expression system often
20 depends not only on the ability of the host cell to produce adequate yields of the protein in an active state, but, to a large extent, may also be governed by the intended end use of the protein.

Typically improved yields obtained in fungi have been obtained by improving the stability of the protein product by using different protease mutants or increasing the expression by using a strong promoter for controlling the expression or improving the secretion pathway by using the
25 most efficient signal peptides.

Fungal host cells used for heterologous protein expression are usually grown under nutrient rich conditions typically found in laboratory and industrial fermentors. Such conditions may, however, be very different from the natural environment of the said host cell.

It is an object of the present invention to provide factors that will affect the optimization of
30 host cell growth in an industrial production setting and to provide methods for improving yield by modifying the expression of such factors.

SUMMARY OF THE INVENTION

The inventors of the present invention have discovered that one such factor is the
35 endogenous fungal enzymes responsible for the production of NADP(H). Furthermore it has been

found that filamentous fungi contain such an ATP dependent NADH kinase (ATP:NADH 2-phosphotransferase, EC 2.7.1.86) and that increasing the expression level from the endogenous gene will result in a significant increase in biomass yield and growth rate.

5 The present invention therefore in a first aspect relates to a recombinant fungal host cell, wherein the activity of a NAD(H) kinase is increased compared to an otherwise identical wild type host cell cultured under identical conditions, and said recombinant host cell further expresses a polypeptide of interest.

10 In a second aspect the present invention relates to a method of producing a polypeptide of interest, comprising culturing the recombinant host cell of the invention under conditions conducive for the production of the polypeptide.

15 In a third aspect the present invention relates to a method of producing a polypeptide of interest, comprising culturing the recombinant filamentous fungal host cell, wherein an NADH kinase activity is increased compared to an otherwise identical wild type host cell cultured under identical conditions, and said recombinant filamentous fungal host cell further expresses a polypeptide of interest.

In a fourth aspect the present invention relates to a method for increasing growth rate of a fungal host cell comprising increasing the activity of an NADH kinase compared to an otherwise identical wild type host cell cultured under identical conditions.

20 BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows an alignment of the *Aspergillus nidulans* NADH kinase (SEQ ID NO: 2), the putative *Aspergillus niger* NADH kinase (SEQ ID NO: 6) and the putative *Aspergillus oryzae* NADH kinase (SEQ ID NO: 8).

25 DEFINITIONS

NADH kinase activity: The term "NADH kinase activity" is defined herein as an ATP dependent NADH kinase (ATP:NADH 2'-phosphotransferase, EC 2.7.1.86) that catalyzes the reaction: $ATP + NAD^+/H \rightarrow ADP + NADP^+/H$. For purposes of the present invention, NADH kinase activity is determined according to the procedure described in the examples.

30 The NADH kinases of the present invention have at least 20%, preferably at least 40%, more preferably at least 50%, more preferably at least 60%, more preferably at least 70%, more preferably at least 80%, even more preferably at least 90%, most preferably at least 95%, and even most preferably at least 100% of the NADH kinase activity of the mature polypeptide of SEQ ID NO: 2, 6, or 8.

35 **Isolated polypeptide:** The term "isolated polypeptide" as used herein refers to a

polypeptide that is isolated from a source. In a preferred aspect, the polypeptide is at least 1% pure, preferably at least 5% pure, more preferably at least 10% pure, more preferably at least 20% pure, more preferably at least 40% pure, more preferably at least 60% pure, even more preferably at least 80% pure, and most preferably at least 90% pure, as determined by SDS-PAGE.

Substantially pure polypeptide: The term “substantially pure polypeptide” denotes herein a polypeptide preparation that contains at most 10%, preferably at most 8%, more preferably at most 6%, more preferably at most 5%, more preferably at most 4%, more preferably at most 3%, even more preferably at most 2%, most preferably at most 1%, and even most preferably at most 0.5% by weight of other polypeptide material with which it is natively or recombinantly associated. It is, therefore, preferred that the substantially pure polypeptide is at least 92% pure, preferably at least 94% pure, more preferably at least 95% pure, more preferably at least 96% pure, more preferably at least 96% pure, more preferably at least 97% pure, more preferably at least 98% pure, even more preferably at least 99%, most preferably at least 99.5% pure, and even most preferably 100% pure by weight of the total polypeptide material present in the preparation. The polypeptides of the present invention are preferably in a substantially pure form, *i.e.*, that the polypeptide preparation is essentially free of other polypeptide material with which it is natively or recombinantly associated. This can be accomplished, for example, by preparing the polypeptide by well-known recombinant methods or by classical purification methods.

Mature polypeptide: The term “mature polypeptide” is defined herein as a polypeptide having NADH kinase activity that is in its final form following translation and any post-translational modifications, such as N-terminal processing, C-terminal truncation, glycosylation, phosphorylation, etc.

The mature polypeptide may be predicted using e.g. SignalP 3.0 software.

Mature polypeptide coding sequence: The term “mature polypeptide coding sequence” is defined herein as a nucleotide sequence that encodes a mature polypeptide having NADH kinase activity.

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

For purposes of the present invention, the degree of 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 in Genetics* 16: 276-277), preferably version 3.0.0 or later. The optional 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)

5 For purposes of the present invention, the degree of 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 3.0.0 or later. The optional parameters used are gap open penalty of
10 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)

15 **Homologous sequence:** The term "homologous sequence" is defined herein as a predicted protein that gives an E value (or expectancy score) of less than 0.001 in a tfasty search (Pearson, W.R., 1999, in *Bioinformatics Methods and Protocols*, S. Misener and S. A. Krawetz, ed., pp. 185-219) with the enzyme of SEQ ID NO. 2, 6, or 8.

20 **Polypeptide fragment:** The term "polypeptide fragment" is defined herein as a polypeptide having one or more (several) amino acids deleted from the amino and/or carboxyl terminus of the mature polypeptide of SEQ ID NO: 2, 6, or 8; or a homologous sequence thereof; wherein the fragment has NADH kinase activity.

25 **Subsequence:** The term "subsequence" is defined herein as a nucleotide sequence having one or more (several) nucleotides deleted from the 5' and/or 3' end of the mature polypeptide coding sequence of SEQ ID NO: 1, 5, or 7; or a homologous sequence thereof; wherein the subsequence encodes a polypeptide fragment having NADH kinase activity.

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

35 **Isolated polynucleotide:** The term "isolated polynucleotide" as used herein refers to a polynucleotide that is isolated from a source. In a preferred aspect, the polynucleotide is at least 1% pure, preferably at least 5% pure, more preferably at least 10% pure, more preferably at least

20% pure, more preferably at least 40% pure, more preferably at least 60% pure, even more preferably at least 80% pure, and most preferably at least 90% pure, as determined by agarose electrophoresis.

Substantially pure polynucleotide: The term "substantially pure polynucleotide" as used
5 herein refers to a polynucleotide preparation free of other extraneous or unwanted nucleotides
and in a form suitable for use within genetically engineered protein production systems. Thus, a
substantially pure polynucleotide contains at most 10%, preferably at most 8%, more preferably at
most 6%, more preferably at most 5%, more preferably at most 4%, more preferably at most 3%,
even more preferably at most 2%, most preferably at most 1%, and even most preferably at most
10 0.5% by weight of other polynucleotide material with which it is natively or recombinantly
associated. A substantially pure polynucleotide may, however, include naturally occurring 5' and
3' untranslated regions, such as promoters and terminators. It is preferred that the substantially
pure polynucleotide is at least 90% pure, preferably at least 92% pure, more preferably at least
94% pure, more preferably at least 95% pure, more preferably at least 96% pure, more preferably
15 at least 97% pure, even more preferably at least 98% pure, most preferably at least 99%, and
even most preferably at least 99.5% pure by weight. The polynucleotides of the present invention
are preferably in a substantially pure form, *i.e.*, that the polynucleotide preparation is essentially
free of other polynucleotide material with which it is natively or recombinantly associated. The
polynucleotides may be of genomic, cDNA, RNA, semisynthetic, synthetic origin, or any
20 combinations thereof.

Coding sequence: When used herein the term "coding sequence" means a nucleotide
sequence, which directly specifies the amino acid sequence of its protein product. The
boundaries of the coding sequence are generally determined by an open reading frame, which
usually begins with the ATG start codon or alternative start codons such as GTG and TTG and
25 ends with a stop codon such as TAA, TAG, and TGA. The coding sequence may be a DNA,
cDNA, synthetic or recombinant nucleotide sequence.

cDNA: The term "cDNA" is defined herein as a DNA molecule that can be prepared by
reverse transcription from a mature, spliced, mRNA molecule obtained from a eukaryotic cell.
cDNA lacks intron sequences that are usually present in the corresponding genomic DNA. The
30 initial, primary RNA transcript is a precursor to mRNA that is processed through a series of steps
before appearing as mature spliced mRNA. These steps include the removal of intron sequences
by a process called splicing. cDNA derived from mRNA lacks, therefore, any intron sequences.

Nucleic acid construct: The term "nucleic acid construct" as used herein refers to a
nucleic acid molecule, either single- or double-stranded, which is isolated from a naturally
35 occurring gene or which is modified to contain segments of nucleic acids in a manner that would

not otherwise exist in nature or which is synthetic. The term nucleic acid construct is synonymous with the term "expression cassette" when the nucleic acid construct contains the control sequences required for expression of a coding sequence of the present invention.

Control sequences: The term "control sequences" is defined herein to include all components necessary for the expression of a polynucleotide encoding a polypeptide of the present invention. Each control sequence may be native or foreign to the nucleotide sequence 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 nucleotide sequence encoding a polypeptide.

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

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

Expression vector: The term "expression vector" is defined herein as a linear or circular DNA molecule that comprises a polynucleotide encoding a polypeptide of the present invention and is operably linked to additional nucleotides that provide for its expression.

Host cell: The term "host cell", as used herein, includes any cell type that is susceptible to transformation, transfection, transduction, and the like with a nucleic acid construct or expression vector comprising a polynucleotide of the present invention.

Modification: The term "modification" means herein any chemical modification of the polypeptide consisting of the mature polypeptide of SEQ ID NO: 2, 6, or 8; or a homologous sequence thereof; as well as genetic manipulation of the DNA encoding such a polypeptide. The modification can be a substitution, a deletion and/or an insertion of one or more (several) amino acids as well as replacements of one or more (several) amino acid side chains.

Artificial variant: When used herein, the term "artificial variant" means a polypeptide having NADH kinase activity produced by an organism expressing a modified polynucleotide sequence of the mature polypeptide coding sequence of SEQ ID NO: 1, 5, or 7; or a homologous sequence thereof. The modified nucleotide sequence is obtained through human intervention by modification of the polynucleotide sequence disclosed in SEQ ID NO: 1, 5, or 7; or a homologous

sequence thereof.

DETAILED DESCRIPTION OF THE INVENTION

The cofactor NADPH is needed in large quantities for biosynthesis. In the cytosol, NADPH
5 is provided primarily by enzymes in the pentose phosphate pathway (PPP), including glucose-6-
phosphate dehydrogenase and 6-phosphogluconate dehydrogenase, which each catalyze the
reduction of NADP⁺. The flux through the PPP has been reported to increase for high NADPH
concentrations in different eukaryotes (Henriksen, C.M., Christiansen, L.H., Nielsen, J., and
10 Villadsen, J., (1996) Growth energetics and metabolic fluxes in continuous cultures of *Penicillium*
chrysogenum. J. Biotechnol. 45:149-164; Obanye, A.I.C., Hobbs, G., Garden, D.C.J., and Oliver,
S.G., (1996) Correlation between carbon flux through the pentose phosphate pathway and
production of the antibiotic methylenomycin in *Streptomyces coelicolor* A3. Microbiol. 142:133-
137; Pedersen, H., Carlsen, M., and Nielsen, J., (1999) Identification of enzymes and
15 quantification of metabolic fluxes in the wild type and in recombinant *Aspergillus oryzae* strain.
Appl. Environ. Microbiol. 65:11-19) and to decrease when the need for NADPH production is low
(dos Santos, M., Thygesen, G., Kotter, P., Olsson, L., and Nielsen, J., (2003) Aerobic physiology
of redox engineered *Saccharomyces cerevisiae* strains modified in the ammonium assimilation for
increased NADPH availability. FEMS Yeast Res. 4:59-68.). In the cytosol the reaction: L-malate +
20 NADP⁺ → pyruvate + CO₂ + NADPH, has been shown to be a major source of NADPH for lipid
synthesis in *Aspergillus nidulans* (Wynn, J.P., and Ratledge, C., (1997) Malic enzyme is a major
source of NADPH for lipid accumulation by *Aspergillus nidulans*. Microbiol. 143:253-257) and
other filamentous fungi (Wynn, J.P., bin Abdul Hamid, A., and Ratledge, C., (1999) The role of
malic enzyme in the regulation of lipid accumulation in filamentous fungi. Microbiol.145:1911-
1917). The sources of NADPH in the mitochondrion, however, are less clear. In mammalian cells,
25 mitochondrial NADP⁺-dependent isocitrate dehydrogenase (NADPH-IDHm) has been reported to
be an important source of NADPH since it cannot be transported across the mitochondrial
membrane. However, deletion of the corresponding gene in baker's yeast *Saccharomyces*
cerevisiae (IDP1) had no effects on cell growth or oxidative stress sensitivity (Minard, Kl.,
Jennings, G.T., Loftus, T.M., Xuan, D., and McAlister-Henn, L., (1998) Sources of NADPH and
30 expression of mammalian NADP⁺ specific isocitrate dehydrogenases in *Saccharomyces*
cerevisiae. J. Biol. Chem. 273:31486-31493). The sources for NADPH production in the
eukaryotic mitochondria are thus not clear.

Catabolic reactions primarily generate reducing power in the form of NADH rather than
NADPH. The NAD⁺/H kinases are the only known enzymes that convert NAD⁺/H to NADP⁺/H in a
35 single reaction (Mori S., Kawai S., Shi F., Mikami B., and Murata, K., (2005) Molecular conversion

of NAD kinase to NADH kinase through single amino acid residue substitution. J. Biol. Chem. 280:24104-24112; Shi, F., Kawai, S., Mori, S., Kono, E., and Murata, K., (2005) Identification of ATP-NADH kinase isoenzymes and their contribution to supply of NADP(H) in *Saccharomyces cerevisiae*. Feder. Eur. Biochem. Soc. J. 272:3337-3349) ($ATP + NAD^+/H \rightarrow ADP + NADP^+/H$).

5 Loss of NAD⁺/H kinase activity in the cell is predicted to have pleiotropic effects. Most multicellular eukaryotic organisms only have one NAD⁺/H kinase gene. All of the NAD⁺/H kinases are highly conserved from simple bacteria such as *Escherichia coli* to mammals. The human enzyme preferentially converts NAD⁺ to NADP⁺. In contrast, the yeast *S. cerevisiae* has three NAD⁺/H kinase genes (*UTR1*, *YEF1*, *POS5*). All of these genes have been shown to encode
10 functional NAD⁺/H kinases by biochemical methods.

In the present invention an ATP dependent NADH kinase (ATP:NADH 2'-phosphotransferase, EC 2.7.1.86), which to date has only been described in yeast and plants, was identified in the filamentous fungus *Aspergillus nidulans*, *A. niger*, *A. oryzae*.

NADH kinase is expected to have a large impact on physiology, and consequently it is
15 unpredictable what the phenotype of a highly active NADH kinase would be in a higher eukaryot. According to the present invention we have found that the phenotypic effect of overexpressing the gene encoding NADH kinase in *Aspergillus* results in a significant increase in biomass yield and growth rate.

20 **Polypeptides Having NADH kinase Activity**

In one aspect, the present invention relates to isolated polypeptides comprising an amino acid sequence having a degree of identity to the mature polypeptide of SEQ ID NO: 2; 6 or 8 of preferably at least 60%, more preferably at least 65%, more preferably at least 70%, more preferably at least 75%, more preferably at least 80%, more preferably at least 85%, even more
25 preferably at least 90%, most preferably at least 95%, and even most preferably at least 96%, at least 97%, at least 98%, or at least 99%, which have NADH kinase activity (hereinafter "homologous polypeptides"). In a preferred aspect, the homologous polypeptides have an amino acid sequence that differs by ten amino acids, preferably by five amino acids, more preferably by four amino acids, even more preferably by three amino acids, most preferably by two amino
30 acids, and even most preferably by one amino acid from the mature polypeptide of SEQ ID NO: 2, 6 or 8

A polypeptide of the present invention preferably comprises the amino acid sequence of SEQ ID NO: 2, 6 or 8 or an allelic variant thereof; or a fragment thereof having NADH kinase activity. In a preferred aspect, the polypeptide comprises the amino acid sequence of SEQ ID
35 NO: 2, 6 or 8. In another preferred aspect, the polypeptide comprises the mature polypeptide of

SEQ ID NO: 2, 6 or 8. In another preferred aspect, the polypeptide consists of the amino acid sequence of SEQ ID NO: 2, 6 or 8 or an allelic variant thereof; or a fragment thereof having NADH kinase activity. In another preferred aspect, the polypeptide consists of the amino acid sequence of SEQ ID NO: 2, 6 or 8. In another preferred aspect, the polypeptide consists of the
5 mature polypeptide of SEQ ID NO: 2, 6, or 8.

In a second aspect, the present invention relates to isolated polypeptides having NADH kinase activity that are encoded by polynucleotides that hybridize under preferably very low stringency conditions, more preferably low stringency conditions, more preferably medium stringency conditions, more preferably medium-high stringency conditions, even more preferably
10 high stringency conditions, and most preferably very high stringency conditions with (i) the mature polypeptide coding sequence of SEQ ID NO: 1, 5, or 7, (ii) the genomic DNA sequence comprising the mature polypeptide coding sequence of SEQ ID NO: 1, 5, or 7, (iii) a subsequence of (i) or (ii), or (iv) a full-length complementary strand of (i), (ii), or (iii) (J. Sambrook, E.F. Fritsch, and T. Maniatis, 1989, *Molecular Cloning, A Laboratory Manual*, 2d edition, Cold Spring Harbor, New York). A subsequence of the mature polypeptide coding sequence of SEQ ID NO: 1 contains
15 at least 100 contiguous nucleotides or preferably at least 200 contiguous nucleotides. Moreover, the subsequence may encode a polypeptide fragment having NADH kinase activity. In a preferred aspect, the complementary strand is the full-length complementary strand of the mature polypeptide coding sequence of SEQ ID NO: 1, 5, or 7.

The nucleotide sequence of SEQ ID NO: 1, 5, or 7; or a subsequence thereof; as well as the amino acid sequence of SEQ ID NO: 2, 6, or 8; or a fragment thereof; may be used to design nucleic acid probes to identify and clone DNA encoding polypeptides having NADH kinase activity from strains of different genera or species according to methods well known in the art. In particular, such probes can be used for hybridization with the genomic or cDNA of the genus or
25 species of interest, following standard Southern blotting procedures, in order to identify and isolate the corresponding gene therein. Such probes can be considerably shorter than the entire sequence, but should be at least 14, preferably at least 25, more preferably at least 35, and most preferably at least 70 nucleotides in length. It is, however, preferred that the nucleic acid probe is at least 100 nucleotides in length. For example, the nucleic acid probe may be at least 200
30 nucleotides, preferably at least 300 nucleotides, more preferably at least 400 nucleotides, or most preferably at least 500 nucleotides in length. Even longer probes may be used, e.g., nucleic acid probes that are preferably at least 600 nucleotides, more preferably at least 700 nucleotides, even more preferably at least 800 nucleotides, or most preferably at least 900 nucleotides in length. Both DNA and RNA probes can be used. The probes are typically labeled for detecting
35 the corresponding gene (for example, with ^{32}P , ^3H , ^{35}S , biotin, or avidin). Such probes are

encompassed by the present invention.

A genomic DNA or cDNA library prepared from such other strains may, therefore, be screened for DNA that hybridizes with the probes described above and encodes a polypeptide having NADH kinase activity. Genomic or other DNA from such other strains may be separated
5 by agarose or polyacrylamide gel electrophoresis, or other separation techniques. DNA from the libraries or the separated DNA may be transferred to and immobilized on nitrocellulose or other suitable carrier material. In order to identify a clone or DNA that is homologous with SEQ ID NO: 1, 5, or 7; or a subsequence thereof; the carrier material is preferably used in a Southern blot.

For purposes of the present invention, hybridization indicates that the nucleotide
10 sequence hybridizes to a labeled nucleic acid probe corresponding to the mature polypeptide coding sequence of SEQ ID NO: 1, 5, or 7; the genomic DNA sequence comprising the mature polypeptide coding sequence of SEQ ID NO: 1, 5, or 7; its full-length complementary strand; or a subsequence thereof; under very low to very high stringency conditions. Molecules to which the nucleic acid probe hybridizes under these conditions can be detected using, for example, X-ray
15 film.

In a preferred aspect, the nucleic acid probe is the mature polypeptide coding sequence of SEQ ID NO: 1, 5, or 7. In another preferred aspect, the nucleic acid probe is a polynucleotide sequence that encodes the polypeptide of SEQ ID NO: 2, 6, or 8, or a subsequence thereof. In another preferred aspect, the nucleic acid probe is SEQ ID NO: 1, 5, or 7. For long probes of at
20 least 100 nucleotides in length, very low to very high stringency conditions are defined as prehybridization and hybridization at 42°C in 5X SSPE, 0.3% SDS, 200 µg/ml sheared and denatured salmon sperm DNA, and either 25% formamide for very low and low stringencies, 35% formamide for medium and medium-high stringencies, or 50% formamide for high and very high stringencies, following standard Southern blotting procedures for 12 to 24 hours optimally.

For long probes of at least 100 nucleotides in length, the carrier material is finally washed
25 three times each for 15 minutes using 2X SSC, 0.2% SDS preferably at 45°C (very low stringency), more preferably at 50°C (low stringency), more preferably at 55°C (medium stringency), more preferably at 60°C (medium-high stringency), even more preferably at 65°C (high stringency), and most preferably at 70°C (very high stringency).

In a third aspect, the present invention relates to isolated polypeptides having NADH
30 kinase activity encoded by polynucleotides comprising or consisting of nucleotide sequences that have a degree of identity to the mature polypeptide coding sequence of SEQ ID NO: 1 of preferably at least 60%, more preferably at least 65%, more preferably at least 70%, more preferably at least 75%, more preferably at least 80%, more preferably at least 85%, even more
35 preferably at least 90%, most preferably at least 95%, and even most preferably 96%, 97%, 98%,

or 99%, which encode an active polypeptide. See polynucleotide section herein.

In still another aspect, the present invention relates to artificial variants comprising a substitution, deletion, and/or insertion of one or more (or several) amino acids of the mature polypeptide of SEQ ID NO: 2, 6 and 8 or a homologous sequence thereof. Preferably, amino acid
5 changes are of a minor nature, that is conservative amino acid substitutions or insertions that do not significantly affect the folding and/or activity of the protein; small deletions, typically of one to about 30 amino acids; small amino- or carboxyl-terminal extensions, such as an amino-terminal methionine residue; a small linker peptide of up to about 20-25 residues; or a small extension that facilitates purification by changing net charge or another function, such as a poly-histidine tract,
10 an antigenic epitope or a binding domain.

Examples of conservative substitutions are within the group of basic amino acids (arginine, lysine and histidine), acidic amino acids (glutamic acid and aspartic acid), polar amino acids (glutamine and asparagine), hydrophobic amino acids (leucine, isoleucine and valine),
15 aromatic amino acids (phenylalanine, tryptophan and tyrosine), and small amino acids (glycine, alanine, serine, threonine and methionine). Amino acid substitutions that do not generally alter specific activity are known in the art and are described, for example, by H. Neurath and R.L. Hill, 1979, *In, The Proteins*, Academic Press, New York. The most commonly occurring exchanges are Ala/Ser, Val/Ile, Asp/Glu, Thr/Ser, Ala/Gly, Ala/Thr, Ser/Asn, Ala/Val, Ser/Gly, Tyr/Phe, Ala/Pro, Lys/Arg, Asp/Asn, Leu/Ile, Leu/Val, Ala/Glu, and Asp/Gly.

20 In addition to the 20 standard amino acids, non-standard amino acids (such as 4-hydroxyproline, 6-N-methyl lysine, 2-aminoisobutyric acid, isovaline, and alpha-methyl serine) may be substituted for amino acid residues of a wild-type polypeptide. A limited number of non-conservative amino acids, amino acids that are not encoded by the genetic code, and unnatural amino acids may be substituted for amino acid residues. "Unnatural amino acids" have been
25 modified after protein synthesis, and/or have a chemical structure in their side chain(s) different from that of the standard amino acids. Unnatural amino acids can be chemically synthesized, and preferably, are commercially available, and include pipercolic acid, thiazolidine carboxylic acid, dehydroproline, 3- and 4-methylproline, and 3,3-dimethylproline.

Alternatively, the amino acid changes are of such a nature that the physico-chemical
30 properties of the polypeptides are altered. For example, amino acid changes may improve the thermal stability of the polypeptide, alter the substrate specificity, change the pH optimum, and the like.

Essential amino acids in the parent polypeptide can be identified according to procedures known in the art, such as site-directed mutagenesis or alanine-scanning mutagenesis
35 (Cunningham and Wells, 1989, *Science* 244: 1081-1085). In the latter technique, single alanine

mutations are introduced at every residue in the molecule, and the resultant mutant molecules are tested for biological activity (*i.e.*, NADH kinase activity) to identify amino acid residues that are critical to the activity of the molecule. See also, Hilton *et al.*, 1996, *J. Biol. Chem.* 271: 4699-4708. The active site of the enzyme or other biological interaction can also be determined by
5 physical analysis of structure, as determined by such techniques as nuclear magnetic resonance, crystallography, electron diffraction, or photoaffinity labeling, in conjunction with mutation of putative contact site amino acids. See, for example, de Vos *et al.*, 1992, *Science* 255: 306-312; Smith *et al.*, 1992, *J. Mol. Biol.* 224: 899-904; Wlodaver *et al.*, 1992, *FEBS Lett.* 309: 59-64. The identities of essential amino acids can also be inferred from analysis of identities with
10 polypeptides that are related to a polypeptide according to the invention.

Single or multiple amino acid substitutions, deletions, and/or insertions can be made and tested using known methods of mutagenesis, recombination, and/or shuffling, followed by a relevant screening procedure, such as those disclosed by Reidhaar-Olson and Sauer, 1988, *Science* 241: 53-57; Bowie and Sauer, 1989, *Proc. Natl. Acad. Sci. USA* 86: 2152-2156; WO
15 95/17413; or WO 95/22625. Other methods that can be used include error-prone PCR, phage display (*e.g.*, Lowman *et al.*, 1991, *Biochem.* 30: 10832-10837; U.S. Patent No. 5,223,409; WO 92/06204), and region-directed mutagenesis (Derbyshire *et al.*, 1986, *Gene* 46: 145; Ner *et al.*, 1988, *DNA* 7: 127).

Mutagenesis/shuffling methods can be combined with high-throughput, automated
20 screening methods to detect activity of cloned, mutagenized polypeptides expressed by host cells (Ness *et al.*, 1999, *Nature Biotechnology* 17: 893-896). Mutagenized DNA molecules that encode active polypeptides can be recovered from the host cells and rapidly sequenced using standard methods in the art. These methods allow the rapid determination of the importance of individual amino acid residues in a polypeptide of interest, and can be applied to polypeptides of unknown
25 structure.

The total number of amino acid substitutions, deletions and/or insertions of the mature polypeptide of SEQ ID NO: 2, 6 or 8, is 10, preferably 9, more preferably 8, more preferably 7, more preferably at most 6, more preferably 5, more preferably 4, even more preferably 3, most preferably 2, and even most preferably 1.

30

Sources of Polypeptides Having NADH kinase Activity

A polypeptide of the present invention may be obtained from microorganisms of any genus. For purposes of the present invention, the term "obtained from" as used herein in connection with a given source shall mean that the polypeptide encoded by a nucleotide
35 sequence is produced by the source or by a strain in which the nucleotide sequence from the

source has been inserted. In a preferred aspect, the polypeptide obtained from a given source is secreted extra-cellularly.

A polypeptide having NADH kinase activity of the present invention may be a fungal polypeptide, and more preferably a yeast polypeptide such as a *Candida*, *Kluyveromyces*, *Pichia*,
5 *Saccharomyces*, *Schizosaccharomyces*, or *Yarrowia* polypeptide having NADH kinase activity; or more preferably a filamentous fungal polypeptide such as an *Acremonium*, *Agaricus*, *Alternaria*,
Aspergillus, *Aureobasidium*, *Botryosphaeria*, *Ceriporiopsis*, *Chaetomidium*, *Chrysosporium*,
Claviceps, *Cochliobolus*, *Coprinopsis*, *Coptotermes*, *Corynascus*, *Cryphonectria*, *Cryptococcus*,
10 *Diplodia*, *Exidia*, *Filibasidium*, *Fusarium*, *Gibberella*, *Holomastigotoides*, *Humicola*, *Irpex*,
Lentinula, *Leptosphaeria*, *Magnaporthe*, *Melanocarpus*, *Meripilus*, *Mucor*, *Myceliophthora*,
Neocallimastix, *Neurospora*, *Paecilomyces*, *Penicillium*, *Phanerochaete*, *Piromyces*, *Poitrasia*,
Pseudoplectania, *Pseudotriconympha*, *Rhizomucor*, *Schizophyllum*, *Scytalidium*, *Talaromyces*,
Thermoascus, *Thielavia*, *Tolypocladium*, *Trichoderma*, *Trichophaea*, *Verticillium*, *Volvariella*, or
Xylaria polypeptide having NADH kinase activity.

15 In a preferred aspect, the polypeptide is a *Saccharomyces carlsbergensis*,
Saccharomyces cerevisiae, *Saccharomyces diastaticus*, *Saccharomyces douglasii*,
Saccharomyces kluyveri, *Saccharomyces norbensis*, or *Saccharomyces oviformis* polypeptide
having NADH kinase activity.

In another preferred aspect, the polypeptide is an *Acremonium cellulolyticus*, *Aspergillus*
20 *aculeatus*, *Aspergillus awamori*, *Aspergillus fumigatus*, *Aspergillus foetidus*, *Aspergillus*
japonicus, *Aspergillus nidulans*, *Aspergillus niger*, *Aspergillus oryzae*, *Chrysosporium*
keratinophilum, *Chrysosporium lucknowense*, *Chrysosporium tropicum*, *Chrysosporium*
merdarium, *Chrysosporium inops*, *Chrysosporium pannicola*, *Chrysosporium queenslandicum*,
Chrysosporium zonatum, *Fusarium bactridioides*, *Fusarium cerealis*, *Fusarium crookwellense*,
25 *Fusarium culmorum*, *Fusarium graminearum*, *Fusarium graminum*, *Fusarium heterosporum*,
Fusarium negundi, *Fusarium oxysporum*, *Fusarium reticulatum*, *Fusarium roseum*, *Fusarium*
sambucinum, *Fusarium sarcochromum*, *Fusarium sporotrichioides*, *Fusarium sulphureum*,
Fusarium torulosum, *Fusarium trichothecioides*, *Fusarium venenatum*, *Humicola grisea*, *Humicola*
insolens, *Humicola lanuginosa*, *Irpex lacteus*, *Mucor miehei*, *Myceliophthora thermophila*,
30 *Neurospora crassa*, *Penicillium funiculosum*, *Penicillium purpurogenum*, *Phanerochaete*
chrysosporium, *Thielavia achromatica*, *Thielavia albomyces*, *Thielavia albopilosa*, *Thielavia*
australeinsis, *Thielavia fimeti*, *Thielavia microspora*, *Thielavia ovispora*, *Thielavia peruviana*,
Thielavia spededonium, *Thielavia setosa*, *Thielavia subthermophila*, *Thielavia terrestris*,
Trichoderma harzianum, *Trichoderma koningii*, *Trichoderma longibrachiatum*, *Trichoderma*
35 *reesei*, or *Trichoderma viride* polypeptide having having NADH kinase activity.

In another preferred aspect, the polypeptide is a *Aspergillus nidulans*, *Aspergillus niger*, or *Aspergillus oryzae* polypeptide.

In a more preferred aspect, the polypeptide is an *Aspergillus nidulans* polypeptide having NADH kinase activity. In a most preferred aspect, the polypeptide is an *Aspergillus nidulans* polypeptide having NADH kinase activity, e.g., the polypeptide comprising the mature polypeptide of SEQ ID NO: 2, obtainable from e.g. *Aspergillus nidulans* AR1 (IBT 27263 from the Glasgow strain collection).

In a more preferred aspect, the polypeptide is an *Aspergillus niger* polypeptide having NADH kinase activity. In a most preferred aspect, the polypeptide is an *Aspergillus niger* polypeptide having NADH kinase activity, e.g., the polypeptide comprising the mature polypeptide of SEQ ID NO: 6, obtainable from e.g. *Aspergillus niger* BO1 (DSM 12665).

In a more preferred aspect, the polypeptide is an *Aspergillus oryzae* polypeptide having NADH kinase activity. In a most preferred aspect, the polypeptide is an *Aspergillus oryzae* polypeptide having NADH kinase activity, e.g., the polypeptide comprising the mature polypeptide of SEQ ID NO: 8, obtainable from e.g. *Aspergillus oryzae* IFO 4177 (also known as A 1560) available from Institute for Fermentation, Osaka or from NITE Biological Resource Center (NBRC) as NBRC 4177.

It will be understood that for the aforementioned species the invention encompasses both the perfect and imperfect states, and other taxonomic equivalents, e.g., anamorphs, regardless of the species name by which they are known. Those skilled in the art will readily recognize the identity of appropriate equivalents.

Strains of these species are readily accessible to the public in a number of culture collections, such as the American Type Culture Collection (ATCC), Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ), Centraalbureau Voor Schimmelcultures (CBS), and Agricultural Research Service Patent Culture Collection, Northern Regional Research Center (NRRL).

Furthermore, such polypeptides may be identified and obtained from other sources including microorganisms isolated from nature (e.g., soil, composts, water, etc.) using the above-mentioned probes. Techniques for isolating microorganisms from natural habitats are well known in the art. The polynucleotide may then be obtained by similarly screening a genomic or cDNA library of such a microorganism. Once a polynucleotide sequence encoding a polypeptide has been detected with the probe(s), the polynucleotide can be isolated or cloned by utilizing techniques that are well known to those of ordinary skill in the art (see, e.g., Sambrook *et al.*, 1989, *supra*).

Polypeptides of the present invention also include fused polypeptides or cleavable fusion

polypeptides in which another polypeptide is fused at the N-terminus or the C-terminus of the polypeptide or fragment thereof. A fused polypeptide is produced by fusing a nucleotide sequence (or a portion thereof) encoding another polypeptide to a nucleotide sequence (or a portion thereof) of the present invention. Techniques for producing fusion polypeptides are known in the art, and include ligating the coding sequences encoding the polypeptides so that they are in frame and that expression of the fused polypeptide is under control of the same promoter(s) and terminator.

A fusion polypeptide can further comprise a cleavage site. Upon secretion of the fusion protein, the site is cleaved releasing the polypeptide having NADH kinase activity from the fusion protein. Examples of cleavage sites include, but are not limited to, a Kex2 site that encodes the dipeptide Lys-Arg (Martin *et al.*, 2003, *J. Ind. Microbiol. Biotechnol.* 3: 568-76; Svetina *et al.*, 2000, *J. Biotechnol.* 76: 245-251; Rasmussen-Wilson *et al.*, 1997, *Appl. Environ. Microbiol.* 63: 3488-3493; Ward *et al.*, 1995, *Biotechnology* 13: 498-503; and Contreras *et al.*, 1991, *Biotechnology* 9: 378-381), an Ile-(Glu or Asp)-Gly-Arg site, which is cleaved by a Factor Xa protease after the arginine residue (Eaton *et al.*, 1986, *Biochem.* 25: 505-512); a Asp-Asp-Asp-Asp-Lys site, which is cleaved by an enterokinase after the lysine (Collins-Racie *et al.*, 1995, *Biotechnology* 13: 982-987); a His-Tyr-Glu site or His-Tyr-Asp site, which is cleaved by Genenase I (Carter *et al.*, 1989, *Proteins: Structure, Function, and Genetics* 6: 240-248); a Leu-Val-Pro-Arg-Gly-Ser site, which is cleaved by thrombin after the Arg (Stevens, 2003, *Drug Discovery World* 4: 35-48); a Glu-Asn-Leu-Tyr-Phe-Gln-Gly site, which is cleaved by TEV protease after the Gln (Stevens, 2003, *supra*); and a Leu-Glu-Val-Leu-Phe-Gln-Gly-Pro site, which is cleaved by a genetically engineered form of human rhinovirus 3C protease after the Gln (Stevens, 2003, *supra*).

25 Polynucleotides

The present invention also relates to isolated polynucleotides comprising or consisting of nucleotide sequences that encode polypeptides having NADH kinase activity of the present invention.

In a preferred aspect, the nucleotide sequence comprises or consists of SEQ ID NO: 1, 5, or 7. In another preferred aspect, the nucleotide sequence comprises or consists of the mature polypeptide coding sequence of SEQ ID NO: 1, 5, or 7. The present invention also encompasses nucleotide sequences that encode polypeptides comprising or consisting of the amino acid sequence of SEQ ID NO: 2, 6, or 8 or the mature polypeptide thereof, which differ from SEQ ID NO: 1, 5, or 7 or the mature polypeptide coding sequence thereof by virtue of the degeneracy of the genetic code. The present invention also relates to subsequences of SEQ ID NO: 1, 5, or 7

that encode fragments of SEQ ID NO: 2, 6, or 8 that have NADH kinase activity.

The present invention also relates to mutant polynucleotides comprising or consisting of at least one mutation in the mature polypeptide coding sequence of SEQ ID NO: 1, 5, or 7, in which the mutant nucleotide sequence encodes the mature polypeptide of SEQ ID NO: 2, 6, or 8.

5 The techniques used to isolate or clone a polynucleotide encoding a polypeptide are known in the art and include isolation from genomic DNA, preparation from cDNA, or a combination thereof. The cloning of the polynucleotides of the present invention from such genomic DNA can be effected, *e.g.*, by using the well known polymerase chain reaction (PCR) or antibody screening of expression libraries to detect cloned DNA fragments with shared structural
10 features. See, *e.g.*, Innis *et al.*, 1990, *PCR: A Guide to Methods and Application*, Academic Press, New York. Other nucleic acid amplification procedures such as ligase chain reaction (LCR), ligated activated transcription (LAT) and nucleotide sequence-based amplification (NASBA) may be used. The polynucleotides may be cloned from a strain of *Aspergillus*, or another or related organism and thus, for example, may be an allelic or species variant of the
15 polypeptide encoding region of the nucleotide sequence.

The present invention also relates to isolated polynucleotides comprising or consisting of nucleotide sequences that have a degree of identity to the mature polypeptide coding sequence of SEQ ID NO: 1, 5, or 7 of preferably at least 60%, more preferably at least 65%, more preferably at least 70%, more preferably at least 75%, more preferably at least 80%, more
20 preferably at least 85%, even more preferably at least 90%, most preferably at least 95%, and even most preferably at least 96%, at least 97%, at least 98%, or at least 99% identity, which encode an active polypeptide.

Modification of a nucleotide sequence encoding a polypeptide of the present invention may be necessary for the synthesis of polypeptides substantially similar to the polypeptide. The
25 term "substantially similar" to the polypeptide refers to non-naturally occurring forms of the polypeptide. These polypeptides may differ in some engineered way from the polypeptide isolated from its native source, *e.g.*, artificial variants that differ in specific activity, thermostability, pH optimum, or the like. The variant sequence may be constructed on the basis of the nucleotide sequence presented as the mature polypeptide coding sequence of SEQ ID NO: 1, 5, or 7, *e.g.*, a
30 subsequence thereof, and/or by introduction of nucleotide substitutions that do not give rise to another amino acid sequence of the polypeptide encoded by the nucleotide sequence, but which correspond to the codon usage of the host organism intended for production of the enzyme, or by introduction of nucleotide substitutions that may give rise to a different amino acid sequence. For a general description of nucleotide substitution, see, *e.g.*, Ford *et al.*, 1991, *Protein Expression and Purification* 2: 95-107.
35

It will be apparent to those skilled in the art that such substitutions can be made outside the regions critical to the function of the molecule and still result in an active polypeptide. Amino acid residues essential to the activity of the polypeptide encoded by an isolated polynucleotide of the invention, and therefore preferably not subject to substitution, may be identified according to
5 procedures known in the art, such as site-directed mutagenesis or alanine-scanning mutagenesis (see, *e.g.*, Cunningham and Wells, 1989, *supra*). In the latter technique, mutations are introduced at every positively charged residue in the molecule, and the resultant mutant molecules are tested for NADH kinase activity to identify amino acid residues that are critical to the activity of the molecule. Sites of substrate-enzyme interaction can also be determined by analysis of the three-
10 dimensional structure as determined by such techniques as nuclear magnetic resonance analysis, crystallography or photoaffinity labeling (see, *e.g.*, de Vos *et al.*, 1992, *supra*; Smith *et al.*, 1992, *supra*; Wlodaver *et al.*, 1992, *supra*).

The present invention also relates to isolated polynucleotides encoding polypeptides of the present invention, which hybridize under very low stringency conditions, preferably low
15 stringency conditions, more preferably medium stringency conditions, more preferably medium-high stringency conditions, even more preferably high stringency conditions, and most preferably very high stringency conditions with (i) the mature polypeptide coding sequence of SEQ ID NO: 1, 5, or 7, (ii) the genomic DNA sequence comprising the mature polypeptide coding sequence of SEQ ID NO: 1, 5, or 7, or (iii) a full-length complementary strand of (i) or (ii); or allelic variants and
20 subsequences thereof (Sambrook *et al.*, 1989, *supra*), as defined herein. In a preferred aspect, the complementary strand is the full-length complementary strand of the mature polypeptide coding sequence of SEQ ID NO: 1, 5, or 7.

The present invention also relates to isolated polynucleotides obtained by (a) hybridizing a population of DNA under very low, low, medium, medium-high, high, or very high stringency
25 conditions with (i) the mature polypeptide coding sequence of SEQ ID NO: 1, 5, or 7, (ii) the genomic DNA sequence comprising the mature polypeptide coding sequence of SEQ ID NO: 1, 5, or 7, or (iii) a full-length complementary strand of (i) or (ii); and (b) isolating the hybridizing polynucleotide, which encodes a polypeptide having NADH kinase activity. In a preferred aspect, the complementary strand is the full-length complementary strand of the mature polypeptide
30 coding sequence of SEQ ID NO: 1, 5, or 7.

Nucleic Acid Constructs

The present invention also relates to nucleic acid constructs comprising an isolated polynucleotide of the present invention operably linked to one or more (several) control
35 sequences that direct the expression of the coding sequence in a suitable host cell under

conditions compatible with the control sequences.

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

The control sequence may be an appropriate promoter sequence, a nucleotide sequence that is recognized by a host cell for expression of a polynucleotide encoding a polypeptide of the present invention. The promoter sequence contains transcriptional control sequences that
10 mediate the expression of the polypeptide. The promoter may be any nucleotide sequence that shows transcriptional activity in the host cell of choice 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 the transcription of the nucleic acid
15 constructs of the present invention in a filamentous fungal host cell are promoters obtained from the genes for *Aspergillus oryzae* TAKA amylase, *Rhizomucor miehei* aspartic proteinase, *Aspergillus niger* neutral alpha-amylase, *Aspergillus niger* acid stable alpha-amylase, *Aspergillus niger* or *Aspergillus awamori* glucoamylase (*glaA*), *Rhizomucor miehei* lipase, *Aspergillus oryzae* alkaline protease, *Aspergillus oryzae* triose phosphate isomerase, *Aspergillus nidulans* acetamidase, *Fusarium venenatum* amyloglucosidase (WO 00/56900), *Fusarium venenatum* Daria (WO 00/56900), *Fusarium venenatum* Quinn (WO 00/56900), *Fusarium oxysporum* trypsin-like protease (WO 96/00787), *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 IV, *Trichoderma reesei* endoglucanase V, *Trichoderma reesei* xylanase I,
20 *Trichoderma reesei* xylanase II, *Trichoderma reesei* beta-xylosidase, as well as the NA2-tpi promoter (a hybrid of the promoters from the genes for *Aspergillus niger* neutral alpha-amylase and *Aspergillus oryzae* triose phosphate isomerase); and mutant, truncated, and hybrid promoters thereof.

30 In a yeast host, useful promoters are obtained from the genes for *Saccharomyces cerevisiae* enolase (ENO-1), *Saccharomyces cerevisiae* galactokinase (GAL1), *Saccharomyces cerevisiae* alcohol dehydrogenase/glyceraldehyde-3-phosphate dehydrogenase (ADH1, ADH2/GAP), *Saccharomyces cerevisiae* triose phosphate isomerase (TPI), *Saccharomyces cerevisiae* metallothionein (CUP1), and *Saccharomyces cerevisiae* 3-phosphoglycerate kinase.
35 Other useful promoters for yeast host cells are described by Romanos *et al.*, 1992, *Yeast* 8: 423-

488.

The control sequence may also be a signal peptide coding sequence that codes for an amino acid sequence linked to the amino terminus of a polypeptide and directs the encoded polypeptide into the cell's secretory pathway. The 5' end of the coding sequence of the nucleotide sequence may inherently contain a signal peptide coding sequence naturally linked in translation reading frame with the segment of the coding sequence that encodes the secreted polypeptide. Alternatively, the 5' end of the coding sequence may contain a signal peptide coding sequence that is foreign to the coding sequence. The foreign signal peptide coding sequence may be required where the coding sequence does not naturally contain a signal peptide coding sequence. Alternatively, the 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 of choice, *i.e.*, secreted into a culture medium, may be used in the present invention.

Effective signal peptide coding sequences for filamentous fungal host cells are the signal peptide coding sequences obtained from the genes for *Aspergillus oryzae* TAKA amylase, *Aspergillus niger* neutral amylase, *Aspergillus niger* glucoamylase, *Rhizomucor miehei* aspartic proteinase, *Humicola insolens* cellulase, *Humicola insolens* endoglucanase V, and *Humicola lanuginosa* lipase.

Useful signal peptides for yeast host cells are obtained from the genes for *Saccharomyces cerevisiae* alpha-factor and *Saccharomyces cerevisiae* invertase. Other useful signal peptide coding sequences are described by Romanos *et al.*, 1992, *supra*.

The control sequence may also be a propeptide coding sequence that codes for an amino acid sequence positioned at the amino terminus of a polypeptide. The resultant polypeptide is known as a proenzyme or propolypeptide (or a zymogen in some cases). A propeptide is generally inactive and can be converted to a mature 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*), *Saccharomyces cerevisiae* alpha-factor, *Rhizomucor miehei* aspartic proteinase, and *Myceliophthora thermophila* laccase (WO 95/33836).

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

It may also be desirable to add regulatory sequences that allow the regulation of the expression of the polypeptide relative to the growth of the host cell. Examples of regulatory

systems are those that cause the 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 yeast, the ADH2 system or GAL1 system may be used. In filamentous fungi, the TAKA alpha-amylase promoter, *Aspergillus niger* glucoamylase promoter, and *Aspergillus oryzae* glucoamylase promoter may be used as regulatory sequences. 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 nucleotide sequence encoding the polypeptide would be operably linked with the regulatory sequence.

10

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 nucleic acids and control sequences described herein may be joined together to produce a recombinant expression vector that may include one or more (several) convenient restriction sites to allow for insertion or substitution of the nucleotide sequence encoding the polypeptide at such sites. Alternatively, a polynucleotide sequence of the present invention may be expressed by inserting the nucleotide sequence or a nucleic acid construct comprising the sequence 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.

15

20

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

25

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.

30

35

The vectors of the present invention preferably contain one or more (several) 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.

Suitable markers for yeast host cells are ADE2, HIS3, LEU2, LYS2, MET3, TRP1, and
5 URA3. Selectable markers for use in a filamentous fungal host cell include, but are not limited to, *amdS* (acetamidase), *argB* (ornithine carbamoyltransferase), *bar* (phosphinothricin acetyltransferase), *hph* (hygromycin phosphotransferase), *niaD* (nitrate reductase), *pyrG* (orotidine-5'-phosphate decarboxylase), *sC* (sulfate adenylyltransferase), and *trpC* (anthranilate synthase), as well as equivalents thereof. Preferred for use in an *Aspergillus* cell are the *amdS*
10 and *pyrG* genes of *Aspergillus nidulans* or *Aspergillus oryzae* and the *bar* gene of *Streptomyces hygroscopicus*.

The vectors of the present invention preferably contain 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.

15 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 nonhomologous recombination. Alternatively, the vector may contain additional nucleotide sequences 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
20 of integration at a precise location, the integrational elements should preferably contain a sufficient number of nucleic acids, such as 100 to 10,000 base pairs, preferably 400 to 10,000 base pairs, and most preferably 800 to 10,000 base pairs, which have a high degree of 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
25 genome of the host cell. Furthermore, the integrational elements may be non-encoding or encoding nucleotide sequences. On the other hand, the vector may be integrated into the genome of the host cell by non-homologous recombination.

More than one copy of a polynucleotide of the present invention may be inserted into a host cell to increase production of the gene product. An increase in the copy number of the
30 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.

35 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*).

Host Cells

5 The present invention also relates to recombinant host cells, which are advantageously used in the recombinant production of a polypeptide of interest. A vector comprising a polynucleotide to be expressed is introduced into a host cell so that the vector is maintained as a chromosomal integrant or as a self-replicating extra-chromosomal vector as described earlier.

10 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.

15 In a preferred aspect, the host cell is a fungal cell. "Fungi" as used herein includes the phyla Ascomycota, Basidiomycota, Chytridiomycota, and Zygomycota (as defined by Hawksworth *et al.*, *In, Ainsworth and Bisby's Dictionary of The Fungi*, 8th edition, 1995, CAB International, University Press, Cambridge, UK) as well as the Oomycota (as cited in Hawksworth *et al.*, 1995, *supra*, page 171) and all mitosporic fungi (Hawksworth *et al.*, 1995, *supra*).

20 In a more preferred aspect, the fungal host cell is a yeast cell. "Yeast" as used herein includes ascosporegenous yeast (Endomycetales), basidiosporegenous yeast, and yeast belonging to the Fungi Imperfecti (Blastomycetes). Since the classification of yeast may change in the future, for the purposes of this invention, yeast shall be defined as described in *Biology and Activities of Yeast* (Skinner, F.A., Passmore, S.M., and Davenport, R.R., eds, *Soc. App. Bacteriol. Symposium Series No. 9*, 1980).

 In an even more preferred aspect, the yeast host cell is a *Candida*, *Hansenula*, *Kluyveromyces*, *Pichia*, *Saccharomyces*, *Schizosaccharomyces*, or *Yarrowia* cell.

25 In a most preferred aspect, the yeast host cell is a *Saccharomyces carlsbergensis*, *Saccharomyces cerevisiae*, *Saccharomyces diastaticus*, *Saccharomyces douglasii*, *Saccharomyces kluyveri*, *Saccharomyces norbensis*, or *Saccharomyces oviformis* cell. In another most preferred aspect, the yeast host cell is a *Kluyveromyces lactis* cell. In another most preferred aspect, the yeast host cell is a *Yarrowia lipolytica* cell.

30 In another more preferred aspect, the fungal host cell 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
35 aerobic. In contrast, vegetative growth by yeasts such as *Saccharomyces cerevisiae* is by

budding of a unicellular thallus and carbon catabolism may be fermentative.

In an even more preferred aspect, the filamentous fungal host cell is an *Acremonium*, *Aspergillus*, *Aureobasidium*, *Bjerkandera*, *Ceriporiopsis*, *Chrysosporium*, *Coprinus*, *Coriolus*, *Cryptococcus*, *Filibasidium*, *Fusarium*, *Humicola*, *Magnaporthe*, *Mucor*, *Myceliophthora*,
5 *Neocallimastix*, *Neurospora*, *Paecilomyces*, *Penicillium*, *Phanerochaete*, *Phlebia*, *Piromyces*,
Pleurotus, *Schizophyllum*, *Talaromyces*, *Thermoascus*, *Thielavia*, *Tolypocladium*, *Trametes*, or
Trichoderma cell.

In a most preferred aspect, the filamentous fungal host cell is an *Aspergillus awamori*,
Aspergillus fumigatus, *Aspergillus foetidus*, *Aspergillus japonicus*, *Aspergillus nidulans*,
10 *Aspergillus niger* or *Aspergillus oryzae* cell. In another most preferred aspect, the filamentous
fungal host cell is a *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*,
15 *Fusarium torulosum*, *Fusarium trichothecioides*, or *Fusarium venenatum* cell. In another most
preferred aspect, the filamentous fungal host cell is a *Bjerkandera adusta*, *Ceriporiopsis aneirina*,
Ceriporiopsis aneirina, *Ceriporiopsis caregiea*, *Ceriporiopsis gilvescens*, *Ceriporiopsis*
pannocinta, *Ceriporiopsis rivulosa*, *Ceriporiopsis subrufa*, *Ceriporiopsis subvermispora*,
Chrysosporium keratinophilum, *Chrysosporium lucknowense*, *Chrysosporium tropicum*,
20 *Chrysosporium merdarium*, *Chrysosporium inops*, *Chrysosporium pannicola*, *Chrysosporium*
queenslandicum, *Chrysosporium zonatum*, *Coprinus cinereus*, *Coriolus hirsutus*, *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*
25 *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 238 023 and Yelton *et al.*, 1984, *Proceedings of the National Academy of Sciences USA* 81:
30 1470-1474. Suitable methods for transforming *Fusarium* species are described by Malardier *et*
al., 1989, *Gene* 78: 147-156, and WO 96/00787. Yeast may be transformed using the procedures
described by Becker and Guarente, *In* Abelson, J.N. and Simon, M.I., editors, *Guide to Yeast*
Genetics and Molecular Biology, Methods in Enzymology, Volume 194, pp 182-187, Academic
Press, Inc., New York; Ito *et al.*, 1983, *Journal of Bacteriology* 153: 163; and Hinnen *et al.*, 1978,
35 *Proceedings of the National Academy of Sciences USA* 75: 1920.

Methods of Production

The present invention also relates to methods of producing a polypeptide of the present invention, comprising: (a) cultivating a cell, which in its wild-type form produces the polypeptide, under conditions conducive for production of the polypeptide; and (b) recovering the polypeptide. In a preferred aspect, the cell is of the genus *Aspergillus*. In a more preferred aspect, the cell is *Aspergillus nidulans*. In a most preferred aspect, the cell is *Aspergillus nidulans* AR1 [pyrG89 argB2; veA1] (IBT 27263 of the Glasgow strain collection). In another preferred aspect the cell is *Aspergillus niger*. In a most preferred aspect, the cell is *Aspergillus niger* BO1, DSM12665. In another preferred aspect the cell is *Aspergillus oryzae*. In a most preferred aspect, the cell is *Aspergillus oryzae* IFO 4177 (also known as A 1560) available from Institute for Fermentation, Osaka or from NITE Biological Resource Center (NBRC) as NBRC 4177.

The present invention also relates to methods of producing a polypeptide of the present invention, comprising: (a) cultivating a recombinant host cell, as described herein, under conditions conducive for production of the polypeptide; and (b) recovering the polypeptide.

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

The polypeptides may be detected using methods known in the art that are specific for the polypeptides. These detection methods may include 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 as described herein.

The resulting 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, centrifugation, filtration, extraction, spray-drying, evaporation, or precipitation.

The polypeptides of the present invention 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
5 extraction (see, e.g., *Protein Purification*, J.-C. Janson and Lars Ryden, editors, VCH Publishers, New York, 1989) to obtain substantially pure polypeptides.

The present invention is also directed to methods for using the polypeptides having NADH kinase activity.

In one aspect of the invention the NADH kinase according to the invention is used to
10 increase the growth rate of the host cell by increasing the NADH kinase activity compared to the wild type cell. NADH kinase activity may be increased in many ways known to the skilled person. One way could be by mutagenesis of the NADH kinase gene selecting for increased activity, however, the simplest way of increasing activity is to increase the level of NADH kinase gene expression. This can be achieved by increasing the promoter strength of the promoter controlling
15 expression of the NADH kinase gene and/or by increasing the copy number of the NADH kinase gene.

Preferably the NADH kinase is expressed recombinantly from a nucleic acid construct comprised in the host cell. The nucleic acid construct may be integrated in the host cell genome or it may be comprised in an expression vector present in the host cell. The NADH kinase
20 according to the invention may be expressed using its native signal sequence or the signal may be replaced by a non-native (exogenous) signal as described below.

The present invention also relates to recombinant expression vectors and recombinant host cells comprising such nucleic acid constructs.

In one aspect the invention therefore relates to a recombinant fungal host cell, wherein an
25 NAD(H) kinase activity is increased compared to an otherwise identical wild type host cell cultured under identical conditions. More preferably the recombinant host cell further encodes a polypeptide of interest. In one embodiment the polypeptide of interest is a heterologous polypeptide. Most preferably the host cell is filamentous fungal cell. NADH kinase activity can be increased in several ways known to the skilled person, however, the simplest way is to increase
30 the transcription level of the gene encoding the NADH kinase. This can in one embodiment be done by e.g. using a stronger promoter.

Particularly the kinase activity is increased at least 25 %, more preferably at least 35 %, more preferably at least 45 %, more preferably at least 55%, more preferably at least 65%, more preferably at least 75 %, more preferably at least 85%, more preferably at least 95 %.

35 The NADH kinase which is expressed in the host cell can be either exogenous or

endogenous to the host cell.

The NADH kinase is normally targeted to the mitochondrion, but may also be present in the cytoplasm. This may particularly be the case when the NADH kinase is over-expressed.

Therefore in one embodiment the NADH kinase is present in the cytoplasm. In a further
5 embodiment the NADH kinase is expressed without its normal mitochondrial targeting signal thereby increasing the level of the kinase present in the cytoplasm. The kinase may even be specifically targeted to the cytoplasm by deleting the mitochondrial targeting signal. In one other
10 embodiment the signal peptide employed is the native signal normally associated with the NADH kinase, however, other signal peptides may also advantageously be used, particularly, when specific targeting is desired. In another embodiment the signal peptide therefore is different from the native signal peptide of the NADH kinase.

In one particular aspect the invention relates to a recombinant fungal host cell, wherein the NADH kinase is selected from the group consisting of:

(a) a polypeptide comprising an amino acid sequence having preferably at least 60%,
15 more preferably at least 65%, more preferably at least 70%, more preferably at least 75%, more preferably at least 80%, more preferably at least 85%, even more preferably at least 85%, most preferably at least 90%, and even most preferably at least 95% identity to the mature polypeptide of SEQ ID NO: 2, 6, or 8;

(b) a polypeptide encoded by a polynucleotide that hybridizes under preferably at least low
20 stringency conditions, more preferably at least medium stringency conditions, even more preferably at least medium-high stringency conditions, and most preferably at least high stringency conditions with (i) the mature polypeptide coding sequence of SEQ ID NO: 1, 5, or 7, (ii) the cDNA sequence contained in SEQ ID NO: 1, 5, or 7, or (iii) a full-length complementary strand of (i) or (ii);

(c) a polypeptide encoded by a polynucleotide comprising a nucleotide sequence having
25 preferably at least 60%, more preferably at least 65%, more preferably at least 70%, more preferably at least 75%, more preferably at least 80%, more preferably at least 85%, even more preferably at least 85%, most preferably at least 90%, and even most preferably at least 95% identity to the mature polypeptide coding sequence of SEQ ID NO: 1, 5, or 7;

(d) a variant comprising a substitution, deletion, and/or insertion of one or several amino
30 acids of the mature polypeptide of SEQ ID NO: 2, 6, or 8.

The recombinant host cell as described previously, in which the NADH kinase activity is increased, is preferably a yeast cell or a filamentous fungal cell. Most preferably the filamentous fungal host cell is selected from the group consisting of *Aspergillus*, *Chrysosporium*, *Fusarium*,
35 *Myceliophthora*, and *Trichoderma*. The *Aspergillus* host cell is preferably *Aspergillus oryzae*,

Aspergillus niger or *Aspergillus nidulans*.

The recombinant host cell can advantageously be used to produce a polypeptide of interest.

In a further aspect the present invention thus relates to a method of producing a polypeptide of interest, comprising culturing the recombinant host cell of the invention further comprising a
5 nucleic acid sequence encoding the polypeptide of interest, under conditions conducive for the production of the polypeptide. The said recombinant host cell of the invention has an increased activity of the NADH kinase compared to an otherwise identical wild type host cell cultured under identical conditions. The said polypeptide of interest may be a heterologous polypeptide or a native polypeptide (endogenous to the host cell).

10 The polypeptide of interest may be expressed using the native promoter or the expression may be recombinant whereby the native promoter has been replaced by a non-native promoter.

In an even further aspect the present invention relates to a method for increasing growth rate of a fungal host cell comprising increasing an NAD(H) kinase activity, e.g. by increasing the level of expression of the NADH kinase, compared to an otherwise identical wild type host cell
15 cultured under identical conditions. The fungal host cell is selected from a yeast cell or a filamentous fungal cell. Particularly the fungal host cell is 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*,
20 *Pleurotus*, *Schizophyllum*, *Talaromyces*, *Thermoascus*, *Thielavia*, *Tolypocladium*, *Trametes*, or *Trichoderma*.

More particularly the host cell is *Aspergillus nidulans*, *Aspergillus niger* or *Aspergillus oryzae*.

For the methods of the invention of producing a polypeptide of interest the growth medium
25 may comprise different carbon sources. In one embodiment the carbon source is selected from the group consisting of starch, maltodextrin maltotriose, maltose, glucose, sucrose, fructose, sorbitol, mannitol, glycerol, acetate. Furthermore the host cells may advantageously be grown under conditions where oxygen is limited. Particularly, wherein the dissolved oxygen tension is lower than 20%.

30 The protein of interest may be native or heterologous to a host cell. The term "protein" is not meant herein to refer to a specific length of the encoded product and, therefore, encompasses peptides, oligopeptides, and proteins. The term "protein" also encompasses two or more polypeptides combined to form the encoded product. The proteins also include hybrid polypeptides that comprise a combination of partial or complete polypeptide sequences obtained
35 from at least two different proteins wherein one or more (several) may be heterologous or native

to the host cell. Proteins further include naturally occurring allelic and engineered variations of the above mentioned proteins and hybrid proteins.

Preferably, the protein of interest is a hormone or variant thereof, enzyme, receptor or portion thereof, antibody or portion thereof, or reporter. In a more preferred aspect, the protein is an oxidoreductase, transferase, hydrolase, lyase, isomerase, or ligase. In an even more preferred aspect, the protein is an aminopeptidase, amylase, carbohydrase, carboxypeptidase, catalase, cellulase, chitinase, cutinase, cyclodextrin glycosyltransferase, deoxyribonuclease, esterase, alpha-galactosidase, beta-galactosidase, glucoamylase, alpha-glucosidase, beta-glucosidase, invertase, laccase, another lipase, mannosidase, mutanase, oxidase, pectinolytic enzyme, peroxidase, phytase, polyphenoloxidase, proteolytic enzyme, ribonuclease, transglutaminase or xylanase.

The gene may be obtained from any prokaryotic, eukaryotic, or other source.

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

15

EXAMPLES

MATERIALS AND METHODS

Chemicals used as buffers and substrates were commercial products of at least reagent grade.

20 Strains

Aspergillus nidulans AR1 (IBT 27263 of the Glasgow strain collection)

Asperillus nidulans gpndk74 is described in example 1

Aspergillus nidulans FGSC A4 Obtained from Fungal Genetics Stock Center, School of Biological Sciences, SBS 404

25 University of Missouri, Kansas City, 5007 Rockhill Road, Kansas City, MO 64110, USA

Aspergillus niger BO1 (DSM 12665, Novozymes A/S, Bagsværd, Denmark)

Aspergillus niger JRoy3 is described in WO/2000/050576

Aspergillus niger C1744 is described in example 4

Aspergillus niger M1025 is described in example 4

30 *Aspergillus niger* Rung76-Rung81 are described in example 4

Aspergillus niger Rung93-Rung96 are described in example 5

Genes:

Aspergillus nidulans NADHkinase (AN8837.2) (Seq ID No:1) is described in Example 1

35 *Aspergillus niger* NADHkinase (An17c0100, bp 2013 to 3492) (SEQ ID NO: 5) is described in

example 3

Aspergillus oryzae NADHkinase (locus_tag AO090009000675 from *Aspergillus oryzae* AP007150 contig) (Seq ID No:7) is described in example 3

5 Synthetic Bacterial promoter (Carsten Olsen, Gene amplification in industrial relevant *Bacillus* species (sp.) using non-antibiotic resistance markers, PhD report, Biocentrum-DTU, Section for Molecular Microbiology, 2003) (Seq ID No:9)

pyrG: This gene codes for orotidine-5'-phosphate decarboxylase, an enzyme involved in the biosynthesis of uridine.

Trametes cingulata AMG gene is described in WO/2006/069289

10

Plasmids

pTr04AN8837.2 is described in example 1

pBARGPE1 Obtained from Fungal Genetics Stock Center, School of Biological Sciences, SBS 404, University of Missouri, Kansas City, 5007 Rockhill Road, Kansas City, MO 64110, USA

15 pTr04 is described in example 1

pJaL721 is described in WO/2003/008575

pJaL554 is described in WO/2007/045248

pJaL485 is described in WO03008575A

pJHR1 is described in example 4

20 pRung9 is described in example 4

pRung 11 is described in example 4

pRung 30 is described in example 4

pRung 35 is described in example 4

pRung 36 is described in example 6

25

Primers

AN8837.2_FseI_fw (Seq ID No:3)

AN8837.2_AscI_re (Seq ID No:4)

Pt1 (Seq ID No:10)

30 Pt2 (Seq ID No:11)

Pt3 (Seq ID No:12)

Pt4 (Seq ID No:13)

Pt5 (Seq ID No:14)

Pt6 (Seq ID No:15)

35 PR35 (Seq ID No:16)

PR36 (Seq ID No:17)

PR37 (Seq ID No:18)

PR38 (Seq ID No:19)

PR99 (Seq ID No:20)

5 PR101 (Seq ID No:21)

PR107 (Seq ID No:22)

PR108 (Seq ID No:23)

PR114 (Seq ID No:24)

PR106 (Seq ID No:25)

10 PR111 (Seq ID No:26)

PR113 (Seq ID No:27)

PR100 (Seq ID No:28)

Media and Solutions

15

Example 1: Construction of an *Aspergillus nidulans* strain overexpressing the NADH kinase.

The gene sequence of the putative gene AN8837.2 was recovered from the Entrez databases as entry NT_107003 from the National Center for Biotechnology Information NCBI (www.ncbi.nlm.nih.gov). This gene sequence is displayed as SEQ ID NO: 1. The function of this
20 was inferred by BLAST comparisons using known NADH kinases deposited in the same database. It is comprised of 1436 nucleotides (from start to stop codon) and two putative introns (56 and 39 nucleotides long, respectively) that are predicted to be close (136 and 328 nucleotides, respectively) to the start of the gene. The putative gene therefore encodes a protein of 446 amino acids with a calculated molecular weight of 49 kDa (SEQ ID NO: 2).

25 Further analysis of the protein sequence revealed that the novel gene contains the PFAM motif ATP-NAD kinase, and the protein localization algorithms TargetP (Emanuelsson, O., Nielsen, H., Brunak, S., and von Heijne, G. (2000) *J. Mol. Biol.* 300:1005-1016) and WoLFPSORT (Horton, P., Park, K.J., Obayashi, T., Fujita, N., Harada, H., Adams-Collier, C.J., and Nakai, K. (2007) *Nucleic Acids Res.* 35:W585-587) predicted that the enzyme most likely is localized in the
30 mitochondrion as is the *S. cerevisiae* NADH kinase. Phylogenetic analysis of proteins obtained by BLASTP comparison against the non-redundant protein database (NR) from NCBI, reveals that the protein encoded by SEQ ID NO: 1 has several homologs throughout the fungal kingdom but also to other eukaryotic organisms. The results show that this NADH kinase falls into the category of conserved kinases.

35 The putative NADH kinase gene from *Aspergillus nidulans*, was PCR amplified from

genomic DNA of *Aspergillus nidulans* AR1 [pyrG89 argB2; veA1] (IBT 27263 of the Glasgow strain collection) prepared as described by Nielsen et. al. (Nielsen, M.L., Albertsen, L., Lettier, G., Nielsen, J., and Mortensen, U.H. (2006) *Fungal Genet.Biol.* 43:54-64) using the Expand High Fidelity PCR System (Roche, Switzerland) using standard PCR conditions with the following
5 primers AN8837.2_FseI_fw (SEQ ID NO: 3) and AN8837.2_AscI_re (SEQ ID NO: 4)

The primers contain the start and stop codons as well as the restriction sites FseI and AscI.

The obtained PCR product of the expected length was then cloned into the available unique restriction sites, FseI and AscI in the pTr04 expression vector, resulting in pTr04AN8837.2
10 which was verified by sequencing. pTr04 was derived from pBARGPE1 (Obtained from Fungal Genetics Stock Center, School of Biological Sciences, SBS 404 University of Missouri, Kansas City, 5007 Rockhill Road, Kansas City, MO 64110, USA) which contains the gpdA promoter from *Aspergillus nidulans*, linked to a multiple cloning site (MCS) and the TrpC terminator. In addition the pTr04 vector contained the *A. fumigates* pyrG gene as a selection marker. *Aspergillus*
15 *nidulans* AR1 was transformed with pTr04AN8837.2 as described in Nielsen et al. (Nielsen, M.L., Albertsen, L., Lettier, G., Nielsen, J., and Mortensen, U.H. (2006) *Fungal Genet.Biol.* 43:54-64) and selection yielded the transformant *Aspergillus nidulans* gpndk74.

Example 2: Fermentation of an *Aspergillus nidulans* strain overexpressing the NADH kinase.

20

Materials and methods.

A defined medium containing trace metal elements was used in all *Aspergillus nidulans* cultivations. The medium used had the following composition (per litre): 15 g (NH₄)₂SO₄, 3 g KH₂PO₄, 2 g MgSO₄•7H₂O, 2 g NaCl, 0.2 g CaCl₂ and 1 ml I-1 trace element solution. Trace
25 element solution composition (per litre): 14.3 g ZnSO₄•7H₂O, 13.8 g FeSO₄•7H₂O and 2.5 g CuSO₄•5H₂O. The carbon sources used were glucose, xylose or acetate (10 g/l). Batch cultivations were carried out in laboratory fermenters and baffled Erlenmeyer flasks. For the auxotrophic strain 0.7 g/L arginine was added in the medium.

The physiological characterization of the strains was performed at 30 °C in well-controlled
30 1.5 l fermenters (working volume of 1.2 l) equipped with two disc-turbine impellers. The stirring rate was 350 rpm and the pH was kept constant at 5.5 by addition of 2 M NaOH or HCl. The fermentor was sparged with air at a constant flow rate of 0.1 vvm (termed "oxygen limitation") or 1.0 vvm (termed "fully aerated").

Fermentation samples were immediately filtered and stored at -20 °C until time of
35 analysis. Medium concentrations of glucose, xylose, glycerol, acetate, succinate and pyruvate

were determined by HPLC analysis using an Aminex HPX-87H column (BioRad). The column was kept at 45 °C and eluted at 0.6 ml min⁻¹ with 5 mM H₂SO₄. A Waters (410) refractive index detector was used together with a UV detector for compound detection.

NADH kinase activity was measured spectrophotometrically at 340 nm in a reaction mixture buffered to pH 7.8, comprising of 120 mM glycylglycine, 12 mM MgCl₂, 3.6 mM phosphoenolpyruvate, 2.4 mM ATP, 0.48 mM NADH, 10 U lactate dehydrogenase, 30 U pyruvate kinase and cell extract. Protein concentrations were measured according to the Bradford method (Bradford, M. (1976) *Anal. Biochem.* 72:248-254.).

Samples for analysis of intracellular metabolites were taken in triplicates at the mid-exponential growth phase. 10 ml fermentation broth was immediately quenched in 20 ml cold (-40 °C) 72% methanol. After quenching, the cells were separated from the quenched solution by centrifugation at 10,000 x g for 20 min at -20 °C and the intracellular metabolites were extracted as described by Villa-Boas et al. (Villas-Boas, S.G., Moxley, J.F., Åkesson, M., Stephanopoulos, G., and Nielsen, J. (2005) *Biochem. J.* 388:669-677.). Finally, the samples were lyophilized and stored at -80 °C until further analysis. Variation of intracellular metabolite levels across replicate samples did not exceed 15%. Organic acids (including amino acids) were analysed by gas chromatography-mass spectrometry (GC-MS). The GC-MS analysis was performed on an Agilent HP 6890 series GC chromatograph (Palo-Alto, CA, USA) coupled to a HP 5973 quadrupole MSD (EI) operated at 70 eV. The GC was equipped with a 4.0 mm i.d. Siltek gooseneck split less deactivated liner (Restek, Bellefonte, PA, USA), and Supelco (Bellefonte, PA, USA) SLB-5 MS column (15m, 0.25mm i.d, 0.25 µm film thickness). The profile of identified intracellular organic acids (including amino acids) was expressed in peak areas normalized to the mass of biomass.

Results.

Aspergillus nidulans gpndk74 and *Aspergillus nidulans* FGSC A4 (wild type strain, Obtained from Fungal Genetics Stock Center, School of Biological Sciences, SBS 404, University of Missouri, Kansas City, 5007 Rockhill Road, Kansas City, MO 64110, USA) was fermented in 250 ml baffled Erlenmeyer flasks containing 100 ml medium for 24 hours at 30°C at 200 RPM on glucose. The cells were harvested and cell extracts were prepared using glass beads and a FastPrep machine. The NADH activity was measured in the extracts of both strains. The NADH kinase activity in the *Aspergillus nidulans* gpndk74 was 2.7 times higher than the activity in the *Aspergillus nidulans* A4 extract verifying that the cloned gene actually codes for a NADH kinase.

Aspergillus nidulans gpndk74 and *Aspergillus nidulans* FGSC A4 was fermented in 1,5 l fermentors. Substrate and product concentrations were determined during growth of the both strains on glucose under fully aerobic (1.0 vvm air flow) and oxygen limited cultivations (0.1 vvm

air flow). The specific growth rate was higher for *Aspergillus nidulans* gpndk74 grown on glucose (0.24 h⁻¹) than for *Aspergillus nidulans* A4 (0.20 h⁻¹) under fully aerobic conditions (Table 1). However, no difference in growth was observed between the two strains when aeration was reduced to 0.1 vvm. The specific growth rate was 0.12 h⁻¹ in both cases. The biomass yields (0.54 and 0.52 g/g for aerobic and oxygen-limited conditions, respectively) of *Aspergillus nidulans* gpndk74 was significantly higher than the ones obtained with *Aspergillus nidulans* A4 (0.44 and 0.42 g/g for aerobic and oxygen-limited conditions, respectively) (Table 1).

Table 1. Growth parameters of the *Aspergillus nidulans* gpndk74 and *Aspergillus nidulans* A4 on glucose.

Strain	gpndk74 (NADH kinase)		A4 (Reference)	
	m1	m0	s1	s0
Air flow rate (vvm)	1.0	0.1	1.0	0.1
Sp. growth rate (h ⁻¹)	0.24	0.12	0.20	0.12
Yield (g DW/g)	0.54	0.52	0.44	0.42

In order to investigate how the cellular metabolism and regulation of the *Aspergillus nidulans* gpndk74 and *Aspergillus nidulans* FGSC A4 strains change as response to the growth conditions applied (fully aerobic and oxygen limited, respectively) we decided to study the metabolic fingerprint using the fermentations described above. The profile of intracellular organic acids (including amino acids) was determined during the exponential growth phase. Our in-house library, covering most of the metabolites in the central carbon metabolism and nearly all amino acids, consists of 78 organic acids (including amino acids) and approximately 30% of these compounds were detected in significant quantities by GC-MS analysis. The relative concentrations of organic acids and amino acids in the mutant and the wild type strains in the different experimental conditions were determined. Aspartate and glutamate were detected in higher levels in the *Aspergillus nidulans* FGSC A4 cultivations, while pyroglutamate was measured in higher concentrations in the *Aspergillus nidulans* gpndk74. Oxygen availability seemed to have different influence on the levels of alanine and GABA in each strain. While the decrease of oxygen supply during wild type strain cultivations resulted in lower concentration of these two metabolites the opposite effect was observed in the mutant. Interestingly, the valine, leucine and isoleucine levels followed the same pattern in response to the applied oxygen conditions and the genotype of the cells. Valine biosynthesis is closely related to the biosynthesis

of leucine and isoleucine (Nuesch, J., Heim, J., and Treichler, H.J. (1987) *Annual Rev. Microbiol.* 41:51-75.). The biosynthetic routes of these three amino acids are compartmentalized into the mitochondrion (Marzulf, G.A. (1997). *Annual Rev. Microbiol.* 51:73-96.).

The level of succinate was significantly higher in cultivations of *Aspergillus nidulans* gpndk74 compared to *Aspergillus nidulans* A4. These observations also point to regulatory and flux changes in the mitochondrion.

Example 3: Identification of an NADH kinase homologue of *Aspergillus niger* and *Aspergillus oryzae*.

10 The DNA sequence of the *Aspergillus nidulans* NADH kinase (SEQ ID NO: 1) was used to search the *Aspergillus niger* genome sequence for a homologue using a BLAST search against the uniprot database

The *Aspergillus niger* An17c0100 contig was found. This contig is a 16704 bp contig. The annotations of this contig predicted a gene from bp 2067 to 3492 and predicted this gene to have the catalytic activity: ATP + NADH \rightleftharpoons ADP + NADPH.

15 Predicted function: the NADH kinase may be involved in maintaining the level of triphosphopyridine nucleotide in the mitochondria. It was noted that the predicted protein had a strong similarity to NADH kinase from patent JP09131185-A - *Pichia membranaefaciens*".

Further analysis using the Agene gene finding program (Munch K and Krogh A. BMC Bioinformatics 2006, 7:263) and Funstart (tool for prediction of translation start) predicted that the coding sequence starts at bp 2013. The complemented genome sequence of the An17c0100 ranging from bp 2013 to 3492 is shown in SEQ ID NO: 5.

20 The putative NADH kinase protein sequence from *Aspergillus niger* was generated by joining bp. 1 to 148 with bp 230 to 343 and bp 408 to 1480 of SEQ ID NO: 5 into a putative mRNA sequence and by translating that into a protein sequence. The protein sequence of the putative NADH kinase of *Aspergillus niger* is given in SEQ ID NO: 6.

30 The *Aspergillus oryzae* AP007150 contig was also found from the blast search. The sequence coding for a protein homologous to the *Aspergillus nidulans* NADH kinase was identified as locus_tag AO090009000675 which is a 1395 bp contig. The coding sequence can be assembled by complement joining bp 1807604 to 1808844 and 1808943 to 1809096. The *Aspergillus oryzae* DNA sequence is given in SEQ ID NO: 7 and the protein sequence in SEQ ID NO: 8.

35 A very high degree of homology is seen between the NADH kinase of *Aspergillus nidulans* (SEQ ID NO: 2), the putative NADH kinase of *Aspergillus niger* (SEQ ID NO: 6), and the putative NADH kinase of *Aspergillus oryzae* (SEQ ID NO: 8) supporting that the *Aspergillus niger* and

Aspergillus oryzae proteins are indeed NADH kinases. An alignment of the three sequences is shown in figure 1.

5 Example 4: Construction of *Aspergillus niger* strains overexpressing the *Aspergillus nidulans* NADH kinase.

An amyloglycosidase producing strain of *Aspergillus niger* termed *Aspergillus niger* BO1 (DSM 12665, Novozymes A/S, Bagsværd, Denmark) is made pyrG negative by transforming with a deletion fragment of the pyrG gene region and selecting on FOA resulting in the strain JRoy3 (WO/2000/050576).

10 This strain is transformed with the plasmid pTr04AN8837.2 as described in example 1, using the pyrG from *A. fumigatus* as the selection marker. A set of 5 randomly selected transformants are selected, re-isolated and preserved.

15 Example 5: Construction of *Aspergillus niger* strains overexpressing the *Aspergillus niger* NADH kinase.

An expression plasmid for the putative *Aspergillus niger* NADH kinase was constructed in the following way. A 73 bp PCR product encoding synthetic promoter for optimized URA3 expression in *E. coli* (Carsten Olsen, Gene amplification in industrial relevant Bacillus species (sp.) using non-antibiotic resistance markers, PhD report, Biocentrum-DTU, Section for Molecular
20 Microbiology, 2003) (SEQ ID NO: 9) was amplified using the primers Pt1 (SEQ ID NO: 10) and Pt2 (SEQ ID NO: 11). Pt1 introduced a RcaI restriction site 5 prime to the promoter sequence for further cloning. Primer Pt2 contained a 14 bp extension for fusion to the URA3 gene using SOE PCR. PCR reactions were performed under standard conditions using the Phusion High-Fidelity DNA Polymerase (Finnzymes Oy, Keilaranta 16 A, 02150 Espoo, Finland). A 894 bp PCR
25 fragment encoding the URA3 gene was amplified by PCR from pJaI721 (WO/2003/008575) using the primers Pt3 (SEQ ID NO: 12) and Pt4 (SEQ ID NO: 13). The PCR products encoding the synthetic promoter and the Ura3 gene were purified from an agarose gel and fused by SOE PCR (Horton, R.M., Hunt, H.D., Ho, S.N., Pullen, J.K. and Pease, L.R. (1989). Gene 77, 61–68) using the primers Pt1 (SEQ ID NO: 10) and Pt4 (SEQ ID NO: 13). The 953 bp fusion PCR product was
30 restriction digested with RcaI and EcoRI, which reduced the size to 940 bp. The 940 bp fragment was ligated with a 1086 bp SphI – RcaI fragment from pJaL721 containing the Lac promoter and origin of replication (WO/2003/008575) and a 5665 bp EcoRI – SphI of pJaI554 containing the pyrG gene (WO/2007/045248). The ligated fragments were transformed into *E. coli* using standard procedures and the resulting 7677bp plasmid was termed pJHR1. A 105 bp linker region
35 was amplified from pJaL485 (WO03008575A) using the primers Pt5 (SEQ ID NO: 14) and Pt6

(SEQ ID NO: 15). The Pt5 primer contained a 5' prime 18 bp extension for SOE PCR based fusion to the URA3 PCR product. The extension also contained an EcoRI restriction site. The Pt6 primer contained a XmnI restriction site for cloning in the pJHR1 plasmid. The PCR fragment was restriction digested with EcoRI and XmnI resulting in a 92 bp fragment that was cloned in a 4298 bp EcoRI-SmaI part of pJHR1. The resulting vector was termed pRung9 and constituted 4390 bp. *Aspergillus niger* BO1 genomic DNA was prepared using the Masterpure™ Yeast DNA Purification Kit (Epicentre Biotechnologies, Madison, WI, USA) and used for PCR amplification of the GPD promoter and terminator.

The *gpdA* (Glyceraldehyde-3-phosphate dehydrogenase) promoter from *Aspergillus niger* (accession number AM270359 from NCBI) was PCR amplified using the *Aspergillus niger* BO 1 DNA as template and the primers, PR35 (SEQ ID NO: 16) and PR36 (SEQ ID NO: 17). The resulting 622 bp PCR fragment is agarose gel purified using standard methods.

The *gpdA* terminator from *Aspergillus niger* is PCR amplified using the *Aspergillus niger* BO 1 DNA as template and the primers, PR 37 (SEQ ID NO: 18) and PR38 (SEQ ID NO: 19).

The resulting 530 bp PCR fragment was agarose gel purified using standard methods.

The 622 bp promoter PCR fragment was mixed with the 530 bp terminator fragment and using these fragments as template, a new PCR reaction was run using the primers PR35 (SEQ ID NO: 16) and PR38 (SEQ ID NO: 19).

The resulting 1152 bp PCR fragment was purified from an agarose gel, and then digested with the restriction enzymes XbaI and KpnI. The digested 1133 bp PCR fragment was ligated to a 4266 bp fragment from pRung9 digested with KpnI and XbaI and was transformed into *E. coli* using standard methods. The resulting vector was termed pRung 11. To remove an undesired BamHI site pRung 11 was digested with XbaI and NheI and a 5162 bp fragment was purified from an agarose gel and religated and transformed into *E. coli* using standard procedures. The resulting vector was termed pRung30.

The NADH kinase gene is PCR amplified using the *Aspergillus niger* BO1 genomic DNA as template and the primers, PR99 (SEQ ID NO: 20) and PR101 (SEQ ID NO: 21)

The formed 1501 bp PCR fragment was agarose gel purified, digested with the restriction enzymes SacI and BamHI and inserted into pRung30 digested with the same enzymes.

The resulting plasmid, pRung35, was a 6644 bp plasmid consisting of the *Aspergillus niger* *pyrG* gene as selective marker and the *Aspergillus niger* putative NADH kinase under control of the *Aspergillus niger* *gpdA* promoter and terminator. pRung35 was transformed into *Aspergillus niger* M1025 by standard protoplast transformation procedures. The *pyrG* marker was used for selection. M1025 was a spontaneous *pyrG* mutant of C1744 obtained by screening for resistance to 5-fluoro-orotic acid (FOA) (Cove D.J., 1996, Biochem. Biophys. Acta. 113:51-56)

C1744 is a standard amylase expression host containing a *Trametes cingulata* AMG gene (WO/2006/069289) inserted in the Neutral amylase I locus and an Acid stable Alpha-Amylase (SP288) in the Neutral amylase II locus. The following genes are disrupted in C1744: Cellobiase, alpha-1,2-mannosidase, oxaloacetate hydrolase, alpha 1,3-transferase, alpha 1,6-transglucosidase, tripeptidyl aminopeptidase as well as the fumonisin gene cluster. Ten randomly picked transformants were reisolated and preserved. Expression of the NADH kinase was determined by Northern blotting and RT PCR. Strains for RNA preparation were grown on 100 ml. cultures of YPD (2 g/l yeast extract, 2 g/l peptone and 2 % glucose) for 72 h at 30° C. Mycelia was harvested and RNA was prepared by the Fast RNA ® Pro Red Kit (MP Biomedicals, LLC, Solon, Ohio 44139, US). Northern blotting was done by standard procedures using 32P labelling and hexanucleotide priming. The probe was a 1501 bp SacI-BamHI fragment from pRung35 encoding the NADH kinase gene. For normalisation of the expression data a probe encoding the *Aspergillus niger* actin gene (XM_001396558.1) (K. Bohle, A. Jungebloud , Y. Göcke, A. Dalpiaz ,C. Cordes , H. Horn and D.C. Hempel, 2007, Journal of Biotechnology Vol 132, p 353-358) was hybridized to the same membrane. The actin probe was a 626 bp PCR fragment amplified from *Aspergillus niger* BO-1 genomic DNA using the primers, PR107 (SEQ ID NO: 22) and PR 108 (SEQ ID NO: 23)

RT PCR was performed to be able to distinguish between expression from the introduced NADH kinase construct and the background expression. RT PCR was performed as described in Protoscript II RT-PCR Kit protocol (New England Biolabs Inc. Ipswich, MA, USA). The NADH kinase cDNA was detected as a 1107 bp PCR product which was amplified using the primers, Pr114 (SEQ ID NO: 24) and PR106 (SEQ ID NO: 25). The Foreward primer PR114 was designed to span the intron between bp 343 and bp 408. The PR106 primer was designed to anneal to the 3'-untranslated region originally derived from the GPD terminator.

The *Aspergillus niger* Actin gene (XM_001396558.1) was used as reference gene for normalization and the Actin cDNA was detected as a 699 bp PCR fragment. The primers PR111 (SEQ ID NO: 26) and PR 113 (SEQ ID NO: 27) were used for detection of actin cDNA. The primer PR113 spans the last intron in the Actin gene to avoid amplification from genomic DNA. Six individual transformants showed increased expression of the introduced NADHkinase. They were named Rung76–Rung81 and were preserved and used for fermentation studies.

Example 6: Construction of an *Aspergillus niger* strain overexpressing the *Aspergillus niger* NADH kinase having the mitochondrial signal sequence deleted.

The WolfPSORT (Paul Horton, Keun-Joon Park, Takeshi Obayashi, Naoya Fujita, Hajime Harada, C.J. Adams-Collier and Kenta Nakai3, Nucleic Acids Research, 2007, 1–3) and TargetP

prediction programs (Olof Emanuelsson, Søren Brunak, Gunnar von Heijne, Henrik Nielsen, 2007 *Nature Protocols* 2, 953-971) predicts the putative *Aspergillus niger* NADH kinase also to be a mitochondrial protein. TargetP predicts that the protein contains a 32 aa mitochondrial localization signal peptide. Removal of the first 32 amino acids of the protein will destroy the function of the mitochondrial signal sequence, and the formed protein is clearly predicted to be targeted to the cytosol using the same predictors.

An expression plasmid for this variant of the NADH kinase of *Aspergillus niger* was constructed in the following way:

The N terminally truncated NADH kinase gene was PCR amplified using the *Aspergillus niger* BO1 genomic DNA as template and the primers PR100 (SEQ ID NO: 28) and PR101 (SEQ ID NO: 21)

The formed 1408 bp PCR fragment was agarose gel purified, digested with the restriction enzymes BamHI and SacI and inserted into pRung35 digested with the same enzymes.

The resulting plasmid pRung36 is a 6551 bp plasmid consisting of the *Aspergillus niger* pyrG gene as selective marker and the *Aspergillus niger* putative N terminally truncated NADH kinase under control of the *Aspergillus niger* gpdA promoter and terminator. The DNA sequence of the coding sequence is given in SEQ ID NO: 29.

The putative protein sequence of the N terminally truncated gene was generated by joining bp. 1 to 19 with bp 101 to 215 and bp 280 to 1351 of SEQ ID NO: 29 into a putative mRNA sequence and by translating that into a protein sequence using the Agene prediction server (Munch K and Krogh A. BMC Bioinformatics 2006, 7:263). The protein sequence of the putative N terminally truncated NADH kinase of *Aspergillus niger* is given in SEQ ID NO: 30

pRung 36 was transformed into *Aspergillus niger* M1025 and 10 transformants were selected, re-isolated and analyzed for expression of the truncated NADH kinase using RT-PCR as described in example 5. Four Transformants showing expression of the introduced NADH kinase without the mitochondrial localization signal were selected and named Rung93-Rung96.

Example 7: Fermentation of *Aspergillus niger* strains overexpressing NADH kinase variants.

30 Materials and methods

Fermentation of strains was performed as described in example 2

Biomass was measured as Dry Cell Weight. About 2 ml of fermentation broth was transferred to a pre-weighted glass tube and weight measured. Then, the broth was washed twice as follows. The broth was diluted up to 10 ml with H₂O and mixed well using a tube mixer (ex. vortex). The well-mixed broth was centrifuged at 3,500 rpm for 10 min and supernatant was

discarded. After washing, the broth was dried out in an electric drying oven at 105°C for over 24 hours and the weight was measured after cooling till room temperature in a desiccator.

Samples for measurement of Amyloglucosidase (AMG) activity were withdrawn from the tanks with regular time intervals.

5 AMG activity was measured by a 3-step coupled colorimetric assay. AMG, EC 3.2.1.3 (exo- α -1,4-glucan-glucohydrolase), hydrolyzes maltose to form α -D-glucose. Glucose is phosphorylated by ATP, in a reaction catalyzed by hexokinase. The glucose-6-phosphate formed is oxidized to 6-phosphogluconate by glucose-6-phosphate dehydrogenase. In this same reaction an equimolar amount of NAD⁺ is reduced to NADH with a resulting increase in absorbance at 340
10 nm. The assays were performed using a Konelab 30 Analyzer. The activity of AMG is defined as amyloglucosidase units pr. grams of culture.

The oxygen uptake and carbon dioxide evolution are measured and used to determine the growth rate as described in example 2.

15 Results

The *Aspergillus niger* strains of example 4, and 6, and an untransformed *Aspergillus niger* C1744 strain were fermented in laboratory tank fermentors similar to the fermentors of example 2 using a similar fermentation medium. It was shown that the growth rate of the *Aspergillus niger* strain expressing the highest level of the *Aspergillus niger* full length NADHkinase had
20 significantly increased specific growth rates compared to the untransformed *Aspergillus niger* C1744. The production of AMG was similar in the transformant and the control. Two transformants showing intermediate expression of the full length NADH-kinase had reduced growth rates compared to the control but accumulated more biomass determined as Dry Cell Weight at later stages of the fermentation. The obtained data indicate that the effect on
25 metabolism of expression of the mitochondrial targeted NADH-kinase vary depending on the level of expression. An explanation to that observation could be that the mitochondrial localization of the NADH-kinase is leaky when the protein is expressed at high levels. Thereby the observed various effects may be due to a differential balance of NADH-kinase activity between the cytosol and the mitochondrion.

30 To test the effect of a cytosolic localization of the *A. niger* NADH-kinase we tested *A. niger* transformants expressing the NADH-kinase without the mitochondrial localization signal (described in example 6) in lab tank fermentations. Two transformants had an increased growth rate compared to the control strain. The specific growth rate of the best transformant (0,24 h⁻¹) was 14% higher than the growth rate for the control strain (0,21 h⁻¹). Also the biomass production
35 was affected and the transformants produced higher levels of biomass. The fastest growing

transformant had a 14 % higher biomass yield at the end of the fermentation (182h). Overexpression of the NADH-kinase without the localization signal had a significant impact on the yield of the AMG enzyme. The fastest growing strain showed increased production of AMG already early in the fermentation and ended up showing a 18 % increase of the AMG yield
5 compared to the control after 182 h of fermentation. Together our data indicate that a positive effect on growth rates and expression of heterologous proteins by overexpression of *A. niger* NADH-kinase in *A. niger* was obtained and that the effect was due to NADH-kinase activity in the cytosol. Cytosolic NADH-kinase activity could be generated in two ways: The NADH-kinase was expressed without the mitochondrial localization signal or it was expressed with the localization
10 signal to a high level resulting in a leaky localization and thereby increased activity of NADH-kinase in the cytosol. Multiple cellular localization may occur by a mechanism involving alternative translational start which may result in transcripts lacking the localization signal (Jakob Christian Mueller, Christophe Andreoli, Holger Prokisch and Thomas Meitinger, 2004, Mitochondrion, Vol 3, p315-325). Two putative alternative translational start codons have been observed in the NADH-
15 kinase sequence, and translation from these sites may disrupt the mitochondrial localization signal. The two positions are 17-19bp and 55-57bp in Seq ID No 5.

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

25

CLAIMS

1. A recombinant filamentous fungal host cell, wherein an NADH kinase activity is increased compared to an otherwise identical wild type host cell cultured under identical conditions, and
5 said recombinant host cell further expresses a polypeptide of interest.
2. The recombinant host cell according to claim 1, wherein the kinase is exogenous to the host cell.
- 10 3. The recombinant host cell according to any of the preceding claims, wherein the kinase is present in the cytoplasm.
4. The recombinant host cell according to any of the preceding claims, wherein the kinase is targeted to the cytoplasm.
- 15 5. The recombinant host cell according to any of the preceding claims, wherein the signal peptide is exogenous to the kinase.
6. The recombinant host cell according to claim 1, wherein the kinase is a mitochondrial
20 kinase.
7. The recombinant host cell according to claim 1, wherein the kinase is selected from the group consisting of:
- (a) a polypeptide comprising an amino acid sequence having preferably at least 60%,
25 more preferably at least 65%, more preferably at least 70%, more preferably at least 75%, more preferably at least 80%, more preferably at least 85%, even more preferably at least 85%, most preferably at least 90%, and even most preferably at least 95% identity to the mature polypeptide of SEQ ID NO: 2, 6, or 8;
- (b) a polypeptide encoded by a polynucleotide that hybridizes under preferably at least low
30 stringency conditions, more preferably at least medium stringency conditions, even more preferably at least medium-high stringency conditions, and most preferably at least high stringency conditions with (i) the mature polypeptide coding sequence of SEQ ID NO: 1, 5, or 7, (ii) the cDNA sequence contained in SEQ ID NO: 1, 5, or 7, or (iii) a full-length complementary strand of (i) or (ii);
- 35 (c) a polypeptide encoded by a polynucleotide comprising a nucleotide sequence having

preferably at least 60%, more preferably at least 65%, more preferably at least 70%, more preferably at least 75%, more preferably at least 80%, more preferably at least 85%, even more preferably at least 85%, most preferably at least 90%, and even most preferably at least 95% identity to the mature polypeptide coding sequence of SEQ ID NO: 1, 5, or 7;

5 (d) a variant comprising a substitution, deletion, and/or insertion of one or several amino acids of the mature polypeptide of SEQ ID NO: 2, 6, or 8.

8. The recombinant host cell according to any of the preceding claims, wherein the host cell is selected from the group consisting of *Acremonium*, *Aspergillus*, *Aureobasidium*, *Bjerkandera*,
10 *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*.

15 9. The recombinant host cell according to claim 8, wherein the host cell is selected from the group consisting of *Aspergillus*, *Chrysosporium*, *Fusarium*, *Myceliophthora*, and *Trichoderma*.

10. The recombinant host cell according to claim 9, wherein the *Aspergillus* host cell is selected from the group consisting of *Aspergillus oryzae*, *Aspergillus niger*, and *Aspergillus nidulans*.

20

11. A method of producing a polypeptide of interest, comprising culturing the recombinant host cell of any of the claims 1 to 10, under conditions conducive for the production of the polypeptide of interest.

25 12. A method of producing a polypeptide of interest, comprising culturing the recombinant filamentous fungal host cell, wherein an NADH kinase activity is increased compared to an otherwise identical wild type host cell cultured under identical conditions, and said recombinant filamentous fungal host cell further expresses a polypeptide of interest.

30 13. The method according to any of the claims 11 and 12, wherein expression of the polypeptide is controlled by a non native promoter.

14. A method for increasing growth rate of a fungal host cell comprising increasing an NADH kinase activity compared to an otherwise identical wild type host cell cultured under identical
35 conditions.

15. The method according to claim 14, wherein the fungal host cell is selected from a yeast cell or a filamentous fungal cell.

- 5 16. The method according to claim 15, wherein the filamentous fungal host cell is an *Aspergillus* host cell selected from the group consisting of *Aspergillus oryzae*, *Aspergillus niger*, and *Aspergillus nidulans*.

10

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2009/056449

A. CLASSIFICATION OF SUBJECT MATTER
 INV. C12N1/15 C12P21/02 C12N9/12

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
 C12N C12P

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

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
 EPO-Internal, Sequence Search, 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.
P, X	PANAGIOTOU GIANNI ET AL: "Overexpression of a novel endogenous NADH kinase in <i>Aspergillus nidulans</i> enhances growth." METABOLIC ENGINEERING JAN 2009, vol. 11, no. 1, January 2009 (2009-01), pages 31-39, XP002503749 ISSN: 1096-7184 the whole document ----- -/--	1-16

Further documents are listed in the continuation of Box C. See patent family annex.

- * Special categories of cited documents :
- *A* document defining the general state of the art which is not considered to be of particular relevance
 - *E* earlier document but published on or after the international filing date
 - *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
 - *O* document referring to an oral disclosure, use, exhibition or other means
 - *P* document published prior to the international filing date but later than the priority date claimed
 - *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
 - *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
 - *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
 - * & * document member of the same patent family

Date of the actual completion of the international search 23 July 2009	Date of mailing of the international search report 31/07/2009
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Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Authorized officer Sonnerat, Isabelle
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INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2009/056449

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>VALKONEN MARI ET AL: "Improvement of foreign-protein production in <i>Aspergillus niger</i> var. <i>awamori</i> by constitutive induction of the unfolded-protein response"</p> <p>APPLIED AND ENVIRONMENTAL MICROBIOLOGY, AMERICAN SOCIETY FOR MICROBIOLOGY, US, vol. 69, no. 12, 1 December 2003 (2003-12-01), pages 6979-6986, XP002348137 ISSN: 0099-2240 the whole document</p>	
A	<p>BIESEBEKE ROB TE ET AL: "Expression of <i>Aspergillus</i> hemoglobin domain activities in <i>Aspergillus oryzae</i> grown on solid substrates improves growth rate and enzyme production"</p> <p>BIOTECHNOLOGY JOURNAL, vol. 1, no. 7-8, August 2006 (2006-08), pages 822-827, XP002504065 ISSN: 1860-6768 the whole document</p>	
A	<p>R POULSEN BJARNE ET AL: "Increased NADPH concentration obtained by metabolic engineering of the pentose phosphate pathway in <i>Aspergillus niger</i>."</p> <p>THE FEBS JOURNAL MAR 2005, vol. 272, no. 6, March 2005 (2005-03), pages 1313-1325, XP002503750 ISSN: 1742-464X abstract</p>	
A	<p>PUNT P J ET AL: "Filamentous fungi as cell factories for heterologous protein production"</p> <p>TRENDS IN BIOTECHNOLOGY, ELSEVIER PUBLICATIONS, CAMBRIDGE, GB, vol. 20, no. 5, 1 May 2002 (2002-05-01), pages 200-206, XP004349665 ISSN: 0167-7799 the whole document</p>	
A	<p>NEVALAINEN ET AL: "Heterologous protein expression in filamentous fungi"</p> <p>TRENDS IN BIOTECHNOLOGY, ELSEVIER PUBLICATIONS, CAMBRIDGE, GB, vol. 23, no. 9, 1 September 2005 (2005-09-01), pages 468-474, XP005039534 ISSN: 0167-7799 the whole document</p>	

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INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2009/056449

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	DATABASE UniProt 6 March 2007 (2007-03-06), PEL H J ET AL: "SubName: Full=Catalytic activity: ATP + NADH = ADP + NADPH; EC= 2.7.1.86" XP002503751 Database accession no. A2R9N0 the whole document -----	
A	DATABASE UniProt 24 January 2006 (2006-01-24), MACHIDA M ET AL: "SubName: Full=Predicted sugar kinase" XP002503752 Database accession no. Q2UTM3 the whole document -----	