A nucleic acid cassette and method to isolate mutants and to clone the complementing gene

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ABSTRACT

The subject invention lies in the field of microorganism mutation and selection of the mutants. In particular, the invention is directed at obtaining metabolic mutants in a simple, direct and specific manner. In a preferred embodiment it is also possible to obtain desired mutants not comprising recombinant DNA, thereby facilitating incorporation thereof in products for human consumption or application, due to shorter legislative procedures. The method according to the invention involves random mutation and specific selection of the desired metabolic mutant. A nucleic acid cassette comprising a nucleic acid sequence encoding a bidirectional marker, said nucleic acid cassette further comprising a basic transcriptional unit operatively linked to the nucleic acid sequence encoding the bidirectional marker and said nucleic acid cassette further comprising an inducible enhancer or activator sequence linked to the basic transcriptional unit in such a manner that upon induction of the enhancer or activator sequence the bidirectional marker encoding nucleic acid sequence is expressed, said inducible enhancer or activator sequence being derived from a gene associated with activity of part of metabolism, said inducible enhancer or activator sequence being derived from a gene associated with metabolism is claimed as is application thereof in a selection method for mutants. In addition a regulator gene xlnR encoding an activating regulator of an inducible enhancer or activator sequence and application of said gene and/or its expression product in overexpression of homologous or heterologous protein or peptide is described. Knockout mutants wherein said gene is absent or inactivated and mutants with increased or decreased DNA binding capacity are also claimed.
The following statement is a full description of this invention, including the best method of performing it known to me/us:
A nucleic acid cassette & method to isolate mutants and to clone the complementing gene

Background of the Invention

All references, including any patents or patent applications, cited in this specification are hereby incorporated by reference. No admission is made that any reference constitutes prior art. The discussion of the references states what their authors assert, and the applicants reserve the right to challenge the accuracy and pertinency of the cited documents. It will be clearly understood that, although a number of prior art publications are referred to herein, this reference does not constitute an admission that any of these documents forms part of the common general knowledge in the art, in Australia or in any other country.
The subject invention lies in the field of microorganism mutation and selection of the mutants. In particular the invention is directed at obtaining metabolic mutants in a simple, direct and specific manner. In a preferred embodiment it is also possible to obtain desired mutants not comprising recombinant DNA, thereby facilitating incorporation thereof in products for human consumption or application, due to shorter legislative procedures. The method according to the invention involves random mutation and specific selection of the desired metabolic mutant. The method can suitably be carried out automatically. Such a mutant can exhibit either increased or decreased metabolic activity. The specificity of the method lies in the selection conditions applied. The mutants obtained are mutated in their regulatory function with regard to a predetermined part of metabolism. Dependent on the selection conditions derepressed mutants can be isolated that will thus exhibit overexpression or mutants can be found in which particular metabolic enzymic activity is eliminated. It thus becomes possible to eliminate undesirable metabolic enzymic activity or to increase desirable metabolic activity.

The methods according to the invention can suitably be carried out on well characterised sources that are already widely used in industry. The overexpressing mutants can for example be used as major sources of enzymes producing huge amounts at low cost. The initial strain to be mutated will depend on several factors known to a person skilled in the art such as: efficient secretion of proteins, the availability of large scale production processes, experience with downstream processing of fermentation broth, extensive genetic knowledge and the assurance of using safe organisms.

In another aspect of the invention it has now also become possible to ascertain and identify specific metabolic gene regulating functions.

To date a method for preparing mutants that was industrially applicable and could be automated was a method of mutating without selection and subsequent analysis of the mutants for the aspect which was to be amended. An alternative method with selection always required an enrichment step, followed by selection on the basis of growth or non growth. This meant a large number of undesired mutants had first to be weeded out. Also the existing method resulted in a high number of mutants with an incorrect phenotype and thus exhibits low selectivity.
Some years ago Gist-Brocades developed and introduced the pluGBug marker gene free technology for *Aspergillus niger*. In the GIST 94/60 p 5-7 by G. Selten a description is given of a vector for *Aspergillus niger* comprising glucoamylase regulatory regions to achieve high expression levels of the gene it regulates. This was selected as regulatory region on the basis of the naturally high expression of glucoamylase by native *Aspergillus niger*. Using recombinant DNA techniques the regulatory region was fused appropriately to the gene of interest as was the selection marker *Aspergillus* AmdS, allowing selection of the desired transformants after transferring the expression cassette to the *A. niger* host. Multiple copies of the expression cassette then become randomly integrated into the *A. niger* genome. The enzyme produced as described in the article was phytase. Subsequently the generation of marker free transformants can be achieved. In the known system the generation of marker free recombinant strains is actually a two step process since the *amdS* gene can be used bidirectionally. First in a transformation round to select initial transformants possessing the offered expression cassette and subsequently in a second round by counterselecting for the final recombinant strain which has lost the *amdS* gene again. The *amdS* gene encodes an enzyme which is able to convert acetamide into ammonium and acetate. Acetamide is used as sole N-source in the transformation round. In the recombination round fluoroacetamide is used as selective N-source, with a second appropriate N source such as e.g. urea. As the product fluoroacetate is toxic for other cells the propagation will be limited to those cells which have lost the *amdS* gene by an internal recombination event over the DNA repeats within the expression cassette. The largest problem with the known method is the fact that the resulting strain is a recombinant strain. The desired characteristic has to be introduced by incorporation of "foreign" nucleic acid, which can lengthen the time required for and sometimes even prevent legislative approval. In addition the method is not suited for developing strains with amended metabolism. Due to the presence of enzyme cascades and multiple feedback loops the mere incorporation of a particular gene cannot always lead to the desired result. Overproduction of a particular product as encoded can be compensated for by concomitant overexpression of another product or down regulated thus annulling the effect of the incorporated gene. The incorporation of DNA will therefore often be a case of trial and error with the incorporation of the desired nucleic acid being selectable but the desired phenotype not necessarily
concomitantly being achieved. Furthermore the loss of the marker gene is a spontaneous process which takes time and cannot be guaranteed to occur for all transformants comprising the nucleic acid cassette.

It is known that strain improvement in microorganisms can be achieved by modification of the organism at different levels. Improvement of gene expression at the level of transcription is mostly achieved by the use of a strong promoter, giving rise to a high level of mRNA encoding the product of interest, in combination with an increase of gene dosage of the expression cassette. Although this can lead to an increase of the product formed, this strategy can have a disadvantage in principle. Due to the presence of multiple copies of the promoter the amount of transcriptional regulator driving transcription can become limited, resulting in a reduced expression of the target gene or genes of the regulator. This has been observed in the case of Aspergillus nidulans strains carrying a large number of copies of the amdS gene (Kelly and Hynes, 1987; Andrianopoulos and Hynes, 1988) and in the case of A. nidulans strains carrying multiple copies of a heterologous gene under the gldA promoter (Gwynne et al., 1987). In the latter case an increase of the gldR gene, encoding the transcriptional regulator of the gldA gene, resulted in the increase of expression of the expression cassette (Gwynne et al., 1987; Davies, 1991). In analogy to the effects found in Aspergillus nidulans, in Aspergillus niger similar limitations were observed in using the glucoamylase (gldA) promoter, due to limitation of the transcriptional regulator driving transcription (Verdoes et al., 1993; Verdoes et al., 1995; Verdoes, 1994). Cloning of the gldR regulatory gene has thusfar been hampered by lack of selection strategy.

In the case of the arabinase gene expression a clear competition for transcriptional regulator was found upon the increase of arabinase gene dosage (Flipphi et al., 1994), reflecting a limitation of a transcriptional regulator common to all three genes studied.

In addition to the abovementioned drawbacks of the state of the art isolation and determination of regulator genes has until now been extremely difficult due to the fact that most of the regulatory proteins exist in very low concentrations in the cell making it difficult to determine which substance is responsible for regulation. In addition generally the regulatory product is not an enzyme and can only be screened for by a DNA binding assay which makes it difficult to determine and isolate and is very time consuming. Thus far the strategies used to clone regulatory genes are e.g.:
by complementation, which however requires a mutant to be available,
by purification of the regulatory protein, which is extremely
laborious, since the protein can only be characterised by its DNA
binding properties. Some of these purifications include affinity
chromatography using a bound DNA fragment as a matrix. One of the
drawbacks in this type of purification is that often more than one
protein binds both specifically as well as non-specifically to the
fragment,
based on gene clustering wherein the regulatory gene is genomically
clustered with the structural genes which are regulated by its gene
product, e.g. the prn cluster, the aic cluster.

Detailed description of the invention

We have now achieved a system that can be used for shortening the
length of time required for registration of mutant microorganisms capable
of overproduction of particular desirable enzymes. The system overcomes
the problem of multiple random inserts of "foreign" nucleic acid and in
particular of the selection marker gene. It does not even require foreign
nucleic acid to achieve the desired characteristic. The resulting mutant
strain will not comprise heterologous nucleic acid. In addition the
system according to the invention enables specific mutation of metabolism
and prevents a large deal of experimentation leading to undesired
phenotypes.

The subject invention is directed at a nucleic acid cassette
comprising a nucleic acid sequence encoding a bidirectional marker,
said nucleic acid cassette further comprising a basic transcriptional
unit operatively linked to the nucleic acid sequence encoding the
bidirectional marker and said nucleic acid cassette further comprising an
inducible enhancer or activator sequence linked to the basic
transcription unit in such a manner that upon induction of the enhancer
or activator sequence the bidirectional marker encoding nucleic acid
sequence is expressed, said inducible enhancer or activator sequence
being derived from a gene associated with activity of part of the
metabolism, said inducible enhancer or activator sequence being derived
from a gene associated with metabolism.

A basic transcription unit comprises any elements required for
transcription of the gene to which the transcription is linked. It can
comprise the promoter with or without enhancer sequences. The basic
transcription unit must be operative in the host organism. The basic
transcription unit must be located such that it is operatively linked to
the bidirectional marker gene for transcription thereof to be possible.
Suitable examples are well known for a number of host cells such as
e.g. Aspergillus, Trichoderma, Penicillium, Fusarium, Saccharomyces,
Kluveromyces and Lactobacillus. In the Examples the basic transcription
unit tGOX derived from the Aspergillus niger goxC transcription unit
(Whittington et al. 1990) is illustrated in an operable embodiment of the
invention.

The inducible enhancer or activator sequence is preferably normally
involved in regulation of an enzyme cascade or involved in a part of
metabolism involved with one or more feed back loops. In a further
embodiment a nucleic acid cassette according to the invention comprises
an inducible enhancer or activator sequence that is normally involved in
carbon metabolism. Suitable examples of inducible enhancer or activator
sequence to be used in a nucleic acid cassette according to the invention
are the Upstream Activating Sequence (UAS) as comprised on any of the
following fragments of nucleic acid:
- a fragment originating from the promoters of the abfA, abfB and abmA
genes encoding respectively arabinofuranosidase A, arabinofuranosidase B
and endoarabinase,
- a fragment originating from the glaA gene encoding glucoamylase,
- a fragment containing the alcR binding site such as on the alcR and
alcA promoter,
- a fragment originating from the CUP1 gene,
- a fragment originating from the PRO5 gene
- a fragment originating from the GAL1, GAL7 or GAL10 genes.
- a fragment originating from the xlnA gene
- a fragment originating from the pgall gene.

By way of example these fragments can be derived from the
following organisms as is described in the literature:
- a fragment originating from the promoters of the abfA, abfB and abmA
genes encoding respectively arabinofuranosidase A, arabinofuranosidase B
and endoarabinase of Aspergillus niger (Flippin M.J.A. et al. 1994)
- a fragment originating from the glaA gene encoding glucoamylase of
Aspergillus niger. (Fowler T. et al 1990)
- a fragment containing the alcR binding site such as on the alcR
promoter of Aspergillus nidulans (Felenbok B. et al. 1994).
- a fragment originating from the CUP1 gene of Saccharomyces cerevisiae
(Hinnen A. et al. 1995)
- a fragment originating from the PHO5 gene of *Saccharomyces cerevisiae* (Hinnen A. et al. 1995)

- a fragment originating from the GAL1, GAL7 or GAL10 genes of *Saccharomyces cerevisiae* (Hinnen A. et al. 1995).

- a fragment originating from the xlnA, xlnB, xlnC or xlnD genes of *Aspergillus nidulans*.

- a fragment originating from the xlnB, xlnC or xlnD genes of *Aspergillus niger* (see elsewhere in this description).

- a fragment originating from the xlnA or xlnD genes of *Aspergillus tubigensis* (de Graaff et al. 1994).

- a fragment originating from the pgall gene of *Aspergillus niger* (see elsewhere in this document).

In the Examples UAS of xlnA is illustrated in an operable embodiment of the invention.

A bidirectional marker is an art recognised term. It comprises a selection marker that can be used to indicate presence or absence of expression on the basis of the selection conditions used. A preferred bidirectional marker will confer selectability on the basis of lethality or extreme reduction of growth. Alternatively different colouring of colonies upon expression or lack of expression of the bidirectional marker gene is also a feasible embodiment. Suitable examples of known bidirectional markers are to be found in the group consisting of the *facB*, the *NiaD*, the *AmdS*, the *Can1*, the *Ura3*, the *Ura4* and the *PyrA* genes. We hereby point out that *PyrA* homologues are also referred to in the literature as *PyrG*, *Ura3*, *Ura4*, *Pyr4* and *Pyrl*. These genes can be found in i.a. the following organisms the *facB* gene in *Aspergillus nidulans*, the *NiaD* gene in *Aspergillus niger*, the *NiaD* gene in *Aspergillus oryzae*, the *AmdS* gene in *Aspergillus nidulans*, the *Can1* gene in *Schizosaccharomyces pombe*, the *Ura3* gene in *Saccharomyces cerevisiae*, the *Ura4* gene in *Saccharomyces pombe* and the *PyrA* genes in *Aspergillus*, *Trichoderma*, *Penicillium*, *Fusarium*, *Saccharomyces* and *Kluveromyces*.

Selection of *facB* mutants i.e. with a negative phenotype can occur on the basis of fluoro-acetate resistance. Selection for FAC B* i.e. a positive phenotype can occur on acetate as a carbon source (Katz, M.E. and Hynes M.J. 1989). Selection of *niaD* mutants i.e. with a negative phenotype can occur on the basis of chlorate resistance. Selection for NIA D* i.e. a positive phenotype can occur on nitrate as a nitrogen source (Unkles S.E. et al. 1989a and 1989b). Selection of *amdS* mutants i.e. with a negative phenotype can occur on the basis of fluor acetamide
resistance. Selection for AMD S* i.e. a positive phenotype can occur on acetamide as a nitrogen source. As most fungi do not have a gene encoding an acetamidase function AMD S is a dominant marker for such fungi. It has been used as such in *Aspergillus niger*, *Aspergillus niger var. tubigenes*, *Aspergillus niger var. awamori*, *Aspergillus foetidus*, *Aspergillus oryzae*, *Aspergillus sydowii*, *Aspergillus japonicus*, *Aspergillus aculeatus*, *Penicillium* species, *Trichoderma* species among others (Kelly and Hynes 1985 and Bailey et al. 1991). Selection of can1 mutants i.e. with a negative phenotype can occur on the basis of canavanine resistance, wherein canavanine is an arginine analogue. Selection for CAN 1* i.e. a positive phenotype can occur on arginine (Ekwall K. 1991). The gene encoding orothidine 5'-P-decarboxylase is known as pyrA, pyrG or ura3. It has been found for various organisms e.g. *Aspergilli*, *Trichoderma*, *Penicillium*, *Fusarium*, *Saccharomyces* and *Kluveromyces*. Selection of pyrA mutants i.e. with a negative phenotype, also described as pyrG or ura3 can occur on the basis of fluoro orotic acid resistance. Selection for PYR A* and homologues thereof i.e. a positive phenotype can be done on the basis of uridine or uracil prototrophy. In the Examples pyrA from *Aspergillus niger* (Wilson et al. 1988) is illustrated in an operable embodiment of the invention. Other examples are known to a person skilled in the art and can be readily found in the literature. The selection marker to be used will depend on the host organism to be mutated and other secondary considerations such as ease of selectability, reliability and cost of substrates to be used amongst others.

The nucleic acid cassette incorporated in a transformation or expression vector is also included in the scope of the invention. Also included is the application of such nucleic acid cassette or vector in transformation and selection methods. In particular in methods for producing mutants exhibiting overexpression of an enzyme involved in a predetermined part of metabolism, methods for producing mutants exhibiting reduced or inhibited expression of an enzyme involved in a predetermined part of metabolism and methods for determining and isolating regulatory genes involved in predetermined parts of metabolism.

Thus a method for preparing and selecting a mutant strain of microorganism, said mutation enhancing a predetermined part of metabolism in comparison to the non mutated strain, said method comprising introducing into a host a nucleic acid cassette according to any of the preceding claims.
said host not exhibiting the phenotype associated with expression of
the bidirectional marker prior to introduction of the nucleic acid
cassette,
- culturing a resulting microorganism under conditions wherein the
enhancer or activator sequence comprised on the nucleic acid cassette
is normally active and under conditions wherein the bidirectional
marker is expressed and wherein preferably expression of said
bidirectional marker will lead to growth and non expression to non
growth.
- selecting a transformant that exhibits the phenotype corresponding to
the expression of the bidirectional marker gene under the
aforementioned culturing conditions,
- subjecting the selected transformant to mutagenesis in a manner known
per se,
- culturing the resulting strain under conditions acceptable for a
strain with a phenotype corresponding to the expression of the
bidirectional marker and under conditions that in the non mutated
parent comprising the nucleic acid cassette result in non-expression
of the bidirectional marker and in the presence of a metabolisable
substrate for the predetermined part of metabolism,
- selecting a strain resulting from the cultivation step following
mutagenesis that exhibits a phenotype corresponding to the expression
of the bidirectional marker gene under selection conditions that for
the non mutated parent comprising the nucleic acid cassette result in
non-expression of the bidirectional marker falls within the scope of
the invention.

In a suitable embodiment of this method
- the inducible enhancer or activator sequence is the Upstream
Activating Sequence (UAS) derived from the gene zlnA,
- the predetermined part of the metabolism is the xylanolytic part of
carbon metabolism,
- the culturing step wherein the resulting microorganisms are cultivated
under conditions wherein the enhancer or activator sequence is
normally active and wherein the bidirectional marker is expressed
comprises cultivation in the presence of inducer of UAS and absence of
repressor of UAS and a metabolisable source of carbon.
- the selecting of a transformant that exhibits the phenotype
corresponding to the expression of the bidirectional marker gene
occurs under the aforementioned culturing conditions.
the culturing step after mutagenesis of the selected transformant occurs under conditions acceptable for a strain with a phenotype corresponding to the expression of the bidirectional marker and under conditions that in the non mutated parent comprising the nucleic acid cassette result in non-expression of the bidirectional marker i.e. in the presence of repressor of UAS and in the presence of a metabolisable source of carbon and optionally also in the presence of inducer of UAS.

the selection of a strain resulting from the cultivation step following mutagenesis of a strain that exhibits a phenotype corresponding to the expression of the bidirectional marker gene occurs under selection conditions that for the non mutated parent comprising the nucleic acid cassette result in non-expression of the bidirectional marker.

In a further embodiment of this method the nucleic acid cassette comprises a nucleic acid sequence encoding the bidirectional marker pyrA.

the host does not exhibit the pyrA+ phenotype associated with expression of the bidirectional marker prior to introduction of the nucleic acid cassette.

the culturing step wherein the resulting microorganisms are cultivated under conditions wherein the enhancer or activator sequence is normally active and wherein the bidirectional marker is expressed comprises cultivation under conditions wherein the enhancer or activator is normally active i.e. in the presence of inducer of the enhancer or activator and in the absence of repressor of the enhancer or activator and under conditions wherein the bidirectional marker is expressed.

the selecting of a transformant that exhibits the phenotype corresponding to the expression of the bidirectional marker gene occurs under the aforementioned culturing conditions.

the culturing step after mutagenesis of the selected transformant occurs under conditions acceptable for a strain with a phenotype corresponding to the expression of the bidirectional marker and under conditions that in the non mutated parent comprising the nucleic acid cassette result in non-expression of the bidirectional marker i.e. in the absence of inducer of the enhancer or activator or in the presence of repressor of the enhancer or activator and in the presence of a metabolisable substrate for the predetermined part of metabolism.
- the selection of a strain resulting from the cultivation step following mutagenesis of a strain that exhibits a phenotype corresponding to the expression of the bidirectional marker gene occurs under selection conditions that for the non mutated parent comprising the nucleic acid cassette result in non-expression of the bidirectional marker i.e. in the absence of inducer of the enhancer or activator or the presence of repressor of the enhancer or activator.

  Suitably the embodiments just mentioned can further be characterised by

- the nucleic acid cassette comprising a nucleic acid sequence encoding the bidirectional marker pyrA,

- the host not exhibiting the pyrA+ phenotype associated with expression of the bidirectional marker prior to introduction of the nucleic acid cassette,

- the inducible enhancer or activator sequence being the UAS derived from the gene zlnA,

- the culturing step wherein the resulting microorganisms are cultivated under conditions wherein the enhancer or activator sequence is normally active and wherein the bidirectional marker is expressed comprising cultivation under conditions wherein UAS is normally active i.e. in the presence of inducer of UAS such as xylose or xylan and in the absence of repressor of UAS i.e. absence of glucose and under conditions wherein the bidirectional marker is expressed,

- the selecting of a transformant that exhibits the phenotype corresponding to the expression of the bidirectional-marker gene occurs under the aforementioned culturing conditions,

- the culturing step after mutagenesis of the selected transformant occurring under conditions acceptable for a strain with a phenotype corresponding to the expression of the bidirectional marker and under conditions that in the non mutated parent comprising the nucleic acid cassette result in non-expression of the bidirectional marker i.e. in the absence of inducer of UAS such as xylose or xylan or in the presence of repressor of UAS i.e. in the presence of glucose and in the presence of a metabolisable source of carbon,

- the selection of a strain resulting from the cultivation step following mutagenesis of a strain that exhibits a phenotype corresponding to the expression of the bidirectional marker gene occurring under selection conditions that for the non mutated parent comprising the nucleic acid cassette result in non-expression of the
bidirectional marker i.e. in the absence of inducer of UAS such as xylose or xylan or the presence of repressor of UAS i.e. in the presence of glucose.

In addition a method for preparing and selecting a non recombinant mutant strain of microorganism, said mutation enhancing a predetermined part of metabolism in comparison to the non mutated strain falls within the preferred scope of the invention. This method comprising carrying out the steps of the method according to the invention as described in the preceding paragraphs followed by crossing out in a manner known per se the nucleic acid of the introduced nucleic acid cassette.

As indicated previously a method for preparing and selecting a mutant strain of microorganism, said mutation inhibiting a predetermined part of the carbon metabolism in comparison to the non mutated strain, said method comprising

- introducing into a host a nucleic acid cassette according to the invention as described above,
  said host not exhibiting the phenotype associated with expression of the bidirectional marker prior to introduction of the nucleic acid cassette and said host exhibiting activity of the type characterising the predetermined part of metabolism to be reduced or inhibited,
- culturing a resulting microorganism under conditions wherein the enhancer or activator sequence of the nucleic acid cassette is normally active and wherein non expression of the bidirectional marker of the nucleic acid cassette will result in growth and detection of the resulting microorganism and wherein expression of said bidirectional marker will preferably be lethal or strongly inhibit growth,
- selecting a transformant that exhibits the phenotype corresponding to the expression of the bidirectional marker gene under the aforementioned culturing conditions
- subjecting the selected transformant to mutagenesis in a manner known per se,
- culturing the strain resulting from the mutagenesis under conditions acceptable for growth of a strain with a phenotype corresponding to the non expression of the bidirectional marker and in the presence of a metabolisable substrate and under conditions that illustrate the reduced or inhibited activity of the predetermined part of metabolism in comparison to the non mutated host either with or without the
nucleic acid cassette.

- selecting a strain resulting from the cultivation step following mutagenesis that exhibits a phenotype corresponding to the reduced or inhibited activity of the predetermined part of the metabolism under selection conditions that illustrate the reduced or inhibited activity of the predetermined part of metabolism in comparison to the non mutated host with or without nucleic acid cassette such as a reduced zone of clearing upon growth on a substrate which serves as a substrate for the part of metabolism for which the activity is to be reduced or inhibited.

In a further embodiment of such a method
- the inducible enhancer or activator sequence is the Upstream Activating Sequence (UAS) derived from the gene xlnA
- the predetermined part of the metabolism is the xylanolytic part of carbon metabolism.
- the culturing step wherein the resulting microorganisms are cultivated under conditions wherein the enhancer or activator sequence is normally active and wherein the bidirectional marker is expressed comprises culturing in the absence of repressor of the UAS of the nucleic acid cassette, in the presence of a metabolisable source of carbon and preferably also in the presence of inducer of the UAS.
- the selecting of a transformant that exhibits the phenotype corresponding to the expression of the bidirectional marker gene occurs under the aforementioned culturing conditions.
- the culturing step after mutagenesis of the selected transformant occurs under conditions acceptable for growth and detection of a strain with a phenotype corresponding to the non expression of the bidirectional marker, under conditions that are unacceptable for growth and detection of a strain with a phenotype corresponding to the expression of the bidirectional marker i.e. in the presence of uridine and fluoro-orotic acid and under conditions that in the non mutated parent comprising the nucleic acid cassette result in activity of the predetermined part of the carbon metabolism i.e. in the presence of inducer of the UAS and a metabolisable carbon source and the absence of repressor of the UAS for example the presence of sorbitol or an alternative non repressing source of carbon in combination with an inducer like xylan or D-xylose.
- the selection of a strain resulting from the cultivation step following mutagenesis that exhibits a phenotype corresponding to the
reduced or inhibited activity of the predetermined part of the carbon metabolism occurs under selection conditions that illustrate the reduced or inhibited activity of the predetermined part of carbon metabolism in comparison to the non mutated host either with or without the nucleic acid cassette such as a reduced zone of clearing upon growth on xylan.

An example of the method according to the preceding paragraph is provided, wherein

- the nucleic acid cassette comprises a nucleic acid sequence encoding the bidirectional marker pyrA,
- the host does not exhibit the PYRA+ phenotype associated with expression of the bidirectional marker prior to introduction of the nucleic acid cassette,
- the culturing step wherein the resulting microorganisms are cultivated under conditions wherein the enhancer or activator sequence is normally active and wherein the bidirectional marker pyrA is expressed i.e. comprises cultivation in the presence of inducer of the enhancer or activator and in the absence of repressor of the enhancer or activator and under conditions wherein the bidirectional marker pyrA is expressed,
- the selecting of a transformant that exhibits the phenotype corresponding to the expression of the bidirectional marker gene pyrA occurs under the aforementioned culturing conditions,
- the culturing step after mutagenesis of the selected transformant occurs under conditions acceptable for growth and detection of a strain with a phenotype corresponding to the non expression of the bidirectional marker i.e. PYRA-phenotype, under conditions that are unacceptable for growth and detection of a strain with a PYRA+ phenotype, such a phenotype corresponding to the expression of the bidirectional marker i.e. such conditions comprising the presence of uridine and fluoro-orotic acid and under conditions that in the non mutated parent comprising the nucleic acid cassette result in activity of the predetermined part of the metabolism i.e. in the presence of inducer of the enhancer or activator and a metabolisable substrate and the absence of repressor of the activator or enhancer or an alternative non repressing substrate in combination with an inducer.

In a preferred embodiment the method according to the preceding 2 paragraphs is a method, wherein furthermore

- the nucleic acid cassette comprises a nucleic acid sequence encoding
the bidirectional marker pyrA.

- the host does not exhibit the PYRA\(^+\) phenotype associated with expression of the bidirectional marker prior to introduction of the nucleic acid cassette.

- the inducible enhancer or activator sequence is the UAS derived from the gene xlnA

- the culturing step wherein the resulting microorganisms are cultivated under conditions wherein the enhancer or activator sequence is normally active and wherein the bidirectional marker pyrA is expressed comprises cultivation under conditions wherein the UAS is normally active i.e. in the presence of inducer of the UAS such as xylose or xylan and in the absence of repressor of the UAS i.e. absence of glucose and under conditions wherein the bidirectional marker pyrA is expressed.

- the selecting of a transformant that exhibits the phenotype corresponding to the expression of the bidirectional marker gene pyrA occurs under the aforementioned culturing conditions.

- the culturing step after mutagenesis of the selected transformant occurs under conditions acceptable for growth and detection of a strain with a phenotype corresponding to the non expression of the bidirectional marker i.e. pyrA\(^-\) phenotype, under conditions that are unacceptable for growth and detection of a strain with a PYRA\(^+\) phenotype, such a phenotype corresponding to the expression of the bidirectional marker i.e. such conditions comprising the presence of uridine and fluoro-orotic acid and under conditions that in the non mutated parent comprising the nucleic acid cassette result in activity of the predetermined part of the carbon metabolism i.e. in the presence of inducer of the UAS and a metabolisable carbon source and the absence of repressor of the UAS for example the presence of sorbitol or an alternative non repressing source of carbon in combination with an inducer like xylan or D-xylose.

- the selection of a strain resulting from the cultivation step following mutagenesis that exhibits a phenotype corresponding to the reduced or inhibited activity of the predetermined part of the carbon metabolism occurs under selection conditions that illustrate the reduced or inhibited activity of the predetermined part of carbon metabolism in comparison to the non mutated host either with or without the nucleic acid cassette such as a reduced zone of clearing upon growth on xylan.
The methods described above can advantageously be carried out with a host characterised in that prior to introduction of the nucleic acid cassette it comprises nucleic acid corresponding at least in part to the nucleic acid sequence encoding the bidirectional marker, said correspondence being to a degree sufficient to allow homologous recombination in the chromosome of the bidirectional marker encoding nucleic acid comprised on the nucleic acid cassette. This aspect ensures the integration of the nucleic acid cassette at a predefined location.

In a further preferred embodiment the nucleic acid cassette will be incorporated in multiple copies to ensure that the mutagenesis step does not inactivate the bidirectional marker as this would result in incorrect results when detecting marker negative phenotypes and a decrease in the number of marker positive phenotypes.

In preferred embodiments of the invention a nucleic acid cassette has been constructed which can be used in a method for producing mutants exhibiting overexpression of an enzyme involved in a predetermined part of metabolism, a method for producing mutants exhibiting reduced or inhibited expression of an enzyme involved in a predetermined part of metabolism and a method for determining and isolating regulatory genes involved in predetermined parts of metabolism. In particular we illustrate the system as used for mutants in carbon metabolism. In the examples mutants with altered xylanolytic characteristics are described as well as arabinase and polygalacturonase mutants leading to mutants in the arabinolytic and pectinolytic pathways. In the examples Aspergillus is used as the strain to be mutated, however any other industrially acceptable microorganism will suffice. Examples of such organisms are Saccharomyces e.g. Saccharomyces cerevisiae, Saccharomyces pombe, Aspergillus e.g. Aspergillus nidulans, Trichoderma, Penicillium, Fusarium Kluveromyces and Lactobacillus. Other examples will be obvious to a person skilled in the art and a number are also mentioned elsewhere in the description. An overexpressing or nulexpressing strain for a predetermined part of the metabolism can now be produced. We can also determine the identity and nucleic acid sequence of the activating regulator of an inducible enhancer or activator sequence. In particular when such activating regulator is involved in metabolism, more specifically when such activating regulator is involved in a part of metabolism with an enzyme cascade or feed back loop or multiple feed back loops. The nucleic acid sequence of such a regulatory gene can subsequently be used to enhance expression of target genes. Said target
gene being a gene that is regulated by the regulatory gene. In a preferred embodiment such a target gene will have a binding site for the expression product of the regulator gene. Combination of a promoter normally associated with a target gene of the regulator with the regulatory gene in an expression cassette said promoter being operably linked to a homologous or heterologous sequence encoding a homologous or heterologous protein or peptide to be expressed can lead to an expression cassette extremely useful for expression of homologous and even heterologous proteins or peptides. The regulator encoding gene can be under control of its native promoter or any other promoter that can be expressed in the host cell of choice. The promoter can be constitutive or inducible, whatever is most desirable for the particular production process. Such a combination expression cassette falls within the scope of the invention as does a vector or a plasmid comprising such a cassette. The degree of expression is no longer restricted by the presence of too small an amount of regulator and thus the degree of expression of the gene to be expressed is much higher than in a corresponding host cell where the gene is expressed under control of the same promoter but without the additional presence of the regulator gene. Such increased expression is preferably achieved in cells of organisms normally comprising components of the part of the metabolic pathway to be influenced. Suitable host cells are filamentous fungi cells. The incorporation of a combined expression cassette of the type just described above in a host cell comprising a target gene of the regulator can lead to increased expression of the target gene or to increased expression of the target genes if multiple target genes are present. Preferably the target gene will be endogenous to the host cell. A host cell comprising the combination expression cassette falls within the scope of the invention.

In the examples the nucleic acid sequence xlnR of the regulator of the xylanolytic pathway xylR is provided. The target genes for this regulator have been found to comprise the genes xlnA, xlnB, xlnC and xlnD as well as axeA. The increase in xylanase A expression is illustrated and can serve to indicate the general applicability of the xylR action on a target gene of the xylR regulator. A number of sequences are known in the state of the art comprising the xlnA, B, C and D genes mentioned and the axeA gene and such information is readily available to a person skilled in the art and is to be considered incorporated herein. The promoters of preferred interest to be used in combination with xlnR nucleic acid can
be selected from xlnA, xlnB, xlnC and xlnD. The use of the axeA promoter also forms a suitable embodiment of the invention. The promoters are known in the state of the art to the person skilled in the art and are considered to be incorporated herein. The xlnA promoter is described in de Graaff et al 1994. The xlnB promoter has been described by Kinoshita et al 1995). The xlnD promoter has been described in EP 95201707.7 and is included in the Sequence Listing in the sequence of sequence id no 8 of this document. The promoter sequences can either be readily synthesized on the basis of known sequences or be derived from organisms or vectors comprising them in standard manners known per se. Where the term promoter, enhancer or regulator is used naturally a fragment comprising the promoter, enhancer or regulator can be employed as long as the operability of such is not impaired. It is not necessary in the constructs according to the invention to merely incorporate the relevant sequence, any flanking non interfering sequences can be present.

Not only is the nucleic acid sequence xlnR encoding the expression product xylR covered by the subject invention but also sequences encoding equivalent expression products and mutant expression products as well as the expression products themselves of the nucleic acid sequences according to the invention. Any application of xylR or xylR encoding sequences (=xlnR) disclosed herein is also applicable to the mutants and the nucleic acid sequences encoding such mutants and is to be considered incorporated mutatis mutandi.

Examples of suitable fungal cells to be used for expression of nucleic acid sequences and cassettes according to the invention-are Aspergilli such as Aspergillus niger, Aspergillus tamarii, Aspergillus aculeatus, Aspergillus awamori, Aspergillus oryzae, Aspergillus nidulans, Aspergillus carbonarius, Aspergillus foetidus, Aspergillus terreus, Aspergillus svdowii, Aspergillus kawachii, Aspergillus japonicus and species of the genus Trichoderma, Penicillium and Fusarium. Other cells such as plant cells are also feasible host cells and elsewhere in the description alternative host cells are also described.

Genes of particular interest for expressing using the expression cassette according to the invention or in combination with a nucleic acid sequence according to the invention are those encoding enzymes. Suitable genes for expressing are genes encoding xylanases, glucanases, oxidoreductases such as hexose oxidase, α-glucuronidase, lipase, esterase, ferulic acid esterase and proteases. These are non limiting examples of desirable expression products. A number of sequences are
known in the state of the art comprising the genes mentioned and such information is readily available to the person skilled in the art and is to be considered incorporated herein. The genes can either be readily synthesized on the basis of known sequences in the literature or databases or be derived from organisms or vectors comprising them in a standard manner known per se and are considered to be knowledge readily available to the person skilled in the art not requiring further elucidation.

An expression product exhibiting 80%-100% identity with the amino acid sequence of xylR according to sequence id no. 9 or as encoded by the nucleic acid sequence of xlnR of sequence id. no. 9 (from nucleotide 948) is considered to be an equivalent expression product of the xylanase regulator (xylR) according to the invention and thus falls within the scope of the invention. The equivalent expression product should possess DNA binding activity. Preferably such DNA binding activity should be such that the expression product binds to the nucleic acid of the target gene to the same degree or better than the expression product binds with the amino acid sequence provided in sequence id no 9. The preferred target genes for determining binding activity are those encoding xylA, xylB, xylC and xylD i.e. the xlnA, xlnB, xlnC and xlnD genes.

Mutants of the xlnR gene expression product xylR and the encoding nucleic acid sequence xlnR according to sequence id no 9 at least maintaining the same degree of target binding activity are also claimed. Mutants considered to fall within the definition of equivalent expression products comprise in particular mutants with amino acid changes in comparison to the amino acid sequence of sequence id no 9. Such equivalents are considered suitable embodiments of the invention as are the nucleic acid sequences encoding them. Mutants with 1-15 amino acid substitutions are suitable and substitutions of for example 1-5 amino acids are also considered to form particularly suitable embodiments of the invention that form equivalent expression products. It is common knowledge to the person skilled in the art that substitutions of particular amino acids with other amino acids of the same type will not seriously influence the peptide activity. On the basis of hydropathy profiles a person skilled in the art will realise which substitutions can be carried out. Replacement of hydrophobic amino acids by other hydrophobic amino acids and replacement of hydrophilic amino acids by other hydrophilic amino acids e.g. will result in an expression product that is a suitable embodiment of the invention. Such substitutions are
considered to be covered by the scope of the invention under the term equivalents. Point mutations in the encoding nucleic acid sequence according to sequence id no 9 are considered to result in nucleic acid sequences that fall within the scope of the invention. Such point mutations can result in silent mutations at amino acid level or in substitution mutants as described above. Substitution mutants wherein the substitutions can be of any type are also covered by the invention. Preferably the identity of the mutant will be 85-100%, more preferably 90-100%, most preferably 95-100% in comparison to the amino acid sequence of sequence id no 9. As already claimed above amino acid sequences exhibiting 80-100% identity with the amino acid sequence of sequence id no 9 are covered by the term equivalent so that deletions and/or substitutions of up to 20% of the amino acid sequence, preferably of less than 15% and more preferably less than 10% most preferably of less than 5% of the amino acid sequence according to the invention are covered. Such a mutant may comprise the deletion and/or substitution in one or more parts of the amino acid sequence. Such a deletion and/or substitution mutant will however comprise an amino acid sequence corresponding to a Zn finger binding region and an amino acid sequence corresponding to the RRRLWW motif. Deletion mutants of 1-5 amino acids are considered to fall within the scope of the invention. Deletion mutants with larger deletions than 5 amino acids and/or with more than 5 substitutions and/or point mutations can also maintain the DNA binding activity. Such larger number of deletions and/or substitutions and/or point mutations will preferably occur in the N terminal-half of the amino acid sequence and the corresponding part of the encoding nucleic acid sequence. The most important regions considered to be involved in regulation and activation are present in the C terminal half of the amino acid sequence from the zinc finger binding region and as such the deletion mutants will preferably comprise at least this portion of the amino acid sequence. Preferably no mutation will be present in the zinc finger binding region corresponding to that encoded in sequence id no 9 from nucleotides at position 1110 to 1260. If a mutation is present preferably it should not involve the spacing between the 6 cysteines coordinating the zinc and most preferably any mutation should not involve any of the 6 cysteines themselves. In addition preferably no mutation is present in the RRRLWW motif present in the amino acid sequence of sequence id no 9. A deletion may occur in one or more fragments of the amino acid sequence. Such a deletion mutant will however comprise an
amino acid sequence corresponding to a Zn finger binding region and an amino acid sequence corresponding to the RRRLWW motif. Deletions of 1-15 amino acids, preferably of 1-10 amino acids and most preferably 1-5 amino acids are suitable embodiments to ensure equivalence.

Embodiments of equivalent nucleic acid sequences are a nucleic acid sequence which encodes an expression product having the same amino acid sequence as xylR of sequence id no. 9 or as encoded by the nucleic acid sequence of xlnR of sequence id. no. 9. A nucleic acid sequence encoding an expression product exhibiting 80%-100% identity with the amino acid sequence of xylR according to sequence id no 9 or as encoded by the nucleic acid sequence encoding xylR of sequence id no 9 is also considered to be an equivalent nucleic acid sequence of xlnR and falls within the scope of the invention. Another embodiment of an equivalent nucleic acid sequence according to the invention is a nucleic acid sequence capable of hybridising under specific minimum stringency conditions as defined in the Examples to primers or probes derived from nucleic acid sequence id no 9, said primers or probes being derived from the non zinc finger binding region and said primers or probes being at least 20 nucleotides in length. Generally suitable lengths for probes and primers are between 20-60 nucleotides, preferably 25-60. Preferably a probe or primer will be derived from the C-terminal encoding half of the sequence of id no 9 from the zinc finger binding region. A preferred embodiment of a nucleic acid sequence according to the invention will be capable of hybridising under specific conditions of at least the stringency illustrated in the examples to the nucleic acid sequence of id no 9. An equivalent nucleic acid sequence will be derivable from other organisms by e.g. PCR using primers based on the nucleic acid sequence id no 9 as defined above. An expression product of an equivalent nucleic acid sequence as just defined is also considered to fall within the scope of the invention. Vice versa a nucleic acid sequence encoding an equivalent amino acid sequence according to the invention is also considered to fall within the scope of the term equivalent nucleic acid sequence. In particular equivalent nucleic acid sequences and the expression products of such sequences derivable from filamentous fungi and plants are preferred embodiments of the invention. Preferably an equivalent nucleic acid sequence will comprise a nucleic acid sequence encoding a zinc finger binding region corresponding to that encoded in sequence id no 9 from nucleotides from position 1110 to 1260. In a preferred embodiment the equivalent nucleic acid sequence should encode
the 6 cysteines coordinating the zinc. In a further embodiment the spacing between the cysteines should correspond to that of sequence id no 9.

Embodiments comprising combinations of the characteristics of the various embodiments of the equivalent nucleic acid sequences and expression products described above also fall within the scope of the invention. Mutants with mutation in the zinc finger binding domain are also claimed as these could exhibit increased DNA binding. Mutants exhibiting decreased DNA binding are also considered to fall within the scope of the invention. Such mutants may possess a mutation in the zinc finger binding domain. In particular mutants of the amino acid sequence provided in sequence id no 9 are claimed.

Fragments of the nucleic acid sequence according to sequence id no 9 of at least 15 nucleotides also fall within the scope of the invention. Such fragments can be used as probes or primers for detecting and isolating equivalent sequences. In particular a combination of two or more such fragments can be useful in a kit for detecting and/or isolating such equivalent sequences. Preferably a fragment will be derived from the C terminal half of the amino acid sequence of sequence id no 9. In a suitable embodiment of the kit one fragment will not comprise a part of the nucleic acid sequence forming the zinc finger domain. In the examples a suitable combination of fragments to be used as primers is illustrated. Any sequence obtainable through PCR as illustrated in the examples with these primers is considered to fall within the scope of the invention.

The hybridisation conditions used in the examples provide the minimum stringency required for selectivity. More stringent conditions can be applied such as the stringent hybridisation conditions described by Sambrook et al for increased homology of the obtained sequences with the sequence id no 9. A lower salt concentration generally means more stringent conditions. Any fragment of the sequence according to sequence id no 9 being or encoding a polypeptide exhibiting the target gene binding activity of the complete sequence is also included within the scope of the invention as are equivalent nucleic acid sequences or amino acid sequences thereof, with equivalent being defined as defined above with regard to hybridisation and/or mutation and/or degeneracy of the genetic code for the complete sequence.

A vector or plasmid comprising the nucleic acid sequence encoding xylR or an equivalent sequence thereof as defined above also falls within the scope of the invention, as do a vector or plasmid comprising such a
sequence and a host cell comprising such an additional sequence. A transformed host cell such as a microorganism or a plant cell carrying at least one additional copy of an encoding nucleic acid sequence according to sequence id no 9 or an equivalent thereof falls within the scope of the invention. Preferably the various embodiments are organised such that the sequence can be expressed in the vector, plasmid or host cell. The regulatory gene can comprise the complete sequence of id no 9 or merely the encoding sequence thereof in combination with an alternative promoter that is operable in the host cell. Suitable examples of host cells have been provided elsewhere in the description. Suitable operable promoters for the various host cells that can be incorporated with the encoding sequence of sequence id no 9 will be clear to a person skilled in the art. In particular for the host cells explicitly mentioned in the description constitutive promoters or inducible promoters are known and available to work the invention without undue burden.

A process for production of homologous or heterologous proteins or peptides is provided, said process comprising expression of a nucleic acid sequence encoding the homologous protein or peptide in a host cell, said host cell further comprising an additional copy of a nucleic acid sequence encoding a regulatory gene such as xlnR or an equivalent thereof which is also expressed. A process as just described is preferably carried out with a combination nucleic acid expression cassette comprising the regulatory gene operably linked to a first promoter and said cassette further comprising a second promoter, said second promoter normally being associated with a target gene of the regulator, said target gene promoter being operably linked to the nucleic acid sequence encoding the homologous or even heterologous protein or peptide to be expressed. The first promoter can be the promoter natively associated with the regulator gene but may also be a promoter of choice that is operable in the host cell. The degree of expression is no longer restricted by the presence of too small an amount of regulator and thus the degree of expression of the gene to be expressed is much higher than in a corresponding host cell where the gene is expressed without the additional presence of the regulator gene. Such increased expression is preferably achieved in cells of organisms normally comprising components of the part of the metabolic pathway to be influenced. Suitable host cells are a plant cell or a microorganism. suitably the microorganism can be a fungus in particular it can be a filamentous fungus. Examples of suitable host cells have been given elsewhere in the description and
may be considered to be incorporated here. The incorporation of a combined expression cassette of the type just described above in a host cell comprising a target gene of the regulator can lead to increased expression of the target gene or to increased expression of the target genes if multiple target genes are present. Preferably the target gene will be native to the regulator. Preferably such a target gene will be endogenous to the host cell. In the examples the nucleic acid sequence of the regulator of the xylanolytic pathway xlnR is provided. The native target genes for this regulator have been found to comprise the genes xlnA, xlnB, xlnC and xlnD as well as axeA and as such these genes are preferred target genes. Other targets exist and are considered to be included in the term target gene. Various embodiments of the host cells according to the invention are covered in the claims. If both regulator sequence and target gene are natively present in the host cell the regulator sequence will be present in multiple copies. Such a microorganism will over express the gene regulated by the target gene promoter in comparison to the native microorganism.

Because now the sequence for the xylanase regulator is known it has become possible to knock out the xylanase regulator. The creation of a knockout host cell once the nucleic acid sequence of the gene to be knocked out is known is standard technology. This method can be carried out analogously to that described in Berka et al (1990) and example 11 of EP 95201707.7, which is a copending European patent application of which a copy has been included upon filing the subject document and the example itself has also been copied into this document in the examples. Such a knockout renders a host cell which can be free of xylanolytic activities. A host cell free of xylanolytic activity due to knocking out the xlnR gene can be used to produce homologous or heterologous expression products of choice free of xylanolytic activity. A host cell with a knocked out xlnR gene falls within the scope of the invention. Such a host cell is preferably a plant cell or a filamentous fungus. Such a filamentous fungus is preferably an Aspergillus. Examples have been provided elsewhere in the description of numerous suitable host cells. A host cell with a mutation in the regulator gene which can be arrived at using the selection and mutation method of the invention can be subjected to complementation with a regular active copy of the regulator gene. Such a complemented strain will subsequently express the products of any target genes that are regulated by the regulator. These target gene products will be absent in the case of the non complemented
Regulator negative mutant. Upon comparison of protein bands obtained in a manner known per se from both of the strains it will become apparent what target products are regulated by the regulator and subsequently the corresponding novel target genes can be determined in a manner known per se once its expression product has been determined. In this manner other target genes than those already known can be found for the xylanese regulator xyIR in the instant examples.

For the purposes of this specification it will be clearly understood that the word “comprising” means “including but not limited to”, and that the word “comprises” has a corresponding meaning.
Example 1: Construction of the plasmids

Example 1.1: Construction of the selection plasmid pIM130

The selection plasmid pIM130 was constructed as depicted in Fig. 1. In PCR1 a fragment was generated from the plasmid pIM120 (de Graaff et al., 1994) using oligonucleotide 1 (SEQ ID NO: 1)

5'-CACAATGCATCCCCTTATCCGGGCGTG-3' (Formula 1)

and oligonucleotide A (SEQ ID NO: 2)

5'-CAATTGCGACTTGAGAGGACATGATGGGCAGATGAGGG-3' (Formula 2)

Oligonucleotide 1 was derived from the *Aspergillus tubigensis* xlnA promoter (de Graaff et al., 1994) positions 600-619 (SEQ ID NO: 5) to which 10 nucleotides containing a *NsiI* site were added. The 3’ end of oligonucleotide A was derived from the *Aspergillus niger* gogC transcription unit (Whittington et al., 1990) ending just before the translation initiation site (positions 708-723)(SEQ ID NO: 6), while the 5’ end was derived from the coding region of the *A.niger* pyrA gene (Wilson et al., 1988) (starting at the translation initiation site (positions 341 to 359, SEQ ID NO: 7).

Fragment A was generated by a PCR containing 10 µl 10x reaction buffer (100 mM Tris-Cl, pH 8.3, 500 mM KCl, 15 mM MgCl₂, 0.01% gelatin), 16 µl 1.25 mM of each of the four deoxynucleotide triphosphates, 1 ng of the plasmid pIM120 DNA and 1 µg of each of the oligonucleotides 1 and A in a final volume of 100 µl. This reaction mixture was mixed and 1 µl TAQ polymerase (5 U/µl) (Life Technologies) was added. The DNA was denatured by incubation for 3 min at 92°C followed by 25 cycles of 1 min 92°C, 1 min 48°C and 1 min 72°C. After these 25 cycles the mixture was incubated for 5 min at 72°C. Analysis of the reaction products by agarose electrophoresis revealed a fragment of about 250 bp, which corresponds to the size expected based on the sequence of the genes.

In PCR2 a fragment was generated from the plasmid pGW635 (Goosen et al., 1987) using oligonucleotide 2 (SEQ ID NO: 3)
5'-AGAGAGGATATCGATGTGGG-3' (Formula 3)
and oligonucleotide B (SEQ ID NO: 4)
5'-CCCTCATCTGCCCATCATGTCCTCCAAGTCGCAATTG-3' (Formula 4)
The 5' end of oligonucleotide B was derived from the A.niger goxC basic transcription unit (positions 708-723, SEQ ID NO:6)(Whittington et al., 1990), while the 3' end was derived from the coding region of the A.niger pyrA gene starting at the translation initiation site. Oligonucleotide 2 was derived from the pyrA coding region (positions 339-359, SEQ ID NO:7) and is spanning an EcoRV restriction site at position 602 (SEQ ID NO:7).

Fragment B was generated in an identical manner as fragment A except that in this case the reaction mixture contained 1 μg each of oligonucleotide 2 and B and 1 ng of plasmid pGW635 DNA. Analysis of the reaction products by agarose electrophoresis revealed a fragment of about 250 bp, which corresponds to the size expected based on the sequence of the pyrA gene.

Fragments A and B were isolated from agarose gel after electrophoresis. The fragments were cut from the agarose gel, after which they were recovered from the piece of agarose by electro-elution using ISCO cups. Both on the large and the small container of this cup a dialysis membrane was mounted, the cup was filled with 0.005 x TAE (50xTAE buffer per 1000 ml: 242.0 g Trizma base (Sigma), 7.1 ml glacial acetic acid, 100 ml 0.5 M EDTA pH 8.0) and the piece of agarose was placed in the large container of the cup. Subsequently the cup was placed in the electro-elution apparatus, with the large container in the cathode chamber containing 1*TAE and the small container at the anode chamber containing 1*TAE/3 M NaAc. The fragments were electro-eluted at 100 V during 1 h. After this period the cup was taken from the electro-elution apparatus and the buffer was removed from the large container, while from the small container the buffer was only removed from the upper part. The remaining buffer (200 μl) containing the DNA fragment was dialyzed in the cup against distilled water during 30 min. Finally the DNA was precipitated by the addition of 0.1 vol. 3 M NaAc, pH 5.6 and 2 vol. cold (-20°C) ethanol. The DNA was collected by centrifugation (Eppendorf centrifuge) for 30 min. at 14,000 x g. at 4°C. After removal of the supernatant the DNA pellet was dried using a Savant Speedvac vacuum centrifuge. The DNA was dissolved in 10 μl TE buffer (TE: 10 mM Tris/HCl pH7.2, 1 mM EDTA pH 8.0) and the concentration was determined by agarose gel electrophoresis, using lambda DNA with a known concentration as a reference and ethidiumbromide staining to detect the DNA.
Fragments A and B were fused in PCR3 which was identical to PCR1 except that in this case the reaction mixture contained 1 μg of each of the oligonucleotides 1 and 2 and approximately 50 ng of each of the fragments A and B. Analysis of the reaction products by agarose gel electrophoresis revealed a fragment of about 500 bp, which corresponds to the size expected based on the sequences of the genes.

The resulting fragment C was isolated from agarose gel as described and subsequently digested using the restriction enzymes NsiI and EcoRV. The DNA was digested for 3 h. at 37°C in a reaction mixture composed of the following solutions: 5 μl (≈ 0.5 μg) DNA solution; 2 μl of the appropriate 10 x React buffer (Life Technologies); 10 U restriction enzyme (Life Technologies) and sterile distilled water to give a final volume of 20 μl. After digestion the DNA fragment was analysed by agarose gel electrophoresis and subsequently isolated from the gel as described.

For the final construction of pIM130 5 μg of the plasmid pGW635 was digested as described using 50 U of the restriction enzymes EcoRV and XbaI in a final volume of 500 μl. After separation of the products a 2.2 kb EcoRV/XbaI fragment (fragment D) was isolated from the agarose gel by electro-electro-elution. Analogously 1 μg vector pGEM-7Zf(+) (Promega) was prepared by digestion with the restriction enzymes NsiI/XbaI, which was after digestion electrophoresed and isolated from the agarose gel by electro-elution.

The plasmid pIM130 was constructed by the following ligation reaction: 100 ng pGEM-7Zf(+) NsiI/XbaI fragment was mixed with 50 ng fragment C and 50 ng fragment D and 4 μl 5 μl ligation buffer (composition: 500 mM Tris-HCl, pH 7.6; 100 mM MgCl₂; 10 mM ATP; 10 mM dithiotreitol; 25% PEG-6000) and 1 μl (1.2 U/μl) T₄ DNA ligase (Life Technologies) was added to this mixture in a final volume of 20 μl. After incubation for 16 h at 14°C the mixture was diluted to 100 μl with sterile water. 10 μl of the diluted mixture was used to transform E. coli DH5α competent cells, prepared as described by Sambrook et al., 1989.

Two of the resulting colonies were grown overnight in LB medium (LB medium per 1000 ml: 10 g trypticase peptone (BBL), 5 g yeast extract (BBL), 10 g NaCl, 0.5 mM Tris-HCl pH 7.5) containing 100 μg/ml ampicillin. From the cultures plasmid DNA was isolated by the alkaline lysis method as described by Maniatis et al. (1982), which was used in restriction analysis to select a clone harbouring the desired plasmid pIM130. Plasmid DNA was isolated on a large scale from 500 ml cultures E. coli DH5α containing pIM130 grown in LB medium containing 100 μg/ml.
The plasmid was purified by CaCl₂ centrifugation, phenolyzed, ethanol precipitated and dissolved in 400 µl TE. The yield was approximately 500 µg.

Example 1.2: Construction of the plasmid pIM135

From the plasmid pIM120 a second plasmid was constructed which contains the goxC basic transcription unit fused to the pyrA coding region and termination region. In PCR4 a fragment was generated from the plasmid pIM120 using oligonucleotide 3, 5'-CACAATGCATCGTATAACTAACCTCGTTCG-3' (Formula 5) which was derived from the goxC basic transcription unit (positions 640-660, SEQ ID NO:6) to which 10 nucleotides containing a Nsil site were added, and oligonucleotide 2 (Formula 3). The fragment generated was isolated from gel, digested with Nsil and EcoRV and cloned together with the 2.2 kb EcoRV/XbaI fragment of pGW635 in the plasmid pGEM-7Zf(+) which was digested with XbaI/Nsil, as described in Example 1.1., resulting in the plasmid pIM135.

The plasmid pIM135 can be used as construction vehicle for preparing vectors according to the invention with any desirable inducible enhancer or activator sequence, a UAS of a gene involved in metabolism. pIM135 comprises a basic transcription unit (tGOX) operatively linked to a bidirectional marker gene (pyrA).

Example 2: Transformation of A. niger using the plasmid pIM130

250 ml of culture medium, which consists of Aspergillus minimal medium (MM) (contains per liter: 6.0 g NaNO₃, 1.5 g KH₂PO₄, 0.5 g MgSO₄·7H₂O, 0.5 g KCl. Carbon source as indicated, pH 6.0 and 1 ml Vishniac solution (contains per liter 10 g EDTA, 4.4 g ZnSO₄·7H₂O, 1.0 g MnCl₂·4H₂O, 0.32 g CoCl₂·6H₂O, 0.32 g CuSO₄·5H₂O, 0.22 g (NH₄)₆Mo₇O₂₄·4H₂O, 1.47 g CaCl₂·2H₂O, 1.0 g FeSO₄·7H₂O, pH 4.0) supplemented with 2 % glucose, 0.5 % Yeast Extract, 0.2 % Casamino acids (Vitamin free), 10 mM L-arginin, 10 mM nicotinamide, 10 mM uridine, was inoculated with 1 * 10⁶ spores per ml of strain NW205 (cspA1, pyrA6, nicA1, argB13) and mycelium was grown for 16 – 18 hours at 30 °C and 250 rpm in an orbital New Brunswick shaker. The mycelium was harvested on Myrcloth (nylon gauze) using a Büchner funnel and mild suction and was washed several times with SP6 (SP6: 0.8 % NaCl, 10 mM Na-phosphate buffer pH 6.0). 150 mg Novozyme 234 was dissolved in 20 ml SMC (SMC: 1.33 M Sorbitol, 50 mM CaCl₂, 20 mM MES buffer, pH 5.8) to which 1 g (wet weight) mycelium was added and which was carefully
resuspended. This suspension was incubated gently shaking for 1 - 2 hours at 30 °C, every 30 minutes the mycelium was carefully resuspended and a sample was taken to monitor protoplast formation using a haemocytometer to count the protoplasts. When sufficient protoplasts were present (more then $1 \times 10^8$) these were carefully resuspended and the mycelial debris was removed by filtration over a sterile glass wool plug. The protoplasts were collected by 10 minutes centrifugation at 3000 rpm and 4 °C in a bench centrifuge and were carefully resuspended in 5 ml STC (STC: 1.33 M Sorbitol, 50 mM CaCl$_2$, 10 mM Tris/HCl, pH 7.5). This wash step was repeated twice and the protoplasts were finally resuspended in STC at a density of $1 \times 10^8$ per ml.

The transformation was performed by adding 1 μg of pIM130 DNA (dissolved in a 10 - 20 μl TE to 200 μl of protoplast suspension together with 50 μl of PEG buffer (PEG Buffer: 25 % PEG-6000, 50 mM CaCl$_2$, 10 mM Tris/HCl pH 7.2), mixed gently by pipetting up and down a few times, and incubated at room temperature for 20 minutes. After this period 2 ml PEG buffer was added, the solution was mixed gently and incubated at room temperature for another 5 minutes and subsequently 4 ml of STC was added and mixed gently on a vortex mixer. This was also done using 5μg pIM130 and 1 and 5 μg of plasmid pGW635 DNA. As a negative control 20 μl of TE was added to the protoplasts.

One ml portions of this suspension were then added to 4 ml of osmotically stabilised top agar and poured on plates (swirl gently to cover plate with top agar) containing MMS having either 100 mM D-glucose or 100 mM D-xylose as a carbon source. These media (MMS) were osmotically stabilised using 0.8 M KCl or by using 1.33 M Sorbitol.

To determine the percentage regeneration serial dilutions of the protoplasts were prepared before transformation (untreated protoplasts, kept on ice) and after transformation (obtained from the negative control). 100 μl of the $10^{-3}$, $10^{-4}$, $10^{-5}$ and $10^{-6}$ dilutions were plated in duplicate on 10 mM uridine supplemented MMS plates.

For the positive control, in which the fungus was transformed using the plasmid pGW635, colonies were found on all plates. However, the transformation frequency was much lower (1-10 transformants per μg plasmid DNA) on KCl stabilised medium than on sorbitol stabilised medium (100-1000 transformants per μg plasmid DNA). This was due to the much higher regeneration frequency on the latter medium, which was about 90% in comparison to 2-5% on the KCl stabilised medium.

In case of the transformations using the plasmid pIM130 transformants
were found on the medium containing D-xylose as a carbon source but not on medium containing D-glucose. The frequency on sorbitol stabilised medium was about 100 per µg of plasmid DNA while the frequency on the KCl stabilised medium was less than one per µg of DNA.

Example 3: Analysis of transformants

The transformants from pIM130 obtained in Example 2 were analysed phenotypically by plating on MM containing 100 mM D-glucose, 100 mM D-glucose/1% Oat spelt xylan (Sigma #X0627) and 1% Oat spelt xylan. A selection of the transformants were replica plated to these media and incubated at 30°C. About 75% of the transformants were growing on xylan containing medium, while no growth was found on media containing D-glucose. The remaining 25% of the colonies grew on all three media tested.

A selection of five transformants having the expected phenotype (growth on xylan containing medium, non-growth on D-glucose containing media) was analysed by Southern analysis. Fungal DNA was isolated by a modified procedure used to isolate plant RNA essentially as described by de Graaff et al., 1988). Mycelium, which was grown overnight in culture medium, was harvested, washed with cold saline, frozen in liquid nitrogen and stored at -80°C. Nucleic acids were isolated by disrupting 0.5 g frozen mycelium using a microdismembrator (Braun). The mycelial powder obtained was extracted with freshly prepared extraction buffer. The extraction buffer was prepared as follows: 1 ml tri-isopropynaphtalene sulfonic acid (TNS) (20 mg/ml) was thoroughly mixed with 1 ml p-aminosalicylic acid (PAS) (120 mg/ml) and 0.5 ml 5 x RNB buffer was added (5 x RNB contains 121.10 g Tris, 73.04 g NaCl and 95.10 g EGTA in 1 l, pH 8.5). After the addition of 1.5 ml phenol, the extraction buffer was equilibrated for 10 min. at 55°C. The warm buffer was then added to the mycelial powder, and the suspension was thoroughly mixed for 1 min. using a vortex mixer. After addition of 1 ml chloroform the suspension was remixed for 1 min. After centrifugation at 10^4 x g for 10 min., using a Sorvall high speed centrifuge, the aqueous phase was extracted once more with an equal volume of phenol/chloroform (1:1) and was then extracted twice with chloroform. DNA was isolated from the aqueous phase using the following procedure; the DNA was immediately precipitated from the aqueous phase with 2 vol. ethanol at room temperature and subsequently collected by centrifugation using a Sorvall high speed centrifuge at 10^6 x g for 10 min. and washed twice by redissolving the
DNA in distilled, sterile water and precipitating it again with ethanol. RNA was removed by adding RNase A (20 \( \mu \)g/ml) to the final solution.

High molecular weight DNA (1-2 \( \mu \)g) isolated from \textit{A. niger} N402 (cspA1) as a wild-type and five pIM130 transformants as described was digested with \textit{HpaI} (Life Technologies) according to the manufacturers instructions. The resulting fragments were separated by agarose gel electrophoresis, and transferred to High-bond \( N \) membrane as described by Maniatis et al. (1982). Hybridisation using a \( ^{32}\)P-labelled 3.8 kb \( XbaI \) fragment, prepared as described by Sambrook et al., 1989, containing the \textit{A. niger} \textit{pyrA} gene as a probe was done according to the following procedure (Sambrook et al.): prehybridization in 6 \( \times \) SSC (20xSSC per 1000 ml: 175.3 g NaCl, 107.1 g sodiumcitrate, 5.5 \( \times \) H\(_2\)O, pH 7.0), 0.1\% SDS, 0.05\% sodium pyrophosphate, 5* Denhardt's solution (100xDenhardts solution per 500 ml: 10 g Ficoll-400, 10 g polyvinylpyrrolidone, 10 g Bovine Serum Albumin (Pentax Fraction V) and 20 \( \mu \)g/ml denatured herring sperm DNA at 68\(^\circ\)C for 3-5 hrs and hybridization in an identical buffer which contained the denatured radiolabelled probe at 68\(^\circ\)C for 15-18 hrs, followed by two washes in 3 \( \times \) SSC, 0.1\% SDS at 68\(^\circ\)C and two washes in 0.2 \( \times \) SSC, 0.1\% SDS at 68\(^\circ\)C. The membrane was covered with Saran wrap and autoradiographed overnight at -70\(^\circ\)C using Konica X-ray films and Kodak X-Omatic cassettes with regular intensifying screens.

As a result a 10 kb hybridising band is found in the N402 lane, while this band is missing in the transformants NW205::130#1, NW205::130#2 and NW205::130#3. In the transformants NW205::130#1 and NW205::130#3 a 15kb hybridising fragment is found, while in NW205::130#2 a 20 kb band is found. These results correspond respectively to a single and a double copy integration at the homologous \textit{pyrA} locus. In the transformants NW205::130#4 and NW205::130#5 the plasmid was integrated at a non-homologous locus. Transformant NW205::130#2 was selected for mutagenesis.

The UAS fragment of pIM130 comprises the binding site required for the positive regulator to exhibit the stimulatory activity of the UAS. Thus inhibition of \textit{xlnR} in the host cell comprising pIM130 will result in negative expression of the bidirectional marker present on pIM130. Expression of \textit{xlnR} is induced by xylan or xylose. Thus the presence of such substrates should result in expression of the bidirectional marker of pIM130 if the host possesses \textit{xlnR}.

The UAS fragment of pIM130 does not comprise the site required for inhibitory activity of \textit{creA} that is present on the native UAS of the \textit{Aspergillus niger} \textit{xlnA} gene. Thus the presence of glucose which renders
A. niger CRE A’ and subsequently inhibits the UAS of xlnA and the other xylanolytic enzyme encoding genes such as xlnD and axeA and also inhibits the xlnR gene encoding the activator of the UAS of the aforementioned xylanolytic enzyme encoding genes i.e. represses xylanolytic enzyme expression which results in negative expression of pIM130.

Example 4: Selection of mutants

Example 4.1: Selection of derepressed mutants

Spores of NW205::130#2 were harvested in 5 ml ST (ST: 0.8 % NaCl, 0.05 % Tween 20), shaken at high frequency and mycelial debris was removed by filtration over a sterile glasswool plug. The spores were collected by centrifugation for 10 minutes at 3000 rpm at room temperature in a bench centrifuge and were resuspended in 5 ml saline. This wash step was repeated twice and the spores were finally resuspended in saline at a density of $1 \times 10^7$ per ml. 10 ml of the spore suspension was dispensed in a glass petridish and was irradiated using UV at a dosage of 18 erg/mm²/min for 2 min. After mutagenesis $10^5$ and $10^6$ spores were plated (10 plates each) on MM+ 10 mM L-arginine + 10 μM nicotinamide containing 3% D-glucose and on plates containing 3% D-glucose/3% oat spelts xylan (Sigma #X0627).

After 4-7 days 5-10 mutant colonies per plate ($10^6$ spores inoculated) were found which on basis of their morphology could be divided into three classes; large, well sporulating colonies, intermediate-sized, well sporulating colonies and small poorly sporulating colonies. A random selection of 20 of these mutants was made and the selected mutants were tested on media containing different carbon sources or substrates. The mutant colonies were found to be able to grow on media containing D-glucose, D-glucose/xylan and xylan, while the parental strain NW205::130#2 only was able to grow on medium containing xylan as a carbon source. In addition these mutants were tested on different chromogenic substrates; 4-methylumbelliferyl-β-D-xyloside (β-xylosidases, endo-xylanases)(Sigma #M7008), 4-methylumbelliferyl acetate (acetyl-xylan esterases)(Sigma #M0883), 4-methylumbelliferyl-α-L-arabinofuranoside (arabinofuranosidases) (Sigma #M9519) and on Remazol Brilliant blue modified xylan (endo-xylanases)(Sigma #M5019). The methylumbelliferyl derivatives were added in a 1 mM final concentration to media containing D-glucose, D-glucose/xylan and xylan, while the RBB-xylan was added in a
concentration of 1 mg/ml to media containing D-glucose/xylan and xylan. For all these substrates tested enzyme activity was found in the mutants after growth on D-glucose containing media, while no expression was found in the parental strain NW205::pIM130#2. On media containing xylan an increased expression of these enzymes was found in comparison to NW205::pIM130. Of the mutants tested mutant 5B had the highest expression levels. In the instant case selection occurred on a substrate normally active as inhibitor of xylanolysis i.e. glucose. To be certain that expression could occur in the absence of repression an inducer of xylanolytic genes xylan was also included. Such a control test is preferably included in a method according to the invention. The mutant clearly exhibited derepression.

For the comparison of the activity levels produced, both A. niger N402 and mutant 5B were cultured on MM containing 1.5% crude Wheat arabinobio- xylan as a carbon source. Samples were taken at 24, 42, 72 and 96 hrs and the activities of α-L-arabinofuranosidase, endo-xylanase and β-xylosidase were measured. The results (Fig. 2 A,B,C) indicated an increase in activity for the mutant strain for all three enzymes. α-L-arabinofuranosidase and endo-xylanase activity was most strongly increased.

Example 4.2: Selection of non-expressing mutants
Spores of strain NW205::130#2 were harvested and mutated as described in Example 4.1 and subsequently plated on MM containing 100 mM D-xylose supplemented with 10 mM uridine, 10 mM L-arginine and 0.8 mg/ml 5-fluoroorotic acid (Sigma #F5013). These plates were incubated for 4-7 days at 30°C. 64 of the growing colonies, having a PYR phenotype, were analysed for xylanase expression by plating on MM containing 1% xylan +10 mM uridine + 10 mM L-arginin + 10 μM nicotinamide. Of these 64 mutants tested 10 gave a reduced zone of clearing on these xylan containing plates and had potential reduced xylanase levels. The phenotype of these mutants was verified on D-glucose, D-glucose/xylan and xylan containing media in the presence and absence of uridine. All 10 mutants did not grow on media without uridine.

For further analysis these mutants were precultured 18 hrs at 30°C on MM containing 50 mM fructose + 10 mM uridine + 10 mM L-arginin + 10 μM nicotinamide after which the mycelium was harvested and 1 g wet mycelium was transferred to MM containing 1% xylan and to MM containing 10 mM D-xylose + 10 mM uridine + 10 mM L-arginine + 10 μM nicotinamide. After 5.5
hrs incubation at 30°C both the mycelium and the culture filtrate were harvested. The culture filtrate was dialysed against 1 mM NaPi, pH 5.6 after which the xylanase (Bailey et al. (1991)) was determined and β-xylosidase activities were determined (Table 1). Both the xylanase as well as the β-xylosidase expression levels were found to be strongly reduced in these selected mutants.
<table>
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<tr>
<th>Strain</th>
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<td>(nkat ml⁻¹)</td>
<td>(nkat ml⁻¹)</td>
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Example 5. Complementation of non-expressing mutants

5.1 Construction of an \textit{A.\textit{niger}} genomic plasmid library

For the construction of a plasmid library 10 \mu g of genomic DNA of \textit{A.\textit{niger}} NW128 (\textit{cspA1, nicA1, pyrA6, goxC17}), isolated as described in Example 3, was partially digested for 30 min at 37°C according to the manufacturer's instructions using 3.5 U \textit{Sau3A} (Life-Technologies) in a final volume of 100 \mu l. After separation of the fragments by agarose electrophoresis, fragments ranging in size from 6.7 kb to 9.4 kb were cut from the low melting point agarose gel and were isolated as described in Sambrook et al., (1989). From totally 6 digestions 4 \mu g of fragments were isolated in a final concentration of 100 ng/\mu l. 600 ng of the resulting fragments were ligated in 100 ng \textit{BamHI} digested pUC18 (Pharmacia #27526201) according to the manufacturer's instructions. After ligation 4\mu l of the resulting ligation mixture was used to transform 100 \mu l \textit{E.coli DH5a-Max Efficiency} competent cells (Life Technologies #18258-012) according to the manufacturer's instructions. Six subsequent transformation experiments resulted in about 5*10^8 colonies. After resuspension of these colonies and growth for 3 hrs at 37°C in TY medium (medium per 100 ml: 16 g Select Peptone 140 (Life Technologies), 10 g NaCl and 10 g Yeast extract) containing 100 \mu g/ml Ampicillin, plasmid DNA was isolated and purified by CsCl centrifugation.

5.2 Complementation of non-expressing mutants

For the complementation in non-expressing mutants a selection of three mutants, NW205::130 \textit{Acl}-4, NW205::130 \textit{Acl}-15 and NW205::130 \textit{Ac4}-4, protoplasts were prepared of these strains and transformed as described in Example 2. In these transformation experiments 10^8 protoplasts were used and combined with; 20 \mu g DNA of the \textit{A.\textit{niger}} plasmid library as described in Example 5.1, and with 10 \mu g DNA of the plasmid library combined with 10 \mu g DNA of the autonomously replicating plasmid \textit{pHELP1} (Gess and Clutterbuck, 1993) and with 20 \mu g DNA of the plasmid library combined with 10 \mu g DNA of the autonomously replicating plasmid \textit{pHELP1}. As a positive control 2 \mu g \textit{pGW635} was used. After the transformation procedure the mixtures were plated on MM stabilised with 1.33 M Sorbitol containing 50 mM D-xylose as a carbon source supplemented with 10 \mu M nicotinamide and 10 mM L-arginin. After about 4 days colonies appeared on the positive control plates and were counted, while after 6 days colonies could be picked from the complementation plates.
The resulting transformant colonies were analysed by plating on medium containing 1% Oat spelt xylan, 1 mM 4-methylumbellyferyl-β-D-xyloside, 10 μM nicotinamide and 10 mM L-arginin. After 6-7 hrs incubation at 30°C xx fluorescent colonies were detected. After 2-3 days a clearing of the xylan around these colonies appeared.

Example 6: Cloning of the \textit{A.}	extit{niger} \textit{zinR} gene

Example 6.1: Isolation of the \textit{A.}	extit{niger} \textit{zinR} gene

The \textit{A.}	extit{niger} \textit{zinR} gene was isolated from transformants obtained and selected as described in Example 5.2. From 13 transformants obtained from NW205::130 Ac 1-4, NW205::130 Ac 1-15 and NW205::130 Ac 4-4 total DNA was isolated, as described by de Graaff et al., 1988. After mycelium was cultured under selective (i.e. inducing) conditions for pyrA and xylanolytic expression on 1.5% crude wheat arabino-xylan as C source from which free replicating plasmids were isolated using Nucleobond AX100 columns (Macherey & Nagel). 200 μg of total DNA dissolved in 400 μl of sterile water was mixed with 2 ml of S1 buffer (50 mM Tris-HCl pH 8.0, 10 mM EDTA, 100 μg RNase A), 2 ml of S2 (200 mM NaOH, 1% SDS), followed by an incubation at room temperature for 5 minutes, and 2 ml of S3 (2.60 M KAc, pH 5.2), followed by an incubation on ice for 5 minutes. After clearing of the suspension at 15,000 g for 30 minutes adsorption, washing, elution and precipitation of the plasmids was done all according to the manufacturers' instructions for "working procedure for the purification of plasmids and cosmids" (5.3 modified alkaline/SDS lysis). 20 μl of the resulting plasmid DNA, dissolved in 150 μl sterile water, was used in \textit{E.}	extit{coli} DH5α transformation. (Sambrook et al., 1989). 12 \textit{E.}	extit{coli} colonies resulting from each of these plasmid preparations were grown for 9-12 hrs at 37°C in 250 ml LB medium containing 50 μg/ml Ampicillin, after which a miniprep plasmid DNA isolation was performed on 1.5 ml of the culture as described for the boiling lysis protocol by Sambrook et al., 1989 and the cells were pelleted by centrifugation and stored at -20°C. Analysis of these DNA preparations, after HinDIII digestion, by agarose electrophoresis revealed three classes of plasmids: pHELP1 type plasmids, genomic library type plasmids and large complex type plasmids. From colonies containing the latter type of plasmids, a large scale plasmid isolation, using Nucleobond AX100 columns according to the manufacturers' instructions for the modified alkaline/SDS lysis (Macherey-Nagel) was performed on the frozen pellet of the 250 ml
culture. This resulted in the isolation of two plasmid types, A and B, complementant A and B respectively. Both these plasmids were digested, using SalI, PstI, EcoRI, HinfIII, and after agarose electrophoresis, the fragments were analysed in triplicate by Southern analysis using denatured radiolabelled pHELP1, plasmid A and plasmid B. This showed that both plasmids A and B, besides the pHELP part, shared the same genomic region. Based upon the differences between the hybridisation signals found using the pHELP plasmid, showing the vector and AMA1 sequences, and the plasmid A and B signals, fragments hybridising with both plasmid A and B, but not with pHELP1, were identified and subcloned. A 4 kb EcoRI fragment and a 6.5 kb HinfIII fragment of plasmid B, and a 3 kb PstI fragment of plasmid A were found to hybridise with both plasmids A and B and were subcloned in pGEM7/EcoRI, pGEM7/HinfIII and pBluescript/PstI, resulting in plasmid pNP1, 2 and 3 respectively. After propagation and purification these plasmids were used in complementation experiments using the mutant NW205::130 Ac 4-4 as described in Example 5.2. In these experiments the mutant was directly transformed without using pHELP1. In these experiments the plasmid pNP2, containing the 6.5 kb HinfIII fragment gave rise to complementation of the mutation. Further subcloning and transformation of pNP2-derived plasmids revealed a 5 kb BamHI-XbaI fragment, subcloned in pBluescript and resulting in plasmid pNP8, giving rise to complementation of strain NW205::130 Ac 4-4.

Example 6.2:
Subcloning of the *A. niger* ztnR gene

For the subcloning of the ztnR gene, the *A. niger* genomic library, constructed as described by Harmsen et al., 1990, was screened for phages containing the ztnR region by using the 4 kb EcoRI fragment of pNP1 as probe. 3 x 10^3 pfu per plate were plated in NZYCM top-agarose containing 0.7% agarose on five 85-mm-diameter NZYCM (1.5% agar) plates as described (Maniatis et al., 1982) using *E. coli* LE392 as plating bacteria. After overnight incubation of the plates at 37°C two replicas of each plate were made on HybondN* filters (Amersham) as described in Maniatis et al. (1982). After wetting the filters in 3xSSC the filters were washed for 60 min. at room temperature in 3xSSC. Hybridisation using the 32P-labelled 4 kb EcoRI fragment, prepared as described by Sambrook et al., 1989, was done according the following procedure (Sambrook et al., 1989); prehybridisation in 6 x SSC (20xSSC per 1000 ml : 175.3 g NaCl,
107.1 g sodium citrate.5.5 H 2 O, pH 7.0), 0.1% SDS, 0.05% sodium pyrophosphate. 5° Denhardt's solution (100x Denhardt's solution per 500 ml: 10 g Ficoll-400, 10 g polyvinylpyrrolidone, 10 g Bovine Serum Albumin (Pentax Fraction V) and 20 μg/ml denatured herring sperm DNA at 68°C for 3-5 hrs and hybridisation in an identical buffer which contained the denatured radiolabelled probe at 68°C for 15-18 hrs, followed by two washes in 2 x SSC, 0.1 % SDS at 68°C and two washes in 0.2 x SSC, 0.1 % SDS at 68°C. The membrane was covered with Saran wrap and autoradiographed overnight at -70°C using Konica X-ray films and Kodak X-Omatic cassettes with regular intensifying screens.

This screening resulted in about 12 positive phages, of which eight were purified. Each positive plaque was picked from the plate using a Pasteur pipette and the phages were eluted from the agar plug in 1 ml of SM buffer containing 20 μl chloroform, as described in Maniatis et al. (1982). The phages obtained were purified by repeating the procedure described above using filter replicas from plates containing 50-100 plaques of the isolated phages.

After purification the phages were propagated by plating 5x10^3 phages on NZYCM medium. After overnight incubation at 37°C confluent plates were obtained, from which the phages were eluted by adding 5 ml SM buffer and storing the plate for 2 h. at 4°C with intermittent shaking.

After collection of the supernatant using a pipette, the bacteria were removed from the solution by centrifugation at 4,000 x g for 10 min. at 4°C. To the supernatant 0.3% chloroform was added and the number of pfu is determined. These phage stocks (A-R/H-R) contain approximately 10^9 pfu/ml.

DNA of five selected phages, A-R,B-R,C-R,E-R,F-R, isolated as described in Sambrook et al. (1989), was analysed by Southern analysis. The DNA was digested for 5 h. at 37°C in a reaction mixture composed of the following solutions; 5 μl (~ 1 μg) DNA solution; 2 μl of the appropriate 10 x React buffer (Life Technologies); 10 U Restriction enzyme (Life Technologies) and sterile distilled water to give a final volume of 20 μl. The samples were incubated for 10 min. at 65°C and rapidly cooled on ice, before loading on a 0.6% agarose gel in 1*TAE buffer. The DNA fragments were separated by electrophoresis at 25 V for 15-18 h.

After electrophoresis the DNA was transferred and denatured by alkaline vacuum blotting (VacuGene XL, Pharmacia LKB) to nylon membrane (Hybond N, Amersham) as described in the VacuGene XL instruction manual.
and subsequently prehybridised and hybridised using the denatured radiolabelled 5 kb BamHI-XbaI fragment of plasmid pNP8 with hybridisation conditions as described. The hybridisation pattern was obtained by exposure of Kodak XAR-5 X-ray film for 18 h. at -70°C using a regular intensifying screen. In all 5 clones, fragments originating from the same genomic region were found, for which a restriction pattern was constructed.

Based on the restriction map a 5 kb BamHI-XbaI fragment was selected for subcloning. 100 ng pBluescript BamHI-XbaI digested vector was mixed with 250 ng 5 kb BamHI-XbaI DNA of phage B-R and 4 ul 5 ul ligation buffer (composition; 500 mM Tris-HCl, pH 7.6; 100 mM MgCl2; 10 mM ATP; 10 mM dithiotreitol; 25% PEG-6000), and 1 ul (1.2 U/ul) T4 DNA ligase (Life Technologies) was added to this mixture in a final volume of 20 ul. After incubation for 16 h at 14°C the mixture was diluted to 100 ul with sterile water. 10 ul of the diluted mixture was used to transform E. coli DH5a competent cells, prepared as described by Sambrook et al. (1989). Six of the resulting colonies were grown overnight in LB medium (LB medium per 1000 ml: 10 g trypticase peptone (BBL). 5 g yeast extract (BBL), 10 g NaCl, 0.5 mM Tris-HCl pH 7.5) containing 100 µg/ml ampicillin. From the cultures plasmid DNA was isolated by the boiling lysis method as described by Maniatis et al. (1982), which was used in restriction analysis to select a clone harbouring the desired plasmid pIM230. Plasmid DNA was isolated on a large scale from 500 ml cultures E. coli DH5a containing pIM230 grown in LB medium containing 100 µg/ml ampicillin (Maniatis et al., 1982 ) The plasmid was purified by CsCl centrifugation. ethanol precipitated and dissolved in 400 µl TE. The yield was approximately 500 µg. E. coli containing pIM230 was deposited at the CBS under the conditions of the Treaty of Budapest on June 1996 under the accession number CBS 678.96

Example 6.3:

Subcloning of the \textit{A. niger xlnR} cDNA

To obtain a cDNA clone of part of the zinc finger region of the \textit{zlnR} gene, a reverse transcriptase and second strand synthesis reaction were carried out on 1 µg of polyA⁺ RNA from an \textit{A. niger} N402 wild-type strain grown on xylan for 30 hrs with an oligonucleotide starting at position 1476-1496 (Seq id no 9), in analogy to the method as described in the ZAP⁺-cDNA synthesis kit (Stratagene). An aliquot (1/50) of the second strand reaction was used as template in PCR with primer R026 and R025 (derived from positions 946-970 of Seq id no 9) in 35 cycles of 60 seconds of
subsequent 95°C, 58°C and 72°C, followed by an incubation of 5 minutes at 72°C. The resulting fragment of 500 bp was subcloned in the pGEM-T vector (Promega) and sequenced.

Example 7:

The primary structure of the zlnR gene

Example 7.1:

Sequence analysis of the zlnR gene

The sequence of the \textit{A. niger} zlnR gene, its promoter/regulation region, the structural part of the gene and the termination region, was determined by subcloning fragments from both pNP8 as pIM230, in combination with the use of specific oligonucleotides as primers in the sequencing reactions.

For nucleotide sequence analysis restriction fragments were isolated and were then cloned in pEMBL, pUC, pBluescript, pGEM DNA vectors, digested with the appropriate restriction enzymes. The nucleotide sequences were determined by the dideoxynucleotide chain-termination procedure (Sanger et al., 1977) using the Pharmacia ALF express automated sequencer and the Thermosequenase sequencing kit (Amersham). In the case of gene specific oligonucleotides the Pharmacia Cy5 internal labelling kit was used in combination with the T7 DNA polymerase sequencing kit (Pharmacia). The reactions and the electrophoresis was performed according to the manufacturers' instructions. Computer analysis was done using the PC/GENE programme (Intelligenetics). The sequence determined is given in SEQ ID NO:9.

Example 7.2:

Description of the zlnR gene

The sequence as given in SEQ ID NO:9, comprising the zlnR structural gene, is preceded by a 947 nucleotide long upstream region. In the upstream non-coding region CT-enriched sequences are found but no TATAA box. The structural part of the zlnR gene ranges from position 948 till position 3690, interrupted by two introns. The intron at position 1174 till 1237 was certified by sequencing the genomic fragment in pIM230, described in example 6.2 and part of the cDNA, as described in example 6.3. A second intron is indicated from position 3496 till position 3549. The second intron sequences follow the conserved intron
sequences, normally found in fungi, for splice junctions and lariat sequence.

The zlnR gene encodes a protein of 875 amino acids in length. The derived polypeptide contains a typical N-terminal zinc binuclear cluster domain encoded by nucleotides from position 1110 till position 1260, with typical six cysteines coordinating the zinc. In this region furthermore a number of similarities with other fungal regulatory proteins are shown as listed in e.g. Fig. 1 of Kraulis et al. (1992) which is herein incorporated by reference.

A typical RRRLWW motif is found from position 2623 till position 2649, this motif is found, with slight variations, in a number of binuclear zinc cluster regulatory proteins as noted by Suárez et al. (1995).

Example 7.3:

Sequence analysis of zlnR in A. niger mutants

To determine whether the mutation, in the case of the A. niger mutants which do not express the xylanolytic system, as described in example 4.2, is located in zlnR, the sequence of the zlnR gene of these mutants was determined. For this a library enriched for 5.5 kb BamHI zlnR containing fragments was made for each of the NW205::130 Ac mutants. For the construction of this zlnR enriched library, 5.5 kb fragments of BamHI, HincIII, XhoI, SstI and KpnI digested genomic DNAs were isolated for each strain. These fragments were mixed with BamHI digested, dephosphorylated pUC18 vector (Ready-To-Go™ pUC18 BamHI/BAP + Ligase, Pharmacia) and ligated. The ligation mixture was used to transform E.coli DH5α (MAX Efficiency DH5α™ Competent Cells, Gibco-BRL) for Amp resistance according to the manufacturers’ protocol, which resulted in a primary library of 5*10^2-10^3 colonies. These A. niger zlnR enriched libraries, after replating the primary library on master plates, were screened by colony filter hybridisation according to standard protocol (Sambrook et al., 1989), with the use of the denatured radiolabelled BamHI-XhoI insert of pIM230 as a probe.

For each mutant strain, positive colonies were picked from the master plate with a toothpick and were grown for 15-18 hrs at 37°C in 5 ml LB medium containing 100 µg/ml Ampicillin, after which a miniprep plasmid DNA isolation was performed as described for boiling lysis by Sambrook et al. (1989). Analysis of these DNA preparations by agarose electrophoresis and comparing digestion patterns with zlnR specific patterns revealed
colonies containing the correct 5.5 kb BamHI zlnR fragment.
The sequence of the A. niger mutants was determined with the use of
specific zlnR oligonucleotides as primers in the sequencing reactions as
described in example 7.2. For mutant NW205::130 Ac 1-15 a single basepair
substitution was determined at position 3479, resulting in the change of
the Leucine at position 823 of SEQ ID NO:9 into a Serine. For mutant
NW205::130 Ac 2-5 a single basepair substitution was determined at
position 3655, resulting in the change of the Tyrosine at position 864 of
SEQ ID NO:9 into an Aspartic acid. These mutations identify both mutants
as zlnR mutants.

Example 8:
Expression of zlnR in A. niger

Example 8.1:
Complementation of A. niger mutants non-expressing the
xylanolytic system

For the complementation in all non-expressing mutants all ten
NW205::130 Ac mutants, as described in Example 4, were transformed as
described in example 2 of this document by combining protoplasts and
pGW635 as a control for transformation frequency and pIM230 DNA for
testing the complementation ability of pIM230.
The resulting transformant colonies were analysed for xylanolytic
activity and compared with their parental mutant strain, by plating them
on appropriate medium containing 1% oat spelts xylan as C source, and
after 2-3 days a clearing of the xylan around the transformant colonies,
but not the parental mutant strain, appeared for all ten, thereby showing
the restoration of xylanolytic activity.

Example 9:
Effect of zlnR gene dosage on the expression of the A. niger
xylanolytic system

To study the potential use of the zlnR gene for strain
improvement for an increased xylanolytic expression, the strain
N902::200-18, harbouring multiple copies (about 6) of the A. niger zlnD
gene encoding β-xylosidase, was transformed to arginine prototrophy in a
co-transformation experiment, as described in Example 2 of this document
using 19 μg of the zlnR harbouring plasmid pIM230 and 2μg of the plasmid
pIM650 harbouring the A. nidulans argB gene (Johnstone et al., 1985). The
transformants obtained were screened for increased endo-xylanase
expression, on MM plates containing 1% oat spelts xylan. Four colonies, having the fastest and largest halo formation, were selected to determine zlnR copy numbers. For this DNA of these transformants and the recipient strain, was isolated and serial dilutions were spotted onto Hybond N membrane. The copy number was estimated from the signals found after hybridisation, using a radiolabelled 4.5 kb SmaI/XbaI fragment spanning the coding sequence of the zlnR gene. Based on comparison to the recipient strain the zlnR copy number was determined to be 8 in N902::200-18-R14 and 32 in N902::200-18-R16. For both these transformants the effect of the increased gene dosage of zlnR was analysed by Northern analysis after strains were grown in liquid culture. This was done in a transfer experiment into 2% oat spelts xylan as a carbon source, after a preculture in 1% fructose for 18 h. Mycelial samples were taken 8 and 24 hrs after transfer, from which total RNA was isolated using TriZol (Life technologies) according to the manufacturers instructions and analysed by Northern blot analysis (Sambrook et al., 1989). Xylanase B expression levels were strongly increased in these transformants in comparison to the recipient strain, as detected after hybridisation using the radiolabelled 1 kb EcoRI/XhoI fragment of A. niger zlnB (Kinoshita et al., 1995).

To further study the potential use of the zlnR gene for strain improvement for an increased endo-xylanase expression, A. niger was transformed, as described in Example 2 of this document according to the following scheme; 1. pGW635 (pyrA) (positive control), 2. pDB-K(XA), 3 pDB-K(XA) + pIM230) and 4 pGW635 + pIM230. The plasmid pDB-K(XA) contains both the A. niger pyrA gene and the A. niger xylanase A gene from A. tubigensis in the vector pEMBL18. Transformants were obtained for all conditions used, strains overexpressing endo-xylanases were selected by halo size in a plate screening on oat spelts xylan (20 transformants from each group were tested).

From each group one transformant was selected and grown for activity assays. The strains were pregrown for 18 hrs on medium containing fructose, after which the mycelium (2.5 g wet weight in 50 ml) was transferred to medium containing 1.4% crude arabino xylan. All cultures were performed in shake flasks. Incubations were done for 40 hrs at 30°C and the xylanase A levels were determined by HPLC analysis, while β-xylanosidase and endo-xylanase activities were determined for both. Chromatography was carried out on standard Pharmacia-LKB HPLC equipment (Uppsala, Sweden) running a SOURCE 15 Q, HR 5/5-column at 1.5 ml/min.
Buffer A = 20 mM TRIS-buffer, pH 7.5 and buffer B = 20 mM TRIS-buffer, pH 7.5 with 1M NaCl. Gradient from 0-50% buffer B over 30 min. Detection at 280 nm. Pharmacia-LKB UV-MII. absorbance range at 0.1-0.2, see figure 3. 100 µl culture media was diluted with 1000 µl 20 mM TRIS-buffer, pH 7.5 and 1000 µl diluted sample was applied to the column. The xylanase A activity is then seen as a peak eluting at approx. 30% B-buffer. From each group of transformants the one showing the highest xylanase A activity/peak in the HPLC analysis is shown in figure 3.

The endo-xylanase activity was determined by measuring the release of dyed fragments from azurine-dyed cross-linked wheat arabinoxylan (Xylazyme tablets) from Megazyme, Warriewood, Australia. The xylanase activity was calculated by comparing with an internal standard enzyme of 100 XU/gram (Xylanase-Unit) at 40°C in 0.1 M acetate buffer, pH 3.4. The same four transformants were assayed on water insoluble arabinoxylan at a pH were only xylanase A contributes to the endoxylanase activity, the results of this analysis is given in table 2.

<table>
<thead>
<tr>
<th>Transformant</th>
<th>XU/gram</th>
</tr>
</thead>
<tbody>
<tr>
<td>pyr+ (control)</td>
<td>4</td>
</tr>
<tr>
<td>DB-K(XA)-15</td>
<td>52</td>
</tr>
<tr>
<td>DB-K(XA)/pIM230-21</td>
<td>111</td>
</tr>
<tr>
<td>pIM230-26</td>
<td>24</td>
</tr>
</tbody>
</table>

From the results shown in figure 3 and table 2 it is clear that transformation with the xylanase A encoding gene as expected gives a large increase in the xylanase A enzyme activity. More surprisingly, also after transformation using the activator gene xlnR, also a large increase in the level of xylanase A is found. This indicates a limitation in the level of activator XYL R (= xlnR gene product) in the untransformed parent. Therefore, it is expected that in a pDB-K(XA) multicopy transformant the amount of transacting regulatory factor will be even more limiting. This is confirmed by the result for the pDB-K(XA)/pIM230 transformant, which has a xylanase A level twice as high as the pDB-K(XA) multicopy transformant.

Example 10: Screening filamentous fungi for the zlnR gene
To analyse whether it is possible to isolate the zlnR counterpart from other fungi by heterologous hybridisation, using the 4.5 kb SmaI/XbaI fragment of the zlnR gene as a probe. DNA was isolated from the following strains: A. niger N902 (argB15, cspA1, fumA1, metB10, pyrA5), A. tubingensis NW184 (cspA1, fumA1, pyrA22), A. nidulans WGO96 (pabA1, yA2) of FGSC 187, A. aculeatus NW240 (pyrA3) of CBS 101.43, A. aculeatus NW217 (fumA1, cspA1, pyrA4, lysA1) of CBS 115.80, A. foetidus (awamori) NW183 (cspA1, fumA1, pyrA13, lysA1) of CBS 115.52, A. japonicus CBS114.51 and Trichoderma reesei QM9414. 1-2 µg DNA was digested with BamHI or with XhoI and subsequently analysed by Southern analysis as described in Example 3. The hybridisation conditions used were:

hybridisation in 6 x SSC (20xSSC per 1000 ml : 175.3 g NaCl, 107.1 g sodium citrate.5.5H2O, pH 7.0), 0.1% SDS, 0.05% sodium pyrophosphate, 5° Denhardt's solution (100 x Denhardt's solution per 500 ml : 10 g Ficoll-400, 10 g polyvinylpyrrolidone, 10 g Bovine Serum Albumin (Pentax Fraction V) and 20 µg/ml denatured herring sperm DNA at 56°C for 18-24 hrs followed by two 30 min. washes in 5 x SSC, 0.1% SDS at 68°C and two 30 min. washes in 2 x SSC, 0.1% SSC at 56°C. After hybridisation the membrane was covered with Saran wrap and autoradiographed overnight at -70°C using Konica X-ray films and Kodak X-Omatic cassettes with regular intensifying screens.

As a result hybridising fragments were found for all fungi analysed, very strong hybridisation signals were found in A. niger, A. tubingensis, A. foetidus, while in the other strains investigated clear hybridisation signals were found.

Example 11: Application of the selection system using other promoter fragments

Plasmids were constructed containing promoter fragments from the A. niger abfA gene (Flipphi et al., 1994) and the A. niger abfB gene (Flipphi et al., 1993). For the construction containing the abfA promoter fragment, a 1.4 kb XhoI/PstI fragment from pIM900 (Flipphi et al., 1994) was ligated in SaI/PstI digested pAlter (Promega), as described in Example 1. Using the Altered Sites II in vitro mutagenesis system (Promega) a XhoI restriction site was created at positions -83 till -88 relative to the translation initiation site (Flipphi et al., 1994). From the resulting plasmid a 953 bp SstI/XhoI fragment was isolated. This fragment and a 1.5 kb XhoI fragment from pIM130 were ligated into pBluescript (Stratagene) digested with SstI/XhoI. Plasmids containing the
correct orientation of the 1.5 XhoI fragment were identified by a digestion using BglII. The resulting plasmid is pAP8.

Analogously a 910 bp PstI fragment from the abfB promoter was isolated from pIM991 (Flipphi et al., 1993) and ligated in PstI digested pEMBL19. The resulting plasmid was digested using Sall and was ligated with the 1.5 kb XhoI fragment from plasmid pIM130. Plasmids containing both fragments in the correct orientation were identified by a digestion using BamHI. The resulting plasmid is pIM132.

A third plasmid containing a fragment from the A.niger pgaII was constructed by cloning a 1145 bp XbaI/XhoI fragment into the vector pAlter. Using the Altered Sites II in vitro mutagenesis system (Promega) a XhoI restriction site was created at positions -107 till -112 relative to the translation initiation site (Bussink et al., 1991). From the resulting plasmid a 1.2 kb XbaI/XhoI fragment was isolated. This fragment and a 1.5 kb XhoI fragment from pIM130 were ligated into pBluescript (Stratagene) digested with XbaI/XhoI. Plasmids containing the correct orientation of the 1.5 XhoI fragment were identified by a digestion using HinDIII. The resulting plasmid is pIIP7. From the same pgaII gene a 223 bp HinDIII/PstI fragment (Bussink et al., 1992) was isolated and ligated in HinDIII/PstI digested pEMBL19. The resulting plasmid was digested using Sall and was ligated with the 1.5 kb XhoI fragment from plasmid pIM130. Plasmids containing both fragments in the correct orientation were identified by a digestion using HinDIII. The resulting plasmid is pHPII.

All four plasmids were introduced in A.niger NW219 by transformation as described in Example 2, using 10 mM L-arabitol as an inducer in transformation experiments using the constructs having the abf promoter fragments (plasmids AP8 and pIM132) and 1% polygalacturonic acid (USB chemicals) in the case of the plasmids harbouring the pgaII fragments. While for the plasmids AP8 and pIM132 transformants were found at high frequency, for the transformation experiment using the pgaII promoter fragment containing plasmids pIIP7 and pHPII only 5 and 7 transformants were found respectively.

The transformants resulting from the transformation experiment using the plasmids pAP8 and pIM132 (abf promoter fragments were analysed phenotypically as described in Example 3 using MM containing 10 mM L-arabitol/50 mM sorbitol, 10 mM L-arabitol/50 mM D-glucose and 50 mM D-glucose. The expected phenotype: growth on 10 mM L-arabitol/50 mM sorbitol, non-growth on 10 mM L-arabitol/50 mM D-glucose and 50 mM D-...
glucose. was found for 10 out of 29 transformants resulting from plasmid pAP8 and 13 out of 30 transformants resulting from plasmid pIM132. The transformants resulting the plasmids pIIP7 and pHPII were tested on MM containing 1% polygalacturonic acid, 1% polygalacturonic acid/50 mM D-glucose and 50 mM D-glucose. Both classes of transformants however, did not show the expected phenotype, all transformants were able to grow on all three media. This suggests that these transformants result from a double cross-over at the pyrA locus.

Based on the results for the pgall promoter fragment containing plasmids, we have tested whether we could improve transformation frequency by improving induction. We assumed that transformation more or less failed due to lack of inducer, since no monomeric inducer for polygalacturonases was available and thus the polymer needs to be degraded to release inducer to give expression. We tested whether supplementation of the medium using a small amount of uridin could overcome this problem. For this the NW219 and a transformant NW219::pIM132#30 were tested on media containing 1% oat spelts xylan, giving induction of abfB, in combination with an increasing amount of uridin; respectively 0, 0.001, 0.005, 0.01, 0.1, 1, 5 and 10 mM. In this experiment the recipient strain NW219 did not grow on media containing less then 0.01 mM uridin, while the NW219::pIM132#30 transformant strain grew under all conditions used. However, the degree of sporulation and colony morphology varied. the lowest uridin concentration giving wild-type-like sporulation being around 0.01 mM uridin.

Based on these results A.niger NW219 transformation using the plasmids pIIP7 and pHPII was repeated using MMS as described in Example 2 containing 1% lemon pectin (degree of esterification 45%) (Copenhagen Pectin factory) and two conditions for uridin supplementation 0.01 mM and 0.005 mM respectively. This resulted in an increased number of transformants found. These transformants were tested on media containing 1% lemon pectin as described above, 1% lemon pectin/50 mM D-glucose and 50 mM D-glucose, for which the expected phenotype is respectively growth, non-growth and non-growth. For the pIIP7 resulting transformants 10 out of 30 transformants and for the pHPII 9 out 30 transformants the expected phenotype was found.

A selection of the transformants showing the expected phenotype were analysed by Southern analysis. DNA was isolated and analysed as described in Example 3. For the transformants resulting from the abfA promoter fragment containing plasmid pAP8 the DNA was digested using
Clal, for pIM132 (abfB) resulting transformants using Clal and for the 
transformants resulting from pgall plasmids pIIP7 and pHPII using Clal.
Based on the autoradiograph obtained after hybridisation using the 
following radiolabelled fragments; the 3.8 kb XbaI and the 1.2 kb Clal 
fragment of the pyrA gene, transformants were selected based on estimated 
copy number. The following transformants were selected: AP8/16 (abfA),
NW219::132#8 (2 copies) and NW219::132#30 (3-4 copies)(abfB),
NW219::pIIP7#3 (2 copies) and NW219::pHPII#9 (2 copies)(pgall)
The selected transformants were subjected to mutagenesis as 
described in Example 4. Arabinofuranosidase derepressed mutants were 
selected on MM + 50 mM D-glucose and on MM + 10 mM L-arabitol/50 mM D-
glucose, while polygalacturonase derepressed mutants were selected on MM 
+ 50 mM D-glucose and on MM + 1% lemon pectin/50 mM D-glucose. After 4-7 
days 5-10 mutant colonies per plate were found, which on basis of their 
morphology could be divided into three classes; large, well sporulating 
colonies, intermediate sized, well sporulating colonies and small poorly 
sporulating colonies. A random selection of 20 of these mutants was made 
and the selected mutants were tested on media containing different carbon 
sources or substrates. The arabinofuranosidase mutant colonies were found 
to be able to grow on media containing D-glucose, D-glucose/L-arabitol 
and L-arabitol, while the parental strains only were able to grow on 
medium containing L-arabitol as a carbon source. Analogously the 
polygalacturonase mutants also were able to grow on MM + 50 mM D-glucose 
and on MM + 1% lemon pectin/50 mM D-glucose and MM + 1% lemon pectin. The 
parental strains could only grow on MM + 1% lemon pectin. In addition the 
arabinofuranosidase mutants (20 of each) were tested on the chromogenic 
substrate methylumbelliferyl-α-L-arabinofuranoside as described in 
Example 4. While the parental strains did not show any 
arabinofuranosidase expression of D-glucose/L-arabitol containing media, 
as detected by fluorescence of the substrate, the mutants showed variable 
levels of expression, the highest levels were found in mutants selected 
on the D-glucose medium.
The polygalacturonase mutants were tested on MM + 1% lemon pectin/50 mM 
glucose, two and three days after inoculation of the plates 
polygalacturonase activity was visualized by staining as described by 
Ried and Collmer (1985). In this case for the parental strain a small 
halo was found, while in the mutants various degrees on increased halo 
formation was detected.

According to Example 4 also non-expressing mutants were
selected on media containing FOA. For this strains NW219::132N30(abfB) were mutated and plated on MM + 10 mM L-arabitol + 1 mg/ml FOA + 10 mM uridin. After 7-10 days mutants were selected and plated to MM containing 10 mM L-arabitol/50 mM sorbitol, 10 mM uridin and having a top agar containing 0.5% AZCL-arabinan (Megazyme, Sydney, Australia) for the detection of endo-arabinan expression. Two of the 30 transformants tested, 132/30 F12 and F26, were not able to release the dye from the substrate, an indication for the absence of endo-arabinan activity. Upon cultivation of these transformants in liquid MM containing 10 mM L-arabitol and 10 mM uridin and subsequent measurement of arabinofuranosidase activity in the culture filtrates, an at least 4-fold decrease in activity was found.

The strain NW219::pIP7#3 was mutated and plated on MM containing 1% lemon pectin/50 mM sorbitol, 10 mM uridin and 1 mg/ml FOA. After incubation of the plates for 6-10 days mutants were picked and screened for polygalacturonase activity as described above. Of 65 mutants selected three mutants were found to have a decreased haloformation after polygalacturonase activity staining, indicating a decrease in polygalacturonase expression.

Examples 7, 8 and 11 of EP 95201707.7, which is a copending European patent application of which a copy has been included upon filing the subject document and which examples have also been copied into this document.

Example 7 of EP 95201707.7: Transformation of A. niger using the plasmid pIM200

250 ml of culture medium, which consists of MM supplemented with 2% glucose, 0.5% Yeast Extract, 0.2% Casamino acids (Vitamin free), 2 mM leucine, 10 μM nicotinamide, 10 mM uridine, was inoculated with 1 * 10^6 spores per ml of strain NW155 (cspA1, argB13, pyrA6, ntcA1, leuA1, prtP28) (derived from NW228. Van den Hombergh et al, 1995) and mycelium was grown for 16 - 18 hours at 30 °C and 250 rpm in an orbital New Brunswick shaker. The mycelium was harvested on Myracloth (nylon gauze) using a Büchner funnel and mild suction and was washed several times with SP6 (SP6: 0.8% NaCl, 10 mM Na-phosphate buffer pH 6.0). 150 mg Novozyme 234 was dissolved in 20 ml SMC (SMC: 1.33 M sorbitol, 50 mM CaCl₂, 20 mM MES buffer, pH 5.8) to which 1 g (wet weight) mycelium was added and which was carefully resuspended. This suspension was incubated
gently shaking for 1 - 2 hours at 30 °C, every 30 minutes the mycelium was carefully resuspended and a sample was taken to monitor protoplast formation using a haemocytometer to count the protoplasts. When sufficient protoplasts were present (more than $1 \times 10^8$) these were carefully resuspended and the mycelial debris was removed by filtration over a sterile glasswool plug. The protoplasts were collected by 10 minutes centrifugation at 3000 rpm and 4 °C in a bench centrifuge, the supernatant was removed and the pellet was carefully resuspended in 5 ml STC (STC: 1.33 M Sorbitol, 50 mM CaCl$_2$, 10 mM Tris/HCl, pH 7.5). This wash step was repeated twice and the protoplasts were finally resuspended in STC at a density of $1 \times 10^8$ per ml.

The transformation was performed by adding 20 μg of pIM200 DNA and 5 μg pGW635, containing the A. niger pVrA gene (dissolved in a 10 - 20 μl TE), to 200 μl of protoplast suspension together with 50 μl of PEG buffer (PEG Buffer: 25% PEG-6000, 50 mM CaCl$_2$, 10 mM Tris/HCl pH 7.2), mixed gently by pipetting up and down a few times, and incubated at room temperature for 20 minutes. After this period 2 ml PEG buffer was added, the solution was mixed gently and incubated at room temperature for another 5 minutes and subsequently 4 ml of STC was added and mixed gently on a vortex mixer. One ml portions of this suspension were then added to 4 ml of 0.95 M sucrose osmotically stabilised top agar and poured on osmotically stabilised plates. As a control A. niger was also transformed using pGW635.

Example 8 of EP 95201707.7: Analysis of transformants

The transformants from pIM200 obtained in Example 7 were analysed phenotypically by plating on MM containing 1% Oat spelt xylan and 1 mM 4-methylumbelliferyl-β-D-xyloside. Of the 26 transformants tested, five had an increased fluorescence. These transformants, together with a PYR+ transformant as a reference, were grown on MM containing 1% Oat spelt xylan for 20, 27 and 42 hrs, after which the β-xylosidase activity towards PNP-X was measured. The results are summarised in Table C.

An increased level of β-xylosidase activity was found in all five transformants selected, the highest level being more than 30 times the wild-type activity. These results were confirmed by Western blot analysis, using the anti β-xylosidase antibody, prepared as described in Example 3 of the EP 95201707.7, and the Bio-Rad Immun-blot GAR-AP assay kit following the suppliers instructions.
Table C

β-xylosidase activities in *A. niger* transformants
activity (mU/ml culture filtrate) after:

<table>
<thead>
<tr>
<th></th>
<th>20 hr</th>
<th>27 hr</th>
<th>42 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>pgW 635</td>
<td>15</td>
<td>16</td>
<td>17</td>
</tr>
<tr>
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<td>82</td>
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<td>74</td>
</tr>
<tr>
<td>XIS A12</td>
<td>96</td>
<td>295</td>
<td>527</td>
</tr>
</tbody>
</table>

Example 11 of EP 95201707.7: Disruption of the *A. niger* zlnD gene

Example 11.1: Construction of the disruption plasmids pIM203 and pIM204

The gene disruption plasmids pIM203 and pIM204 were constructed by generating an internal fragment of the zlnD gene by PCR. The fragment was generated using the oligonucleotides derived from the zlnD sequence (SEQ ID NO: 8). Xylos001 was derived from positions 1157 till 1176 and xylos004 was derived from positions 3147 till 3164. The fragment was generated by PCR containing 10 µl 10× reaction buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl, 15 mM MgCl2, 0.01% gelatine), 16 µl 1.25 mM of each of the four deoxynucleotide triphosphates, 1 ng of the plasmid pIM200 DNA and 1 µg of each of the oligonucleotides in a final volume of 100 µl. This reaction mixture was mixed and 1 µl TAQ polymerase (5 U/µl) (Life Technologies) was added. The DNA was denatured by incubation for 3 min at 92°C followed by 25 cycli of 1 min 92°C, 1.5 min 52°C and 1.5 min 72°C. After these 25 cycli the mixture was incubated for 5 min at 72°C.

Analysis of the reaction products by agarose electrophoresis revealed a fragment of about 2000 bp, which corresponds to the size expected, based on the sequence of the gene. The resulting fragment was subcloned in the vector pGEM-T (Promega) resulting in the plasmid pIM202. Plasmid pIM203 was constructed by ligation of a Smal/PstI fragment of pILJ16 (Johnstone et al., 1985), containing the *A. nidulans* *argB* gene (Upshall et al., 1986), in the EcoRV/PstI digested pIM202 vector. Plasmid pIM204 was constructed by ligation of the NsiI/XbaI fragment of pIM130 (this document, EP 95202346.3), containing the *pyrA* gene under the control of the UAS of the zlnA promoter of *A. tubigensis*, in the Spel/NsiI digested pIM202 vector.

Example 11.2: Disruption of the zlnD gene in *A. niger*
The plasmids containing the \( \text{xlnD} \) internal fragment as well as the 
\( \text{argB} \) gene (pIM203) or the \( \text{pyrA} \) gene (pIM204), as described in Example 11.1 of the copending EP application, as a selection marker in 
transformation, were used to disrupt the \( \text{A. niger} \) \( \text{xlnD} \) gene. For this \( \text{A. niger N902} \) (\( \text{argB}15, \text{cshA1}, \text{fumA1}, \text{metB10}, \text{pyrA5} \)) was transformed, as 
described in Example 2 of this document, using the plasmids pIM203 and 
pIM204 selecting for arginine or uridine prototrophy respectively. The 
resulting transformants were screened for activity on methylumbelliferyl-
\( \beta \)-D-xyloside on a 1 % xylan plate as described in Example 8 of the 
copending EP application. For both groups of transformants twenty were 
screened. Of these transformants one of each group had a severe decreased 
level of MUX activity after 24 h of growth. Southern analysis of the 
selected transformants, as described in Example 3 of the copending 
application, demonstrated for the pIM203 transformant a multicopy 
integration at the homologous \( \text{xlnD} \) locus. In case of the pIM204 
transformant a single homologous integration at the \( \text{xlnD} \) locus had 
ocurred. Analysis for PNP-X activity, as described in Example 8 of the 
copending EP application, of these transformants revealed an at least 
100-fold decrease in \( \beta \)-xylosidase activity.

Example 11.3: Effect of overexpression and inactivation of \( \text{xlnD} \) gene on 
the expression of xylanolytic system of \( \text{A. niger} \).

To determine the effect of \( \text{xlnD} \) expression on the expression of 
the xylanolytic spectrum, \( \text{A. niger N902} \), two \( \text{xlnD} \) multicopy-transformants 
in N902 and the \( \text{xlnD} \) gene disruption strains were grown in liquid 
culture. This was done in a transfer experiment into 2% oat spelt xylan 
or 3 % D-xylose as a carbon source, after a preculture in 1 % fructose 
for 18 h. Beta-xylosidase activity was determined as PNP-X activity in 
the culture filtrate. With both C sources a clear overexpression could be 
seen for the pIM200 transformants against an almost absence of PNP-X 
activity for both (pIM203 and pIM204) inactivation transformants. The 
\( \text{xlnD} \) gene disruption transformants showed an initial decreased level of 
endo-xylanase expression, which however increased in time finally after 
16 hrs resulting in increased activity levels in comparison to the \( \text{A. niger} \) 
wild-type. Thus resulting in xylanase preparations free of \( \beta \)-
xylosidase.

The culture filtrates were subsequently analysed by HPLC analysis, 
using a Dionex system and Pulsed Amperometric Detection. For this 1 ml of 
culture filtrate was boiled immediately after harvesting, to inactivate
the xylanolytic enzymes, after which the sample was centrifuged for 10 min. (14,000 rpm at 4°C, Eppendorf centrifuge). The resulting supernatant was diluted 5-fold in bidest and 20 μl was analysed by HPLC using a Dionex CarboPac 100 column. The analysis indicated that, while in the wild-type and in the over-expression transformants only in the initial stage xylose oligomers could be detected in the culture filtrate, in the disruption mutant xylobiose and to a lesser extent xylotriose accumulated in the culture filtrate, thus resulting in a source for xylooligomers, in particular xylobiose and xylotriose.

The entire disclosure in the complete specification of our Australian Patent Application No. 62443/96 is by this cross-reference incorporated into the present specification.
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   (C) CITY: Wageningen
   (E) COUNTRY: The Netherlands
   (F) POSTAL CODE (ZIP): 6701 BH

(ii) TITLE OF INVENTION: A novel method to isolate mutants and to clone the complementing gene

(iii) NUMBER OF SEQUENCES: 9

(iv) COMPUTER READABLE FORM:
   (A) MEDIUM TYPE: Floppy disk
   (B) COMPUTER: IBM PC compatible
   (C) OPERATING SYSTEM: PC-DOS/MS-DOS
   (D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
   (A) LENGTH: 30 base pairs
   (B) TYPE: nucleic acid
   (C) STRANDEDNESS: single
   (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

CACAATGCAT CCCCTTTATC CGCCTGCCGT

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
   (A) LENGTH: 37 base pairs
   (B) TYPE: nucleic acid
   (C) STRANDEDNESS: single
   (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

CAATTGCGAC TTGGAGGACA TGATGGGCAG ATGAGGG

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
   (A) LENGTH: 20 base pairs
   (B) TYPE: nucleic acid
   (C) STRANDEDNESS: single
   (D) TOPOLOGY: linear
(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:
AGAGAGGATA TCGATGTGGG

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 37 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:
CCCTCATCTG CCCATCATGT CCTCCAAGTC GCAATTG

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2054 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Aspergillus tubigensis

(ix) FEATURE:
(A) NAME/KEY: TATA signal
(B) LOCATION: 848..854

(ix) FEATURE:
(A) NAME/KEY: exon
(B) LOCATION: 950..1179

(ix) FEATURE:
(A) NAME/KEY: intron
(B) LOCATION: 1179..1228

(ix) FEATURE:
(A) NAME/KEY: exon
(B) LOCATION: 1229..1631

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: join(950..1179, 1229..1631)
(D) OTHER INFORMATION: /EC_number= 3.2.1.8.
/product= "1,4-beta-xylanxylanohydrolase"
/gene= "xlnA"
/standard_name= "endo-xylanase"

(ix) FEATURE:
(A) NAME/KEY: mat_peptide
(B) LOCATION: 1031..1631

(ix) FEATURE:
(A) NAME/KEY: sig_peptide
(B) LOCATION: 950..1031

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

AACGTCTGCA GTCCCTGACT GTTTACCAAA ATGCCAGCCC ACTGTTGQAT ATACAACCTT
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GTAATACGTT GCCGGAGTCG GCCCCTACTC CCTGATGGGT TCCCACTCCC TAGTTACTTC
120

CTACTGGGTA GTAGGCTCTC AGAGTTGGGT AAAGTTTGGC AAGGTTTAGG CCCAGGCCTT
180

GTTTATGCTT GGCTAGGCGG GACCTGGGTA AGTTGATGGC TCCTGGATTC TCACCTGAGT
240

ATTTCCAGCT ATAAAGGAGA TTTGCCATAC TCTTCAGGGA GTCCGGATGG TCCGGCGCGA
300

GGITGACCTT GCCTTCCATC CCTACCAAAA GAACCTCCG GCCAACTCCC GGCAGCCTTC
360

GAGCTCCAAA GTACCTCCGC GACCTGGGCT CAGTGTCTTC CGCAAGGTTT ACTGAGCCTA
420

AGGCTTGGTA CAATAAAATA AGAGACATAA CCTGCAAGTA CATAGGTTTT GTATGAGCCGA
480

GGAACCTGCT TCAGTACTAG ATCAGTTGGT AGAATACAT GAACAATGAT TCTGAGCCAG
540

AAAACCTGCTT GCAGGGAAAC CGTGAAGAAA CCCCACCTCC CGGCTCCAC TAAGGCGGAG
600

CCCTTTATCC GCCGGCCCTC CATTTAGCCA ATATGATCCC ATTTAGCCCA GTCCGGTGCCA
660

TTTAGGCAAG TCCAGTGCTT AGGTTGAGTT CTACACAGGA AAGGGCCATG AATGAGAGCA
720

CAACTATAGA ACTGTCCCTA GAAATAGGCT CGAGTGTGTT AGAGCTTTAA AGGTGATGCG
780

GCAAAATCCA TATGACTGAT TTTGCTCAAC GTCCAGGGGA AAGGGATAAA TAGTCTTTTT
840

CGCAGAAATAT AAATAGAGGT AGAGCGGGCT CGCAGCAATA TGGACCAGGA CAAGGGCTCT
900

TTTCCAGTTG CATAACATCA TTCCACAGAT TCAGCTTTCT TCAATCATC ATG AAG
955

Met Lys

-27

GTC ACT GCG GCT TTT GCA GGT CTT TTG GTC ACG GCA TTC GCC GCT CCT
Val Thr Ala Ala Phe Ala Gly Leu Leu Val Thr Ala Phe Ala Ala Pro

-25 -20 -15 -10

GCC CCA GAA CCT GAT CTG GTG TCG CGA AGT GCC GGT ATC AAC TAC GTG
Ala Pro Glu Pro Asp Leu Val Ser Arg Ser Ala Gly Ile Asn Tyr Val

-5 1 5

GTTATGCTT GGCTAGGCGG GACCTGGGTA AGTTGATGGC TCCTGGATTC TCACCTGAGT
240

ATTTCCAGCT ATAAAGGAGA TTTGCCATAC TCTTCAGGGA GTCCGGATGG TCCGGCGCGA
300

GGITGACCTT GCCTTCCATC CCTACCAAAA GAACCTCCG GCCAACTCCC GGCAGCCTTC
360

GAGCTCCAAA GTACCTCCGC GACCTGGGCT CAGTGTCTTC CGCAAGGTTT ACTGAGCCTA
420

AGGCTTGGTA CAATAAAATA AGAGACATAA CCTGCAAGTA CATAGGTTTT GTATGAGCCGA
480

GGAACCTGCT TCAGTACTAG ATCAGTTGGT AGAATACAT GAACAATGAT TCTGAGCCAG
540

AAAACCTGCTT GCAGGGAAAC CGTGAAGAAA CCCCACCTCC CGGCTCCAC TAAGGCGGAG
600

CCCTTTATCC GCCGGCCCTC CATTTAGCCA ATATGATCCC ATTTAGCCCA GTCCGGTGCCA
660

TTTAGGCAAG TCCAGTGCTT AGGTTGAGTT CTACACAGGA AAGGGCCATG AATGAGAGCA
720

CAACTATAGA ACTGTCCCTA GAAATAGGCT CGAGTGTGTT AGAGCTTTAA AGGTGATGCG
780

GCAAAATCCA TATGACTGAT TTTGCTCAAC GTCCAGGGGA AAGGGATAAA TAGTCTTTTT
840

CGCAGAAATAT AAATAGAGGT AGAGCGGGCT CGCAGCAATA TGGACCAGGA CAAGGGCTCT
900

TTTCCAGTTG CATAACATCA TTCCACAGAT TCAGCTTTCT TCAATCATC ATG AAG
955

Met Lys

-27

GTC ACT GCG GCT TTT GCA GGT CTT TTG GTC ACG GCA TTC GCC GCT CCT
Val Thr Ala Ala Phe Ala Gly Leu Leu Val Thr Ala Phe Ala Ala Pro

-25 -20 -15 -10

GCC CCA GAA CCT GAT CTG GTG TCG CGA AGT GCC GGT ATC AAC TAC GTG
Ala Pro Glu Pro Asp Leu Val Ser Arg Ser Ala Gly Ile Asn Tyr Val

-5 1 5

GTTATGCTT GGCTAGGCGG GACCTGGGTA AGTTGATGGC TCCTGGATTC TCACCTGAGT
240

ATTTCCAGCT ATAAAGGAGA TTTGCCATAC TCTTCAGGGA GTCCGGATGG TCCGGCGCGA
300

GGITGACCTT GCCTTCCATC CCTACCAAAA GAACCTCCG GCCAACTCCC GGCAGCCTTC
360

GAGCTCCAAA GTACCTCCGC GACCTGGGCT CAGTGTCTTC CGCAAGGTTT ACTGAGCCTA
420

AGGCTTGGTA CAATAAAATA AGAGACATAA CCTGCAAGTA CATAGGTTTT GTATGAGCCGA
480

GGAACCTGCT TCAGTACTAG ATCAGTTGGT AGAATACAT GAACAATGAT TCTGAGCCAG
540

AAAACCTGCTT GCAGGGAAAC CGTGAAGAAA CCCCACCTCC CGGCTCCAC TAAGGCGGAG
600

CCCTTTATCC GCCGGCCCTC CATTTAGCCA ATATGATCCC ATTTAGCCCA GTCCGGTGCCA
660

TTTAGGCAAG TCCAGTGCTT AGGTTGAGTT CTACACAGGA AAGGGCCATG AATGAGAGCA
720

CAACTATAGA ACTGTCCCTA GAAATAGGCT CGAGTGTGTT AGAGCTTTAA AGGTGATGCG
780

GCAAAATCCA TATGACTGAT TTTGCTCAAC GTCCAGGGGA AAGGGATAAA TAGTCTTTTT
840

CGCAGAAATAT AAATAGAGGT AGAGCGGGCT CGCAGCAATA TGGACCAGGA CAAGGGCTCT
900

TTTCCAGTTG CATAACATCA TTCCACAGAT TCAGCTTTCT TCAATCATC ATG AAG
955

Met Lys

-27

GTC ACT GCG GCT TTT GCA GGT CTT TTG GTC ACG GCA TTC GCC GCT CCT
Val Thr Ala Ala Phe Ala Gly Leu Leu Val Thr Ala Phe Ala Ala Pro

-25 -20 -15 -10

GCC CCA GAA CCT GAT CTG GTG TCG CGA AGT GCC GGT ATC AAC TAC GTG
Ala Pro Glu Pro Asp Leu Val Ser Arg Ser Ala Gly Ile Asn Tyr Val

-5 1 5
CAA AAC TAC AAC GCC AAC CTT GGT GAT TTT ACC TAC GAC GAG AGT GCC
Gln Asn Tyr Asn Gly Asn Leu Gly Asp Phe Thr Tyr Asp Glu Ser Ala
10 15 20
GGA ACA TTT TCC ATG TAC TGG GAA GAT GGA GTC TCC GAC TTT GTC
Gly Thr Phe Ser Met Tyr Trp Glu Asp Gly Val Ser Ser Asp Phe Val
25 30 35
GTT GGT CTG GCC TGG ACC ACT GGT TCT TCT
Val Gly Leu Gly Trp Thr Thr Gly Ser Ser Ser Asn
40 45 50
GTATTTTTA ACCAAGGCT AGGATCTAAC CTCTTTCAG C GCT ATC ACC TAG TCT
Ala Ile Thr Tyr Ser
55
GCC GAA TAC AGC GCT TCT GCC TCC GCT TAC CTC GCT GTG TAC GGC
Ala Glu Tyr Ser Ala Ser Gly Ser Ala Ser Tyr Leu Ala Val Tyr Gly
60 65 70
TGG GTC AAC TAT CCT CAA GCT GAG TAC TAC ATC GTC GAG GAT TAC GTT
Trp Val Asn Tyr Pro Glu Ala Glu Tyr Tyr Ile Val Glu Asp Tyr Gly
75 80 85
GAT TAT AAC CCT TGC AGT TCG GCC ACA AGC GCT GTG TAC GTC TCT
Asp Tyr Asn Pro Cys Ser Ser Ala Thr Ser Leu Gly Thr Val Tyr Ser
90 95 100
GAT GGA AGC ACC TAC CAA GTC TGC ACC GAC ACT CGA ACA AAC GAA CCG
Asp Gly Ser Thr Tyr Glu Val Cys Thr Asp Thr Arg Thr Asn Glu Pro
105 110 115
TCC ATC ACG GGA ACA AGC AGC TTC ACG CAG TAC TCC GTT CGA GAG
Ser Ile Thr Gly Ser Thr Phe Thr Glu Tyr Phe Ser Val Arg Glu
120 125 130 135
AGC ACG GCC ACA TCT GGA AGC AGC GTG ACT GTT GCC AAC CAT TTC AAC TCC
Ser Thr Arg Thr Ser Gly Thr Val Thr Val Ala Asn His Phe Asn Phe
140 145 150
TGG GCG CAC CAT GGG TTC GCC AAT AGC GAC TTC AAT TAT CAG GTC GTG
Trp Ala His Gly Phe Gly Asn Ser Asp Phe Asn Tyr Glu Val Val
155 160 165
GGG GCG GAA GCA TGG AGC GGT GCT GCC AGC GCT AGT GTC ACA ATC TCT
Ala Val Ala Trp Ser Gly Ala Gly Ser Ala Ser Val Thr Ile Ser
170 175 180
TCT TGAGAGATTA GTCGCCCTACT AGTCGGAAGA TATCAACOCG GCAGTTTGCT
Ser
1801
CTCAGGCTGT GTGATGACTG GATCCCCTCT CTGGGCTTCT ATGGAGCCTG TATAAGT
1741
TGTGGGGCCC AGCTGTCAGC GOCTGCG7TI' TCAGCTCA CACATMATCA ACTCTC='1
1801
TCTATCTCTT GCCCTTCTC GCCTCTATAT C TATCCATAG ATAAATTATTT TGCCCACTAC
1861
CACAACCTGT TCGGTCGCAG TAGTCACTCC GAGCAAGGCA TTGGGAAATG GGGATGCGG 1921
GCTGCTGCGT ACCCTCTAAC CTAGGGCAT GGGGTCAAC ATAGATTTCTA 1981
TAGATAAGA CTTCTTAGGA CTGCAGGAAG AAGGATTTCTA CAGTTGATG 2041
CAGTTCAATG CGA 2054

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 3026 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Aspergillus niger
(B) STRAIN: N400O (CBS 120.49)

(ix) FEATURE:
(A) NAME/KEY: TATA_signal
(B) LOCATION: 643..6648

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 7214..2538
(D) OTHER INFORMATION: /EC_number= 1.1.3.4.
/product= "glucose oxidase"
/gene= "goxC"

(ix) FEATURE:
(A) NAME/KEY: mat_peptide
(B) LOCATION: 790..2538

(ix) FEATURE:
(A) NAME/KEY: sig_peptide
(B) LOCATION: 724..790

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

CTGCAGGTAC CTGAAGCCTG CCTAGTTTGA TCACCTGAA ACCAGCAGT GCTGCTTGA 60
CCTGCTGTG GAGTTTCGAC CTGGCGCGCG TGTCAAATA AACTCCTCAA TTGACCCCTT 120
CCCGTGGGAG AACACAGCAG AACACTAGG CTTCTAGGAG AGGATAGGAC GAGGACCTA 180
TGTTTGTGCG ACTGGCGGAG GCGTACCCCG AGCAGGAAC CGGAAGGCA GAACCTCAGAA 240
63

TCGCGGTTC TGCGAGGU GCCAGCTCT ATCCCTAGG GCCACCCATGA TTTCGGTCC

GGATCGGT ATGGTCAGG TGCTACCTCC GCCTTCCGCT AGCGTGGGCT ACTGAGCTAA

TAGGGGCCA ATAGCCACG CAGGAGGATG CATGGCCTCT ACAAGAAGG AGACCCAGAG

GATCAGGAGC CCAATCTGCG CTGACCCTCT GCTAGGATT GCTTCTTTAGA CTATCCAGGG

ATTAGGGCT CGGATTATTC TATTCGGGAT ACCGACGGCT GAGACACCG AGGATGGGCT

TCAGCTCAGG GCCCTCTAGA GTATCCATT ACGGATGCC TTCTTGGAAA GCAGAGGAAT

TGATTATCG AACAGGGTG TCTCGGACCA TGACTGGAG CGTATAAGTA ACCTCGCTGG

GTCCTCCTGT CACCTTCTGA TCAGCAGCGA CCCTTTTCTC TACTCTGCCC

ATC ATG CAG ACT CTC CTT GTG AGC TCG TTT GTG TCC TCC GCT GCG
Met Gln Thr Leu Leu Val Ser Ser Leu Val Ser Leu Ala Ala
-22 -20 -15 -10

GCC CTG CCA CAC TAC ATG AGG ACC AAT GCC ATT GAA GCC AGC CTC CTG
Ala Leu Pro His Tyr Ile Arg Ser Asn Gly Ile Glu Ala Ser Leu Leu
-5 1 5

ACT GAT CCC AAC GAG AGT GTG GCC ACC GCG ATG TTC ATG ACG TAC TCC GCC
Thr Asp Pro Lys Asp Val Ser Gly Arg Thr Val Asp Tyr Ile Ile Ala
10 15 20 25

GGT GTA GGT GTG ACT GGA GTC ACC ACC GCT GCT GTG ACG GAG AAC
Gly Gly Leu Thr Gly Leu Thr Ala Ala Arg Leu Thr Glu Asn
30 35 40

CCC AAC ATC AGT GTG CTC GTC ATC GAA AGT GCC TCC TAC GAG TCG GAC
Pro Asn Ile Ser Val Leu Ile Glu Ser Gly Ser Tyr Gly Ser Asp
45 50 55

AGA GGT CCT ATC ATT GAG GAC CTG AAG GCC TCG TAC GCC TCT TCC GCC
Arg Gly Pro Ile Ile Glu Asp Ala Tyr Gly Leu Ile Phe Gly
60 65 70

AGC AGT GTA GAC CAC GCC TAC GAG ACC GTG GAG CTC GCT GCT ACC AAC AAT
Ser Ser Asp His Ala Tyr Glu Thr Val Glu Leu Ala Thr Asn Asn
75 80 85

CAA ACC GCG CTG ATC GCC TGG GAG ACT GGT CTC GCT GCC TCT ACT CTA
Gln Thr Ala Leu Ile Arg Ser Gly Asn Gly Leu Gly Gly Ser Thr Leu
90 95 100 105

CTG AAT GGT GCC ACC TGG ACT CCG CCC CAC AAG GCA CAG GTT GAC TCT
Val Asn Gly Gly Thr Trp Thr Arg Pro His Lys Ala Gin Val Asp Ser
110 115 120

TGG GAG ACT GTC TTT GGA AAT GAG GCC TGG AAC TGG GAC AAT GGG GCC
Trp Glu Thr Val Phe Gly Asn Glu Gly Trp Asn Trp Asp Asn Val Ala
125 130 135
GCC TAC TCC CTC CAG GCT GAG CTT GCT CGC GCA CCA AAT GCC AAA CAG
Ala Tyr Ser Leu Gln Ala Glu Arg Ala Arg Ala Pro Asn Ala Lys Gln
140 144 150

ATC GCT GCT GCC CAC TAC TTC AAC GCA TCC TGC CAT GGT TGT AAT GGT
Ile Ala Ala Gly His Tyr Phe Asn Ala Ser Cys His Gly Val Asn Gly
155 160 165

ACT GTC CAT GCC GGA CCC CCG GAC ACC GGC GAT GAC TAT TCT CCC ATC
Thr Val His Ala Gly Pro Arg Asp Thr Gly Asp Tyr Ser Pro Ile
170 175 180 185

GTC AAG GCT CTC ATG AGC GCT GTC GAA GAC CCG GGC GTT CCC ACC AAG
Val Lys Ala Leu Met Ser Ala Val Glu Asp Arg Gly Val Pro Thr Lys
190 195 200

AAA GAC TTC GGA TGC GGT GAC CCC CAT GGT TCG ATG TTA TCT CCC AAC
Lys Asp Phe Gly Cys Gly Asp Pro His Gly Val Ser Met Phe Pro Asn
205 210 215

ACC TTG CAC GAA GAC CAA GTG CCG TCC GAT GCC GGC GCT GCC GAA TGG CTA
Thr Leu His Glu Asp Gln Val Arg Ser Asp Ala Ala Arg Glu Trp Leu
220 225 230

CTT CCC AAC TAC CAA CGT CCC ACC CTG CAA GTC ACC ACC GGA CAG TAT
Leu Pro Asn Tyr Glu Arg Pro Asn Leu Gln Val Leu Thr Gly Gln Tyr
235 240 245

GTC GAA ACC GTC TTT CAC GGC ACC ACC ACC CCT CTT GCC GCC GCC GAT
Val Gly Lys Val Leu Leu Ser Gln Asp Gln Thr Thr Pro Arg Ala Val
250 255 260 265

GCC GTG GAA TTC GCC ACC CAC AAG GCC ACC ACC CAC AAG GTC TAC GCT
Gly Val Glu Phe Gly Thr His Lys Gly Asn Thr His Asn Val Tyr Ala
270 275 280

AAG CAC GAG GTG CTC CTC GCC GGC GCC GCC GCC GCC GTC GTG TCT CCC ACA ATC
Lys His Glu Val Leu Leu Ala Ala Gly Ser Ala Val Ser Pro Thr Ile
285 290 295

CTC GAA TAT TCC GCT ATC GGA ATG AAC TTC ATC CTG GAG GCC CTT GCT
Leu Glu Tyr Ser Gly Ile Gly Met Lys Ser Ile Leu Gly Pro Leu Gly
300 305 310

ATC GAC ACC GTC GGT GAC CTG CCC GTC GCC TGC CGC TTG AAC CTG CAG GAC CAG
Ile Asp Thr Val Val Asp Leu Pro Val Gly Leu Asn Leu Gln Asp Gln
315 320 325

ACC ACC GCT ACC GTC CGC TCC CGC ATC ACC TCT GCT GGT GCA GGA CAG
Thr Thr Ala Thr Val Arg Ser Arg Ile Thr Ser Ala Gly Ala Gly Gln
330 335 340 345

GGA CAG GCC GCT TGG TTC GCC ACC TCC AAC GAG ACC TTT GGT GAC TAT
Gly Gln Ala Ala Trp Phe Ala Thr Phe Asn Glu Thr Phe Gly Asp Tyr
350 355 360
GGTATAATCA TAGATTGGAT AGAATTGGTA GTTACATAG ACAGTTACA TGAATAGACG
TTGCTTATAT GTGACCAGAC ATTACTACCA AACAAGGGCA TTGTTCAAGT AGTCGAACQA
TAGTCATATG TTTGTACGG GAAGAAAGTT TCACCTAATTA TTAAGCAAAC GGATCAGGGG
TTGCCAGCTA AAATACAATC ATCCGATGTT CTATTTTCTT CAATTTGATG GACCAAGTCAG
TTAATGAATG CATGAGACCA ACTCTGCGCA TCTCTCAGCT ATCTAGTCAA TAAATAAGCAT
GGTCCTTAAG ATGAAACACC GGCATAGACCA TATCTGGTG CTGGTAAGAC AAGCCCTCAG
TTAAATAGCT GATAACCTCC TATGCCAGTA GAATATTTTC GCACCTCGCT GCCGCTCIC
AAAAGCCTT

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:
   (A) LENGTH: 1517 base pairs
   (B) TYPE: nucleic acid
   (C) STRANDEDNESS: double
   (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ORIGINAL SOURCE:
   (A) ORGANISM: Aspergillus niger
   (B) STRAIN: L112

(ix) FEATURE:
   (A) NAME/KEY: exon
   (B) LOCATION: 339..495

(ix) FEATURE:
   (A) NAME/KEY: intron
   (B) LOCATION: 496..563

(ix) FEATURE:
   (A) NAME/KEY: exon
   (B) LOCATION: 564..1237

(ix) FEATURE:
   (A) NAME/KEY: CDS
   (B) LOCATION: join(339..495, 564..1237)
   (D) OTHER INFORMATION: /product= "oroticine-5'-phosphate decarboxylase"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

GCAGGGAAAA ATACGAGCTC CAATGAACCT GGGTGTGGCA ACTTCAATGG AAAGGAACTG
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GGCTGGTCCG TGAGGGTCCG CGATGATTAT CGAGCTCCCT CTGGTGCCTGC ACAATGAATG

60
120
180
TGCATTGGTC ACCTCATATA AGG GCCAGTC GCTGCTAAAT TATTCGCTAG TATTTGCGCa
TCTCTGGATC TACCAATTAG GCCATATCAG TGGAAACTCC AAGCTACTCA TATCAGACAA
GCTCTTTTCA TCCCCGGCAT AACCCTTCCA CGCAGACC ATG TCC TGC AAG TGG
Met Ser Ser Lys Ser

CAA TTG ACC TAC ACT GCC GTG GCC AGG AAG CAC CCC AAT GCT CTG GCC
Gln Leu Thr Tyr Thr Ala Arg Ala Ser Lys His Pro Asn Ala Leu Ala

AAG CGG CTG TTC GAA ATT GCT GAG GCC AAG AAG ACC AAT GTG ACC GTC
Lys Arg Leu Phe Glu Ala Ala Lys Pro Leu Thr Val

TCT GCC GAC GTT ACC ACC ACT AAG GAG CTA CTA GAT CTT GCT GAC C
Ser Ala Asp Val Thr Thr Thr Lys Glu Leu Asp Arg

CTAGCCGAC CCGCCATTCT GCTGTTTAT GCTGACATA AACTATTTAA CGTGATACCC
GGACTGAG GT TCT GGT CCC TAC ATC GCC GTG ATC AAA ACC CAC ATC GAT
Arg Leu Gly Pro Tyr Ile Ala Val Ile Lys Thr His Ile Asp

ATC TCT TCT GAC TTC ACC GAC GAG ACC ATT GAG GGC CTC AAG GCT CTT
Ile Leu Ser Asp Phe Ser Asp Glu Thr Ile Glu Gly Leu Lys Ala Leu

GCC CAG AAC CAC ATT TTC CTC ATC TTC GAC GCC CCG AAA TAC ATC GAC
Ala Gln Lys His Asn Phe Leu Ile Phe Glu Asp Arg Lys Phe Ile Asp

ATT GCC AAC ACT GTC CAG AAG CAA TAC CAC GTG GTT ACC CTC GCC ACG
Ile Gly Asn Thr Val Gln Lys Gly Tyr His Arg Gly Thr Leu Arg Ile

TCA GAA TGG GCC CAT ATC ATC AAC TGC AGC ATC CTG CCT GCC GAG GGT
Ser Glu Trp Ala His Ile Ile Asn Cys Ser Ile Leu Pro Gly Glu Gly

ATC GTG GAG CTT GCT CTC GCT AGC TGG CAC CGC CAG GCC ATC
Ile Val Glu Ala Ala Gln Thr Ala Ser Ala Pro Asp Phe Ser Tyr

GCC CCC GAA GTT CTG TTT ATC TTG GCC GAA ATG ACC TCT AAG GGT
Gly Pro Glu Arg Gly Leu Leu Ile Leu Ala Glu Met Thr Ser Lys Gly

TCC TTG GCC ACC GGC CAG TAC ACT ACT TCT TCG GTT GAT TAT GCC CAG
Ser Leu Ala Thr Gly Gin Tyr Thr Ser Ser Val Asp Tyr Ala Arg

AAA TAC AAG AAC TTC GTC ATG GGA TTT GTG TCG ACC CGC TCG TTG GGT
Lys Tyr Lys Asn Phe Val Met Gly Phe Val Ser Thr Arg Ser Leu Gly

67

240
300
353
401
449
495
555
604
652
700
748
796
844
892
940
988
GAG GTG CAG TCG GAA GTC AGC TCT CCT TCC GAT GAG GAC TTT GTG
Glu Val Gln Ser Glu Val Ser Ser Pro Ser Asp Glu Glu Asp Phe Val 195
200

GTC TTC ACG ACT GGT GTG AAC ATT TCG TCC AAG GGA GAT AAG CTC GGT
Val Phe Thr Thr Gly Val Asn Ile Ser Ser Lys Gly Asp Lys Leu Gly 215
220

CAG CAG TAC CAG ACT CCC GCA TCG GCT ATC GGT CCG GGT GCT GAC TTC
Gln Gln Tyr Gln Thr Pro Ala Ser Ala Ile Gly Arg Gly Ala Asp Phe 230
235
240

ATT Ile Ile Arg Gly Ile Tyr Ala Ala Pro Asp Pro Val Gln Ala 245
250
255

GCG CAG CAC TAC CAG AAG GAA GGT TGG GAG GGC CAG TAC CGC GGT GCT
Ala Gln Glu Tyr Glu Lys Glu Gly Trp Glu Ala Tyr Leu Ala Arg Val 260
265
270

GGC GGA AAC TAATACTATA AAATGAGGAA AAAAGTTTTG ATGGTTATGA
Gly Gly Asn 275

ATGATATAGA AATGCAACTT GCGGCTACGA TACCGATACA AACTAATGTC GAGCAGGGGT
1337

AGTCAGACTG CGGCATCGGA TGTTCAAAACG GTATTGATCC TGAGGGCTAT TATAGGTTGG
1397

CAGGGATTA ATGCGGTACC ACGATTGAT GCAGATAAACG AGGCTGCCGA ATACTTAGTC
1457

CTGTAACCTC TGCGTAGACC AAATGCGCGAC GGTGCGCTGA TGAGGGACGG TGATAAGC
1517

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4108 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) ORIGINAL SOURCE:
(A) ORGANISM: Aspergillus niger (CBS 120.49)
(B) STRAIN: NW147

(ix) FEATURE:
(A) NAME/KEY: TATA_signal
(B) LOCATION: 787..794

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 855..3266
(D) OTHER INFORMATION: /EC_number= 3.2.1.37
/product= "1,4-beta-D-xylan xylanohydrolase"
/gene= "xlnD"
/standard_name= "beta-xylosidase"

(ix) FEATURE:
(A) NAME/KEY: sig_peptide
(B) LOCATION: 855..932

(ix) FEATURE:
(A) NAME/KEY: mat_peptide
(B) LOCATION: 933..3266

(ix) FEATURE:
(A) NAME/KEY: polyA_site
(B) LOCATION: 3383

(ix) FEATURE:
(A) NAME/KEY: polyA_site
(B) LOCATION: 3404

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

CTCAGAGCCA TGATATCTG GAAATAGGTT GAGTGAAGAA AAATCGTCAA GATTGAGGCG
GAGATCCCGA GGGCCCGGAT AAAGAAGGTT GCGGCGCAC CGGAGAGCCC AGCCACCGCC
ACGCCCGGCC AGATAGTAT ACCGTATCCG GTGCCGCTCG AGTCCCTCGA TGGGGAGATT
GTGCGGGGCG AGTITGGCSC CGGCAATGGTAA ATCTGGGAAGG AGTATGGGA TGTGATTAGG
CTGATGGAAT GTGCAGGGCC CAAGGACCTG CATAACTAGT GGGGCAGAGG GGTCAAGATC
ACGTTTGGAG ATGGGGAGGT GGTGGAGAAG TGTGTGAAAG CTCCGAAAGG AGTCCATCCT
GGGTGACGA ATGAGGAGGT GTGCAAGAAG TGGCCGGGCTG TGACGAAAGG GGTAAATTGC
GAAAGGAGGC AGAAGAAGAT CGAGGAGATT GTTGGTAATT TGGAGAGATT GGAAGGATTG
GCTGTGTTTT TGGGCGGATT GTTGGAGGAA GAGACGGTGA ATGTGGCTCCA GTAGACGGTT
ACCCCATTTG GACGGGATG GCTCTATTAT TCCAAAGCGA TGTCAAGCCCA TAGAAAGGCC
ACATTTAACC GGTGCTGAG CCGAAGCTCTA CTTCAAGACT CAACTGAAAG AATGCCAATG TTTACTATC
TTGGTTTAT TGCTAAAATGC AAACATCCCA GTTCTCTTCT AATCCGGCGT AAATCATTTCA
GGCTAAAACC CGCGGATGAA CTCAATCGGT CATTCTCCGG CCCATCTCGC CATCTCGCCA
AAACCGTATA AAATCTAACC CAGATTACGT CCCCCCCCGAC ATTTACTATCC CCCCCGGCCAC
AGACTGGCTC AACC ATG GCC CAC TCA ATG TCT GTG CCC GTG GCT GCC ACT

Met Ala His Ser Met Ser Arg Pro Val Ala Ala Thr
-26 -25 -20

GCC GCT GCT CTG GCT GCT GCT GCT CTG CAA GCT CTG GCC CAG GCC
Ala Ala Ala Leu Ala Leu Ala Leu Ala Leu Pro Gin Ala Leu Ala Gin Ala
-10 -5 1
AAC ACC ACC TAC GTC GAC TAC AAC ATC GAA GCC ACC CCG GAC TTG TAT  
Asn Thr Ser Tyr Val Asp Tyr Asn Ile Glu Ala Asn Pro Asp Leu Tyr  
  5 10 15

CCT TTG TGC ATA GAA ACC ATC CCG ACC TTT CCC GAC TTC CAG AAT  
Pro Leu Cys Ile Glu Thr Ile Pro Leu Ser Phe Pro Cys Gln Asn  
 20 25 30

GCT CCC CTG CCC ACC CCG CAT CTC ATC GAT TTC GAA ACA GCC ACC CCC TAT  
Gly Pro Leu Arg Ser His Leu Ile Cys Asp Glu Thr Ala Thr Pro Tyr  
 35 40 45 50

GAC CGA GCA TCG CTC ATC TCG CTC TTC ACC CTG GAC CTG ATC  
Asp Arg Ala Ala Ser Leu Ile Ser Leu Phe Thr Leu Asp Glu Leu Ile  
 55 60 65

GCC AAC ACC GCC AAC ACC GCC CTC GGT CTC TTC CGA CTC GCC CTC CCT  
Ala Asn Thr Gly Asn Thr Gly Leu Gly Val Ser Arg Leu Gly Leu Pro  
 70 75 80

GCA TAC CAA GTA TGG AGT GAA GCT CTT CAC GCC CTC GAC CTG GCC AAT  
Ala Tyr Gln Val Trp Ser Glu Ala Leu His Gly Leu Asp Arg Ala Asn  
 85 90 95

TTC ACC GAC TCA GGA GCC TAC AAT TGG GCC ACC TCA TTC CCC CAG CCC  
Phe Ser Asp Ser Gly Ala Tyr Asn Trp Ala Thr Ser Phe Pro Glu Pro  
100 105 110

ATC CTG ACC ACC CCG GCC CTG ACC ACC CTC ACG ACC ACC CTG ACG CAA ATC GCC  
Ile Leu Thr Thr Ala Ala Leu Asn Arg Thr Leu Ile His Gln Ile Ala  
115 120 125 130

TCC ATC ATC TCT ACC CAA GCC GCC CCG CCC TTC ACG AAC GCC GCC CCG TAC  
Ser Ile Ile Ser Thr Gln Gly Ala Asp Asn Ala Gly Arg Tyr  
135 140 145

GCC CTC GAC GTG TAC GCC CCC AAC ATC AAC ACC TTC GCC CAC CCC GTG  
Gly Leu Asp Val Tyr Ala Pro Asn Ile Asn Thr Phe Arg His Pro Val  
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TGG GGT CCG GCA CAA GAA ACC CCA GGA GAG GAC CTG TCT CTC GCC GGC  
Trp Gly Arg Gly Gln Glu Thr Pro Gly Glu Asp Val Ser Leu Ala Ala  
165 170 175

GTC TAC GCC TAC GAA TAC ATC ACC GCC ATC CAG GGT CCC GAC CCA GAA  
Val Tyr Ala Tyr Glu Ile Thr Gly Ile Gln Gly Pro Asp Pro Glu  
180 185 190

TCA AAC CTC AAA CTC GCC GCC ACC CAG GCC AAC CAC TAC GCC GCC TAC GAT  
Ser Asn Leu Lys Leu Ala Thr Ala Lys His Tyr Ala Gly Tyr Asp  
195 200 205 210

ATC GAG AAC TGG CAC AAC CAC TCC GCC GCC CTC GGT GCC AAC GAC ATG AAT  
Ile Glu Asn Trp His Asn His Ser Arg Leu Gly Asn Met Asn Ile  
215 220 225
ACC CAG CAA GAC CTC TCC GAA TAC TAC ACG CCC CAA TTC CAC GTC GCC
Thr Gln Gln Asp Leu Ser Glu Tyr Tyr Thr Pro Gln Phe His Val Ala
230 235 240

GCC GCC GAC GCC AAA GTC CAG AGT GTC ATG TGC GCC TAC AAC GCC GTC
Ala Arg Asp Ala Lys Val Gln Ser Val Met Cys Ala Tyr Asn Ala Val
245 250 255

AAC GGC GTC CCT GCC TGC GCC GAC TCC TAC TTC CTC CAG ACC CTO CTC
Asn Gly Val Pro Ala Cys Ala Asp Ser Tyr Phe Leu Gln Thr Leu Leu
260 265 270

CGC GAC ACC TTC GGA TTT GTC GAC CAC GGA TAC GTC TCC ACC GAC TGC
Arg Asp Thr Phe Gly Phe Val Asp His Gly Tyr Val Ser Ser Asp Cys
275 280 285 290

GAT GCC GCC TAT AAC ATC TAC AAC CCC CAG GCC TAT CCC TCC TCC CAG
Asp Ala Ala Tyr Asn Ile Tyr Asn Pro His Gly Tyr Asn Ser Ser Gln
295 300 305

GCT GCC GCT GCC GCT GAG GCC ATC CTC GCC GCC ACC GAC ATC GAC TGC
 Ala Ala Ala Ala Glu Ala Ala Gly Thr Asp Ile Asp Cys
310 315 320

GGT ACC ACC TAC CAA TGG CAC CTG AAC GAG TCC ATC GCT GCC GGA GAT
Gly Thr Thr Tyr Gln Trp His Leu Asn Glu Ser Ile Ala Ala Gly Asp
325 330 335

CTC TCT CGC GAT GAT ATT GAG CAG GGT GTG ATT CTC TAC ACG ACC
Leu Ser Arg Asp Asp Ile Gly Phe Val Ile Arg Leu Tyr Thr Thr
340 345 350

CTC GTG CAG GCC GGA TAC TTC GAC TCC AAC ACC ACA AAG GCC AAC AAC
Leu Val Gln Ala Gly Tyr Phe Asp Ser Asn Thr Thr Lys Ala Asn Asn
355 360 365 370

CCC TAC GCC GAC CTC TCC TGG GCC GTC CTC CTG GAC GTC CTT GAG AGC GCC GAC TGG
Pro Tyr Arg Asp Leu Ser Trp Ser Asp Val Leu Glu Thr Asp Ala Trp
375 380 385

AAC ATC TCC TAC CAA GCC GCG ACG CAG GCC ATT GTC CTT CTC AAG AAC
Asn Ile Ser Tyr Gln Ala Ala Thr Gln Gly Ile Val Leu Leu Lys Ala
390 395 400

TCC AAC ACC TGC CTC CCC CTC ACC GAG AAA GCT TAC CCA CCA TCC AAC
Ser Asn Ala Val Leu Pro Leu Thr Glu Lys Ala Tyr Pro Ser Asn
405 410 415

ACC ACC GTC GCC CTC ATG GCC TGG GCC AAC GCC ACC AAC CAA CTC
Thr Thr Val Ala Leu Ile Gly Pro Trp Ala Asn Ala Thr Thr Gln Leu
420 425 430

CTG GCC AAC TAC TAC GCC AAC GCT CCC TAC ATG ATC AGC CCC GCG GCC
Leu Gly Asn Tyr Tyr Gly Asn Ala Pro Tyr Met Ile Ser Pro Arg Ala
435 440 445 450
ATC AGG AAC ACT GGA AAG CTG GAA TCG GAT TAC ACC GCT ATG GTA TTC
Ile Arg Asn Thr Gly Lys Leu Glu Ser Asp Tyr Thr Ala Met Val Phe
675 680 685
GCC AAT ACC TCT GAT GCC GGG CCG GCG CCG TAT CCC AAG AAG TGG CTT
Ala Asn Thr Ser Asp Ala Gly Pro Ala Pro Tyr Lys Lys Trp Leu
695 700 705
GTC GGG TGG GAT CGG CTT GGG GAG GTC AAG GTC GGG GAG ACG AGG TAG
Val Gly Trp Asp Arg Leu Gly Val Lys Val Gly Ser Val Arg Gly
710 715 720
TTG AGG GTC CCC GGT GAG GTC GGG ACC TTT CCC GCA GGC GGT AAT CAT
Leu Arg Pro Val Gly Lys Ser Phe Pro Val Asp Leu Leu Leu
725 730 735
GCC GAT TGG GTG GTG TGG CCG CAG GAA CAG TTT CCC GCA AAG CAC CGT
Gly Asp Trp Val Val Phe Pro Gly Thr Phe Leu Ala Asn Leu
740 745 750
GAG AGG AAT CAG AAA AAA TCT AAG GAA AAA AAA AAA TCT GTT AAA AAA
Glu Arg Asp GAT AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA
755 760 765 770
GTC CTG AAG TGG CCG GGG AAG GAG TAGGAATAC TATTTGTTG ATGGCTCTAG
Val Leu Lys Pro Gly Lys Glu
775
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3002
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3098
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3146
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3194
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3242
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3296
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3356
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3416
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3476
CTTGAGACCT TCTCATCTGGCTG TGGTGCAGAAG AGCCTTTTCT TCTGATCTGC
3536
ACGAGATCT CGCTGGCGGC TAGACATGAA GACGATGAG TCTGTGCTCC CAAAGAAGAC
3596
AACCGTTTGC GGAATCAGAA CTTGCAATGCT CTTGCGGAG CAGGCGGATG
3656
GATTTCTTCG CGGATCAGAA CAAACCTTC AAGGCGTGG TCGGACCTTG
3716
ATCTACGCGA CAACACTCAT AAACACCTCC TGCCCCACCC AGACCCCTTC TTCTTCTGAG
3776
GATCAGGAG AATTACCAC TACCTCTGGCTG AAAAAAGTTA AACAACGGCC TGCGGCGAGG
3836
ATCGTCCCGA CTCAAAAGCA ACAAACCTCT CAGTCGACAT AAACCTCTGT CCTGATTTG
3896
CTTGAGACCT TCTCATCTGGCTG TGGTGCAGAAG AGCCTTTTCT TCTGATCTGC
3956
ACGAGATCT CGCTGGCGGC TAGACATGAA GACGATGAG TCTGTGCTCC CAAAGAAGAC
4016
AACCGTTTGC GGAATCAGAA CAAACCTTC AAGGCGTGG TCGGACCTTG
4076
GATTTCTTCG CGGATCAGAA CAAACCTCAT AAACACTGCC TCCCCACCC AGCAACCTTC TCTCAGTCTT
(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:
   (A) LENGTH: 4173 base pairs
   (B) TYPE: nucleic acid
   (C) STRANDEDNESS: double
   (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
   (A) ORGANISM: Aspergillus niger
   (B) STRAIN: CBS 120.49
   (C) INDIVIDUAL ISOLATE: N400

(ix) FEATURE:
   (A) NAME/KEY: CDS
   (B) LOCATION: join(948..1173, 1238..3495, 3550..3690)
   (C) IDENTIFICATION METHOD: experimental
   (D) OTHER INFORMATION: /function= "Transcriptional activator of xylanolytic genes"
                               /product= "Binuclear Zn finger DNA binding protein"
                               /gene= "xlnR"
                               /standard_name= "XYL R"

(ix) FEATURE:
   (A) NAME/KEY: exon
   (B) LOCATION: 948..1173

(ix) FEATURE:
   (A) NAME/KEY: intron
   (B) LOCATION: 1174..1237

(ix) FEATURE:
   (A) NAME/KEY: exon
   (B) LOCATION: 1238..3495

(ix) FEATURE:
   (A) NAME/KEY: intron
   (B) LOCATION: 3496..3549

(ix) FEATURE:
   (A) NAME/KEY: exon
   (B) LOCATION: 3550..3690

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

CCC GGCGCATGTC TCGGTTCGCTGCCCTGCTCCTTCCCCTCTCAATGTTCTGACTCC
60
CCCAATCTTCCCTTTTTTTTTGTCTCGCCAGGCTGTGTCTT TTTCCCCCTCCCCCCCTCCTCC
120
CCTCGTCTAG TTTTCCTCTCCAGCATGGTCTGTTACCAACTACAAACGCTCTGCTGATCTGAAG
180
ACG AAG GAT CAA CCA CCC TTT GAT AAT GAG AAG AAC CAG AGC ACT GGC
  Thr Lys Asp Glu Pro Pro Phe Asp Asn Glu Asn Ser Thr Gly
  5

TCG GGT TTT AGG GAC GCT CTG CAA AGA GAT CCC CTC GTG GAG GCT GGC
  Ser Gly Phe Arg Asp Ala Leu Gln Arg Asp Pro Leu Val Glu Ala Arg
  20

TCT GCC GTC CCG AAA ACC TCG TCT TCA GCT CGG GTT CGC CCG CGA ATC
  Ser Ala Val Arg Lys Thr Ser Ser Ser Ala Pro Val Arg Arg Ile
  40

AGC CTT GGT CAG TAT AAC CAA TCC GCA ACG AAA TGC GAC GGG
  Ser Arg Ala Cys Asp Glu Cys Asn Gln Leu Arg Thr Lys Cys Asp Gly
  55

CAG CAT CCG TGC GCT GAT CAT GTC ATT
  Gln His Pro Cys Ala His Cys Ile
  70

CGATGATCGC GATGAGCGG ACCCTTGACT GACCTTTCTC GTAG AA TTC GGA CTC
  Glu Phe Gly Leu
  1248

ACC TGC GAG TAT CCG CAG GAA CGG AAG AAG CAG GCT GGA AAA GGC TCG AAG
  Thr Cys Glu Tyr Ala Arg Glu Arg Lys Arg Gly Lys Ala Ser Lys
  80
AAG GAT CTG GCG GCA GCT GCG CCG CTT ACC CAA GGG TCG AAT GGT
Lys Asp Leu Ala Ala Ala Ala Ala Ala Thr Gln Gly Ser Asn Gly
100 105 110

CAT TCC GGG CAG GCC AAC GCG TCG CTA ATG GCC GAG CGA ACG TCG GAA
His Ser Gly Gln Ala Asn Ala Ser Leu Met Gly Glu Arg Thr Ser Glu
115 120 125

GAC ACC CGG CCA GGA CAA GAC GTG AAC GCC ACA TAC GAC TCG GCT TTT
Asp Ser Arg Pro Gly Gln Asp Val Asn Gly Thr Tyr Asp Ser Ala Phe
130 135 140

GAG ACC CAC CAT CTT ACC TGG CAG CCA TCG ATG CAG CAT GCA AGC
Glu Ser His His Leu Ser Ser Gln Pro Ser His Met Gln His Ala Ser
145 150 155

ACT GCA GGG ATA TCC GCC CGC TGG CAC GAG TCT CAG ACG GCA CCG TCG CAT
Thr Ala Gly Ile Ser Gln Leu His Glu Ser Gln Thr Ala Pro Ser His
160 165 170 175

TCG CAA TCA TCG CTA GGA ACG ACT ATC GAT GCG ATG CAT TGG AAT CAT
Ser Gln Ser Ser Leu Gln Thr Thr Ile Asp Ala Met His Leu Asn His
180 185 190

TTC AAC ACG ATG AAC GAT TCC GGT GCC CGG GGA ATG TCC AAT TCC GAT
Phe Asn Thr Met Asn Asp Ser Gly Arg Pro Ala Met Ser Ile Ser Asp
195 200 205

CTG GCT TCG CTA CCC CCG TCC GTC TTA CCA CCG GAA CTA ACG GCT
Leu Arg Ser Leu Pro Pro Ser Val Leu Pro Pro Gln Gly Leu Ser Ser
210 215 220

GGG TAC AAC CGG AGC GCC TTC GCT TTG GTG AAC CGG CAA GAG CGG GGC
Gly Tyr Asn Ala Ser Ala Phe Ala Leu Val Asn Pro Gln Glu Pro Gly
225 230 235

TC

AAC CAG TTT CCG TTG GGA AGC TCA GCG GAA AAC CCA ACC
Se.

Se.

24u 24v 24w 24x 24y 25u 25v 25w 25x 25y 26u 26v 26w 26x 26y 27u 27v 27w 27x 27y

GCA CCG TTT CTT GGT CTC TCG CTT CCA GGA CAG TCG CCT GGA TGG CTC
Ala Pro Phe Leu Gly Ser Pro Pro Gln Ser Pro Gly Trp Leu
260 265 270

CCT CTT CCC TCG CCA TCT CCT GCC AAC TTT CCT TCT TCC AGC TCG CAT
Pro Leu Pro Ser Pro Ser Pro Ala Asn Phe Pro Ser Phe Ser Leu His
275 280 285

CCG TTT TCC AGC ACT TTA CGA TAC CTT GGT TTG CAG CGG GTC CTC CCT
Pro Phe Ser Ser Thr Leu Arg Tyr Pro Val Leu Gln Pro Val Leu Pro
290 295 300

CAC ATC GCC TCC ATT ATT CGG CAG TCC CTA GCG TGT GAC CTT CTG GAT
His Ile Ala Ser Ile Ile Pro Gln Ser Leu Ala Cys Asp Leu Leu Asp
305 310 315
GTT TAC TTC ACT AGT TCC TCT TCG TCC CAC CTG TCT CCC TTG TCC CCA
Val Tyr Phe Thr Ser Ser Ser Ser Ser Ser Ser Ser Ser Thr Ser Pro
320 325 330 335

TAC GTG GTG GGC TAC ATC TTC CCG AAG CAG TCT TCC TCT CAC CCG ACA
Tyr Val Val Gly Tyr Ile Phe Arg Lys Glu Ser Phe Leu His Pro Thr
340 345 350

AAA CCC CGA ATA TGC AGC CCC GGT CTC CTG GCG AGT ATG CTC TGG GTA
Lys Pro Arg Ile Cys Ser Pro Gly Leu Leu Ala Ser Met Leu Trp Val
355 360 365

GCC GCA CAA AGC ACT GAA CCT GCG TAT CTC ACA TCG CCG CCC TCG GCT
Ala Ala Glu Thr Ser Ser Glu Ala Ala Phe Leu Thr Ser Pro Ser Ser Ala
370 375 380

CGG GGG CGT GTA TCC CAG AAA CTG CTA GAA CTG ACC ATT GGT TTG CTC
Arg Gly Arg Val Cys Glu Leu Leu Glu Leu Thr Ile Gly Leu Leu
385 390 395

CGA CCG TTG GTG CAT GGT CCT GCT ACC GGA GAA GCG TCG CCC AAC TAT
Arg Pro Leu Val His Gly Pro Ala Thr Gly Glu Ala Ser Pro Asn Tyr
400 405 410 415

GCC GCG AAT ATG GTC ATC AAT GGC GTC GCT GGC GGA TTT GGG GTC
Ala Ala Asn Met Val Ile Asn Gly Val Ala Gly Gly Phe Gly Val
420 425 430

TCC ATG GAT CAG CTG GGC CCA ACT AGC GCC ACC GGC CCC TGG GAT
Ser Met Asp Glu Leu Gly Ala Glu Ser Ser Ala Thr Gly Ala Val Asp
435 440 445

GAT GTA GCA ACT TAT GTG CAT CTT GCC ACA GTA GTA TCC GCC AGC GAG
Asp Val Ala Thr Tyr Val His Leu Ala Thr Val Ala Ser Ser Glu
450 455 460

TAC AAG CGG GGC AGG ATG CGG TGG TGG ACT GCG GCG TGG TCT CTA CGG
Tyr Lys Ala Ala Ser Met Arg Trp Trp Thr Ala Ala Trp Ser Leu Ala
465 470 475

CGG GAT GTC AAA CTA GCC GCT GAG CTG CCA CCC AAT GTT TCC CAC CAA
Arg Glu Lys Leu Gly Arg Glu Leu Pro Pro Asn Val Ser His Ala
480 485 490 495

CGG CGA GGT GGG CAA GAT CGA GAG CGA GAT GGG GAT GGC GAG CCC CAA CAT
Arg Glu Asp Gly Arg Asp Gly Asp Gly Glu Ala Asp Lys Arg His
500 505 510

CCT CGG ACC CTC ATC AGC TCA CTG GGT CAT GGA TCG GAA AGC TCC GCC
Pro Pro Thr Leu Ile Thr Ser Leu Gly His Gly Ser Gly Ser Ser Gly
515 520 525

ATT AAT CTC ACC GAA GAG GAG CGT GAG GCG CGT CTA TGC TGG TCG
Ile Asn Val Thr Glu Glu Arg Glu Arg Arg Leu Trp Trp
530 535 540
CTC TTA TAT GCG ACC GAT CGG CAC CTG GCG CTG TGC TAC AAC CGG CCC
Leu Leu Tyr Ala Thr Asp Arg His Leu Ala Leu Cys Tyr Asn Arg Pro
545 550 555

CTC ACG CTG CGC GAC AAG GAA TGT GGC GCG CTG CAG CCG ATG AAC
Leu Thr Leu Leu Asp Lys Glu Cys Gly Gly Leu Leu Gln Pro Met Asn
560 565 570

GAT GAT CTG TGG CAG GTC GCC GAC TTT GCA GCC GCT TAC CGC CAG
Asp Asp Leu Trp Gln Val Gly Asp Phe Ala Ala Ala Tyr Arg Gln
580 585 590

GTC GGA CCG CCC GTC GAG TGT ACG GGT CAC ACC ATG TAT GGA TAC TTT
Val Gly Pro Pro Val Glu Cys Thr Gly His Ser Met Tyr Gly Tyr Phe
595 600 605

CTA CCG CTG ATG ACG ATT CTT GGA GGG ATC GTC GAT CTG CAC CAC GCT
Leu Pro Leu Met Thr Ile Leu Gly Gly Ile Val Asp Leu His His Ala
610 615 620

GAG AAT CAT CCG CCC TTT GCC CTG GCG TTC CCC AAT AGC CCG GAG TGG
Glu Asn His Pro Arg Phe Gly Leu Ala Arg Asn Ser Pro Glu Trp
625 630 635

GAG CCG CAG GTA CTG GAC GTC GAC ACG ACA TAT GGG CGC
Glu Arg Gln Val Leu Asp Val Thr Arg Gln Leu Asp Thr Tyr Gly Arg
640 645 650 655

AGC TGG AAG GAA TTC GAG GCC CGC TAC ACC AAC TAC CTG GGG
Ser Leu Lys Gly Phe Glu Ala Arg Tyr Thr Ser Asn Leu Thr Leu Gly
660 665 670

GCT ACG GAT AAC GAG CCT GTC GAA GGT GCC CAC TGG GAT CAC ACG.
Ala Thr Asp Asn Glu Pro Val Val Glu Gly Ala His Leu Asp His Thr
675 680 685

AGT CCT TCG GCC GCC TCC ACC ACC GTC GTA TGG GCA TGG CAC GAG
Ser Pro Ser Gly Arg Ser Ser Ser Thr Val Gly Ser Arg Val Ser Glu
690 695 700

TCC ATC GTC CAC ACG AGG ATG CTG GCC TAC GCC CAC TGG ACG CAT ATG
Ser Ile Val His Thr Arg Met Val Val Ala Tyr Gly Thr His Ile Met
705 710 715

CAC GTC CTG CAT ATT TTG CTC GCC GCA AAA TGG GAC CGG GTG AAT CTG
His Val Leu His Ile Leu Leu Ala Gly Lys Trp Asp Pro Val Asn Leu
720 725 730 735

TTG GAA GAT GAT CTG TGG ATC TCC TCG GAG TCG TTT GTC TCG GCC
Leu Glu Asp His Asp Leu Trp Ile Ser Ser Glu Ser Phe Val Ser Ala
740 745 750

ATG AGC CAT GCG CTC GGT GCC GCA GAA GCA GCC GCA GAA ATC TGG GAG
Met Ser His Ala Val Gly Ala Ala Glu Ala Ala Ala Glu Ile Leu Glu
755 760 765
THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. A combination nucleic acid cassette comprising
   1) a promoter normally associated with a target gene of an
      activating regulator of an inducible enhancer or activator
      sequence,
   2) the nucleic acid sequence encoding the regulator being
      operably linked to a further promoter,
   3) a homologous or heterologous sequence encoding a
      homologous or heterologous protein or peptide,
   said promoter (1) being operably linked to the homologous
   or heterologous encoding sequence, wherein said such
   activating regulator is involved in metabolism.

2. A combination nucleic acid cassette according to claim
   1, wherein said activating regulator is involved in a part
   of metabolism with an enzyme cascade or feed back loop or
   multiple feed back loops and such target gene having a
   binding site for the expression product of the regulator
   gene.

3. A combination nucleic acid cassette according to claim
   1 or 2, wherein the further promoter operably linked to the
   regulator encoding nucleic acid sequence is a promoter
   associated natively with the regulator encoding nucleic
   acid sequence.

4. A combination nucleic acid cassette according to any
   one of claims 1 to 3, wherein the nucleic acid sequence
   encoding the regulator encodes a xylanolytic regulator.

5. A combination nucleic acid cassette according to claim
   4, wherein the nucleic acid sequence encodes xylR.
6. A combination nucleic acid cassette according to any one of claims 1 to 5, wherein the nucleic acid sequence encoding the regulator is any one of:
   a) xlnR encoding the expression product xylR according to the encoding nucleic acid sequence of SEQ ID NO. 9, or
   b) a nucleic acid sequence capable of hybridising under specific minimum stringency conditions as described in the examples to the encoding SEQ ID NO. 9 or primers or probes of nucleic acid SEQ ID NO. 9, said primers or probes being present in the non-zinc finger binding region and said primers or probes being at least 20 nucleotides in length, or
   c) a nucleic acid sequence encoding an expression product exhibiting 80% to 100% identity with the amino acid sequence of xylR according to SEQ ID NO. 9 or as encoded by the nucleic acid sequence encoding xylR of SEQ ID NO. 9.

7. A combination nucleic acid cassette according to any one of claims 1 to 6, wherein the promoter (1) is selected from a promoter associated with the target genes xlnA, xlnB, xlnC, xlnD and axeA.

8. A combination nucleic acid cassette according to any of claims 1 to 7, wherein the promoter (1) is a promoter associated with the target gene xlnD.

9. A combination nucleic acid cassette according to any of claims 1 to 8, wherein the homologous or heterologous sequence encodes an enzyme.

10. A combination nucleic acid cassette according to any of claims 1 to 9, wherein the homologous or
heterologous sequence encodes a xylanase, glucanase, α-glucuronidase, lipase, esterase, ferulic acid esterase, a protease or an oxidoreductase such as hexose oxidase.

11. A vector comprising a combination nucleic acid cassette according to any one of claims 1 to 10.

12. A host cell comprising a combination nucleic acid cassette according to any of claims 1 to 10 and/or a vector according to claim 11.

13. A host cell comprising the components of claim 1, said host cell comprising a target gene of the regulator either natively or through recombinant DNA technology with the proviso that when the target gene and the regulator are native to the host cell, the regulator is present in multiple copies.

14. A host cell comprising multiple copies of the nucleic acid sequence encoding the regulator as defined in claim 1.

15. A host cell according to claim 14, wherein the nucleic acid encoding the regulator is any one of:
   a) xlnR encoding the expression product xylR according to the encoding nucleic acid sequence of SEQ ID NO. 9, or
   b) a nucleic acid sequence capable of hybridising under specific minimum stringency conditions as described in the examples to the encoding SEQ ID NO. 9 or primers or probes of nucleic acid SEQ ID NO. 9, said primers or probes being present in the non-zinc finger binding region and said primers or probes being at least 20 nucleotides in length, or
   c) a nucleic acid sequence encoding an expression
product exhibiting 80% to 100% identity with the amino acid sequence of xylR according to SEQ ID NO. 9 or as encoded by the nucleic acid sequence encoding xylR of SEQ ID NO. 9.

16. A host cell according to any of claims 12 to 15 comprising the nucleic acid sequence encoding the regulator as defined in claim 1.

17. A host cell according to claim 16, wherein the nucleic acid sequence encoding the regulator is any one of:
   a) xlnR encoding the expression product xylR according to the encoding nucleic acid sequence of SEQ ID NO. 9, or
   b) a nucleic acid sequence capable of hybridising under specific minimum stringency conditions as described in the examples to the encoding SEQ ID NO. 9 or primers or probes of nucleic acid SEQ ID NO. 9, said primers or probes being present in the non-zinc finger binding region and said primers or probes being at least 20 nucleotides in length, or
   c) a nucleic acid sequence encoding an expression product exhibiting 80% to 100% identity with the amino acid sequence of xylR according to SEQ ID NO. 9 or as encoded by the nucleic acid sequence encoding xylR of SEQ ID NO. 9 and the promoter (1) as defined in claim 1, said promoter being a promoter associated with the target gene xlnD.

18. A host cell according to any of claims 12 to 17 being selected from the group comprising microorganisms and plant cells.

19. A host cell according to any of claims 12 to 18 being selected from fungal cells, preferably filamentous fungal cells.
20. A host cell according to claim 19, said host cell being selected from the genus *Aspergillus*, *Trichoderma*, *Penicillium* and *Fusarium*.

21. A host cell according to any of claims 12 to 20, said host cell being a strain selected from *Aspergillus niger*, *Aspergillus tubigensis*, *Aspergillus aculeatus*, *Aspergillus awamori*, *Aspergillus oryzae*, *Aspergillus nidulans*, *Aspergillus foetidus*, *Aspergillus terreus*, *Aspergillus sydowii*, *Aspergillus kawachii*, *Aspergillus carbonarius* and *Aspergillus japonicus*.

22. A host cell according to any of claims 12 to 20, said host cell being a strain belonging to a genus selected from *Saccharomyces*, *Kluvyeromyces* and *Lactobacillus*.

23. A host cell according to claim 22, said host cell being a strain selected from *Saccharomyces cerevisiae* or *Saccharomyces pombe*.

24. A host cell according to any of claims 12 to 23, wherein the target gene is endogenous to the host cell.

25. A host cell according to any of claims 12 to 24, wherein the target gene is present in multiple copies.

26. Use of a combination nucleic acid cassette according to any of claims 1 to 10 for production of the homologous or heterologous protein or peptide in a manner known per se for producing protein or peptide from nucleic acid sequences encoding protein or peptide.

27. Use of any one of:
   a) *xlnR* encoding the expression product *xy1R* according to the encoding nucleic acid sequence of SEQ ID NO. 9,
   b) a nucleic acid sequence capable of hybridising under
specific minimum stringency conditions as described in the examples to the encoding SEQ ID NO. 9 or primers or probes of nucleic acid SEQ ID NO. 9, said primers or probes being present in the non-zinc finger binding region and said primers or probes being at least 20 nucleotides in length, or
c) a nucleic acid sequence encoding an expression product exhibiting 80% to 100% identity with the amino acid sequence of xylR according to SEQ ID NO. 9 or as encoded by the nucleic acid sequence encoding xylR of SEQ ID NO. 9 for overexpression of a target gene by expressing the nucleic acid sequence in a host cell comprising the target gene operably linked to a promoter normally associated with a target gene of the activating regulator of an inducible enhancer or activator sequence encoded by the nucleic acid sequence with the proviso that when the target gene and the nucleic acid sequence are native to the host cell the nucleic acid sequence is present in multiple copies in comparison to the wild type host cell.

28. Use of a host cell according to any one of claims 12 to 25, for production of the homologous or heterologous protein or peptide in a manner known per se for production of protein or peptide from a nucleic acid sequence encoding a protein or peptide.
29. A combination nucleic acid cassette according to claim 1, substantially as herein described with reference to any one of the Examples.

30. A host cell according to claim 12, substantially as herein described with reference to any one of the Examples.

31. Use according to claim 26 or 27, substantially as herein described with reference to any one of the Examples.

Dated this 13th Day of March 2002

DANISCO INGREDIENTS A/S (DANISCO A/S)
By their Patent Attorneys
GRiffith Hack
fellows Institute of Patent and
Trade Mark Attorneys of Australia
Construction of pIM130

pIM120

ATG

oligonucleotide A

Nsi I

oligonucleotide 1

oligonucleotide 2

ATG

EcoRV

XbaI

pGW635

pyr A

oligonucleotide B

Digestion with Eco RV and Xbal

Isolation of the 2.2kb EcoRV/Xbal fragment

Fragment D

PCR 1

Mix and PCR using oligo's 1+2

Fragment B

PCR 3

Digestion with Nsi I and Eco RV

Nsi I

Eco RV

Fragment C

Ligation in Nsi I/Xba I digested pGEM 7

pIM130

xin A fragment
gox C basic transcription unit

pyr A coding region and terminator
Aspergillus niger

\( \alpha \)-L-arabinofuranosidase

\( \beta \)-Xylanase

\( \beta \)-Xylosidase

Time (h)