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Expression of proteolytic enzymes in koji mold in the presence of carbon sources

The present invention refers to koji molds capable of expressing proteolytic enzymes in the presence of a carbon source in at least the same amount as in the absence thereof. In particular, the present invention pertains to a modification of the expression of the creA gene product as a tool to increase the amount of a wide spectrum of proteolytic enzymes in the presence of a carbon source.

State of the art

Hydrolyzed proteins, which are widely used in the food industry, may be prepared by hydrolytic degradation of protein material with acid, alkali or enzymes. As regards a treatment of the material with acid or alkaline this procedure has been shown to also destroy essential amino acids generated during hydrolysis thus reducing the nutritional value of the final product. On the other hand hydrolysis by addition of enzymes rarely goes to completion so that the hydrolyzed protein material still contains substantial amounts of peptides. Depending on the nature of the protein and the enzymatic components utilized for proteolysis, the peptides formed may, however, lead to extremely bitter tastes and are thus organoleptically undesirable.

In some methods instead of chemical or isolated biological material microorganisms as such are employed for this purpose. In these cases the proteinaceous material available is hydrolyzed by the action of a large variety of enzymes, such as amylases, proteinases, peptidases etc., that are secreted by the microorganism.

One class of such microorganisms are koji molds that are traditionally used for making koji cultures (see e.g. US 4,308,284). These molds comprise e.g. microorganisms of the genus Aspergillus, Rhizopus and/or Mucor, in particular Aspergillus soyae, Aspergillus

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oryzae, Aspergillus phoenicis, Aspergillus niger, Aspergillus awamori, Rhizopus oryzae, Rhizopus oligosporus, Rhizopus japonicus, Rhizopus formosaensis, Mucor circinelloides, Mucor japanicus, Penicillium glaucum and Penicillium fuscum.

According to the rules of the International Code of Botanical Nomenclature (ICBN), Aspergillus is an anamorphic genus. This means that true Aspergilli only reproduce asexually through conidiophores. However, the typical Aspergillus conidiophore morphology may also be found in fungi that may reproduce sexually via ascospores. Some Aspergillus taxonomists caused confusion, because they did not adhere to ICBN terminology. Instead, they attempted to make various revisions of taxonomical schemes to include Aspergillus nidulans in this genus, despite the fact that its taxonomically correct name is Emericella nidulans (Samson, In: Aspergillus. Biology and Industrial Applications, pp 355-390, ed. by Bennett and Klich, Boston). In effect, the microorganism termed Aspergillus nidulans may be considered not to belong to the genus Aspergillus itself.

In EP 0 417 481 a process for the production of a fermented soya sauce is described, wherein a koji is prepared by mixing a koji culture with a mixture of cooked soya and roasted wheat. The koji thus obtained is then hydrolyzed in an aqueous suspension for 3 to 8 hours at 45 °C to 60 °C with the enzymes produced during fermentation of the koji culture, a moromi is further prepared by adding sodium chloride to the hydrolyzed koji suspension, the moromi is left to ferment and is then pressed with the liquor obtained being pasteurized and clarified.

EP 0 429 760 describes a process for the production of a flavoring agent in which an aqueous suspension of a protein-rich material is prepared, the proteins are solubilized by hydrolysis of the suspension with a protease at pH 6.0 to 11.0, the suspension is heat-treated at a pH of 4.6 to 6 and is subsequently ripened with enzymes of a koji culture.

Likewise, European patent application 96 201 923.8 describes a process for the production of a meat flavor, in which a mixture containing a vegetal proteinaceous source and a vegetal carbohydrates containing source is prepared, said mixture having initially at least 45% dry matter, the mixture is inoculated with a koji culture and by one or more other species of microorganisms involved in the traditional fermentation of meat, and the mixture is incubated until meat flavors are formed.

Yet, all the processes involving the use of different microorganisms also show the disadvantage that the protein material is not hydrolysed completely while a longer incubation of the material with the microorganisms to achieve a substantial hydrolysis may lead to the formation of unwanted metabolic side products.

Thus there exists a need in the art for optimizing said hydrolysis processes. Yet, said optimization and further development of koji processes have been seriously hampered by the lack of knowledge on the nature of the hydrolytic enzymes involved, their regulation and the influence of process parameters on their expression and activity, e.g. temperature, pH, water activity and salt concentration.

From Katz et al., Gene **150** (1994), 287-292 it is known that in the fungi Emericella nidulans the expression and secretion of proteolytic enzymes, that are inherently used by the microorganism to provide the nitrogen-, sulfur- and carbon sources required for its proliferation, is subject to at least three general control circuits including carbon catabolite repression, nitrogen- and sulfur-metabolite repression.

These three regulatory circuits ensure that the available nitrogen-, carbon- and sulfur-sources in a substrate are utilized sequentially according to their nitrogen-, energy- and sulfur-yield. Nitrogen metabolite repression has been found to be inter alia exerted by the areA gene product in Emericella nidulans (Arst et al., Mol. Gen. Genet. **26** (1973), 111-141,), whereas in other fungi it is assumed that possibly other genes are deemed to be responsible for said function. In fact, most fungi that have been studied seem to have an areA homologue performing said function.

In wheat bran fermentations performed with Aspergillus oryzae, proteolytic activity could only be detected when the glucose concentration dropped below a certain threshold. These observations suggest that any expression of proteolytic enzymes in A. oryzae is not induced by the presence of proteins but seems to be merely carbon-derepressed. During a fermentation process utilising soy kojis a significant amount of glucose has been found to be liberated as result of amylase activity which eventually results in a carbon catabolite repression of protease-encoding genes.

Hence, there is a need for an improved method for hydrolyzing proteins leading to high degree of protein hydrolysis and to hydrolysates with excellent organoleptic properties.

Summary of the Invention

This object has been solved by providing a koji mold belonging to the genus Aspergillus, Rhizopus, Mucor or Penicillium, the proteolytic activity of which is not carbon repressed.

According to the invention, in said microorganisms the expression of the creA gene has been modified such that the gene product thereof gives rise to a polypeptide exhibiting a decreased or no binding affinity at all to DNA sequences responsible for blocking the transcription of proteases.

In another preferred embodiment the synthesis of the creA gene is modified in such a way that the corresponding gene product is substantially not transcribed or not transcribed at all or not translated to a functional product. This may e.g. be achieved by means of introducing a construct into the genome of the microorganism that gives rise to a creA anti-sense mRNA thus preventing translation of the creA gene into a functional polypeptide. On the other hand also mutations may be introduced into the creA gene so that no transcription takes place. Eventually, the creA gene may also be entirely deleted so that no repression takes place in the presence of a carbon source.

The mutations leading to the microorganism having the desired traits may be obtained via classical techniques, such as mutation and selection or by using genetic engineering techniques, with which a selective mutation in the creA gene may be achieved.

In addition, a creA mutation may also be combined with the property of an increased production of the areA gene, a positive stimulator for the production of proteases.

Detailed Description of the Invention

In the Figures:

Fig. 1 is a restriction map of a λGem12 clone. The coding region was localised on a 4.3 kB PstI-SpHI fragment that was subcloned in pUC19.

Theoretically, generating mutations in the creA gene, that diminish or even interrupt binding of the gene product thereof to the corresponding DNA sequences should lead to an earlier onset of protease production in wheat bran kojis, resulting in a higher protease yield and thus to an increased secretion of proteases. Also, in soy kojis creA mutations would theoretically alleviate carbon catabolite repression of protease production and should result in higher protease production.

Yet, in Gene **130** (1993), 241-245 M. Drysdale et al. reported that in A. nidulans a deletion of the creA gene together with flanking sequences leads to a lethal phenotype. It was therefore assumed that in addition to its role as a repressor protein creA has still other viable regulatory roles without which the microorganism is not capable to proliferate and grow.

In contrast to this general belief the present inventors have surprisingly found that it is in fact possible to create viable creA mutants, that are capable to express a wide variety of different proteolytic enzymes even in the presence of a carbon source.

In order to achieve this objective the following procedure has been adopted.

It has been assumed that creA mutants may be isolated as areA suppressor mutations. The areA gene is one of several genes involved in the activation of the transcription of a wide variety of proteolytic polypeptides. The areA gene is controlled by the presence or absence of intracellular glutamine, which in effect represents a nitrogen dependent control.

A. oryzae NF2 (CNCM 1882), an areA null-mutant described in detail in EP 97111378.2, which document is incorporated herein by way of reference, has been shown to be unable to grow on minimal medium (see below) containing 0.2% soy protein and 50 mM glucose. The same mutant was also incapable to grow in wheat gluten koji.

In an areA null-mutant, the areA gene product no longer stimulates the transcription of protease encoding genes, resulting in the microorganisms to exhibit a decreased protease secretion.

In addition, in the presence of a carbon source, such as glucose, fructose or saccharose, the creA gene product represses transcription of protease encoding genes eventually resulting in an incapability of the areA null mutant to use protein as a nitrogen source. Consequently, area null mutants with an operative creA gene should be unable to proliferate and grow in such an environment.

In order to isolate creA mutants, areA null mutants of A. oryzae may be subjected to mutagenic agents in the above mentioned medium (0.2 % soy protein, 50 mM glucose), such as e.g. UV irradiation, treatment with EMS (Ethyl methane sulfonate), methyl methane sulfonate or DMSO, nitrosoguanidine, etc..

Theoretically, in at least some colonies that are capable to grow on the medium the creA gene should have been mutated such that the gene product thereof may not exert its normal action thus allowing for the growth in such a medium (see above).

The colonies may then be analysed for the presence of an increased proteolytic activity, which may be achieved e.g. by means of determining the activity of enzymes that are under control of creA, such as alcohol dehydrogenase, amylase, acetamidase etc..

For example, colonies growing in the above referenced medium may be investigated for hypersensitivity towards Fluor-acetate. In wild type strains an active creA protein prevents the induction of acetate utilisation enzymes in the presence of glucose. Under this condition Fluor-acetate is not metabolised. Yet, in creA mutants, in which the creA gene product does not take over its inherent function, these acetate utilisation enzymes are transcribed in an essentially constitutive manner. As a result, Fluor-acetate will be converted to compounds that are toxic for the microorganisms. The visual result resides in that strains, having a mutation in the creA gene which renders the gene product essentially ineffective, will not grow in a medium containing Fluor-acetate and a carbon source.

CreA mutants may also be identified according to their hypersensitivity towards allyl-alcohol in the presence of a carbon source. In wild type strains the active creA protein normally prevents the induction of alcohol dehydrogenase, that oxidises the above substrate to ketone acreoline, a compound toxic for the microorganism. Under repressive conditions, i.e. in the presence of a carbon source, the allyl-alcohol will normally not be oxidised to the toxic compound due to creA exerting its inherent function to repress the transcription of alcohol dehydrogenase. However, in mutants in which the creA gene is not functional any more, alcohol dehydrogenase is essentially expressed constitutively, intoxicating the mould with acreoline even in the presence of the carbon source.

In addition to the above random mutagenesis of an areA null mutant by mutagenic agents and selection for the desired trait the creA gene may also be modified in a suitable way by means of genetic engineering.

To this end, a construct may be incorporated in the moulds' genome, comprising a DNA sequence being transcribed into an anti-sense RNA to creA. This may be achieved by techniques well known in the art such as is e.g. described in Maniatis, A Laboratory manual, Cold Spring Harbor, 1992. This option provides for the advantage that the action of the anti-sense RNA itself may be controlled in a suitable way by rendering the transcription dependent on the presence or absence of particular molecules known to induce transcription in a given system. Vectors to clone a given DNA fragment as well as promotors and their way of induction are well known in the art and may e.g. be found in Maniatis, supra.

Further, the creA gene may well be modified in such a way that the gene product thereof is substantially or even entirely ineffective. This may be effected by introducing mutations into the DNA sequence so that the corresponding polypeptide looses its capability of exerting its regulatory action by e.g. binding to the corresponding regulatory DNA sequences. Moreover, the creA gene may partly or even entirely be deleted so that no repression takes place at all in the presence of a carbon source.

It has now been found that in spite of the difference in relation the creA gene of A. oryzae may be is isolated using a DNA sequence comprising the coding region of the corresponding gene of Aspergillus nidulans as a probe, however, applying low stringent conditions during hybridisation.

Due to the low stringency conditions applied a plurality of different colonies were initially isolated which could subsequently be excluded by increasing the conditions of stringency.

After having isolated DNA of strongly hybridising colonies the complete A. oryzae creA gene could be assigned to a 4.3 KB PstI-SphI fragment, which could be cloned into a suitable vector, such as a plasmid or a viral vector and sequenced. The sequence obtained thereby is shown under SEQ ID NO I, below.

In analysing the DNA sequence a potential open reading frame could be found yielding a polypeptide having the amino acid sequence identified as SEQ ID NO II, below

The DNA sequence thus identified may then be used to introduce specific mutations into the creA gene. This may be effected by e.g. cloning the fragment in a suitable vector, such as the high copy number vector pUC or M13, deleting part of the coding sequences or introducing a stop codon in the reading frame and introducing the modified creA gene into an *are*A mutant, like A. oryzae NF2 (CNCM 1882). *Cre*A-*are*A double mutants can then be selected on minimal medium (below) containing protein (i.e. 0.2% soy) and 50 mM glucose by their ability to grow, whereas an *are*A mutant will not grow.

In determining for an effective transfer of a suitably modified construct in a wild type background a marker such as e.g. a resistance gene may be utilised, that may be deleted from the moulds' genome after having isolated a creA mutant having the desired traits. Techniques for cloning, introducing mutations and/or deletions into a given gene and for introducing DNA sequences into a microorganism are known in the art and may be e.g. found in Maniatis et al., supra.

The following examples further illustrate the invention.

Strains & plasmids

A. nidulans G332 (pabaA1, yA2, xprD1), used to amplify the creA gene,- was obtained from the Glasgow Genetic Stock Centre via Dr. A.J. Clutterbuck. A. oryzae TK3 (aflR1, omtA1), were obtained from the strain collection of the Nestlé Research Center Lausanne. A. oryzae NF1 (pyrG1) is a uridine auxotroph derivative of A. oryzae TK3

in which the pyrG gene, encoding orotidine 5'-phosphate decarboxylase, was inactivated by targeted disruption. A. oryzae NF2 (CNCM 1882) is an *areA* disruption mutant, derived from *A. oryzae* NF1 as described in EP 97111378.2.

The vector LambdaGem-12 was obtained from Promega, pUC19 (Yanisch-Perron C., Vieira, J. and Messing, J. Improved M13 phage cloning vectors and host strains: nucleotide sequences of M13mp18 and pUC19; Gene **33** (1985), 103-119) was obtained from New England Biolabs Inc. USA.

Media

Minimal medium (MM) contains per litre 1.5 KH_2PO_4 (Merck, Darmstadt, FRG), 0.5 g $MgSO_4.7H_2O$ (Merck, Darmstadt, FRG), 0.5 g KCl (Merck). For selection of mutants 50 mM Glucose ((Merck, Darmstadt, FRG), 0.2% Soy Protein (Protein Technologies International) and 2% agar noble were added to MM. Protease plate assays were performed either on MM with 0.08% sodium desoxycholate (Fluka, Buchs, Switzerland) and 0.2% soy protein as sole carbon and nitrogen source or on MM with 1% skimmed milk (Difco) and 2% agar noble (Difco)

Example 1

Isolation of creA mutants

To isolate creA mutants relevant to the production of proteolytic activity, areA null mutants have been created as described in EP 97111378.2. Further, 108 conidiospores of A. oryzae NF2 (CNCM 1882) were UV irradiated (500 mJ/cm2 254 nm, 50% survival) and plated on minimal medium containing 0.2% soy protein, 50 mM glucose and 2% agar noble (Difco). Four sporulating colonies, termed NF14 to NF17 were selected, that were found to be sensitive to 15 mM allyl alcohol in the presence of 50 mM glucose, suggesting that these four mutants were creA mutations. Furthermore, NF14 to NF17 were shown to secrete proteases in the presence of glucose.

Example 2

Isolation of the creA gene

A genomic library of Aspergillus oryzae TK3 (supra) in GEM 12 was screened under low stringency conditions (55° C, 5xSSC, 1% SDS) with a 1.3 KB PCR product encompassing the coding region of the A. nidulans creA gene.

A total of 100 positive clones were propagated and again hybridised with the probe under conditions of slightly increased stringency by increasing the temperature to about 60 °C. In the following three of the most strongly hybridising clones were isolated.

The A. oryzae creA gene was subcloned from a Gem12 clone as a 7.3 KB BamHI fragment. By Southern analysis, the coding region was localised on a 4.3 KB PstI-SphI fragment that was subcloned in pUC19 generating pNFF212 and completely sequenced. The nucleotide and deduced amino acid sequence of the *A. oryzae cre*A gene is given below. Sequence motifs in the putative promoter region that fit the SYGRGG consensus of CREA DNA-binding sites (Kulmburg *et al.*, 1993) are singly underlined and marked in bold. The region encompassing the DNA-binding C_2H_2 Zn-finger region in the CREA protein (Dowzer *et al.*, 1989) is doubly underlined and in bold.

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-1120 CTGCAGTTCCAGTTTCTACCCCGTAAATCCCTATCAACTTAGTCCGCCCCACATTCTTTT   -1061
-1060 TTTTTTTTCCTTTTTTTTTCGCTCCCGGTCAGAGTGATAGTGGGATTTATTACACACCGT   -1001
-1000 GCGTGGTCGAAGAACGACACGGAAGAAGCCCCGGAAGACGCCTTCTCTAGGCAACAAATG    -941
 -940 ATTGTACTCTTATGATACTCAATACGGTAGAAAATAGAGAATTGAGATACGAAAGCTGAC    -881
 -880 TCATCAGAACAGAATAAGGGGAATTTTTGATTAGCAAATAACAATAATAATTATACAAAA    -821
 -820 AAACAAATAAAAAAATTTAGGGGACTCCCCACCCGCTGTAATCCTGGGTGTATCTCAAAG    -761
 -760 CAAAGCAGGCGATCTGGGGGGGAGCACGTTCTTTTTTTTTCTTTTCTCTTTTTTCTATTTT    -701
 -700 TTTTTTTTTTTTTATTTTAGGTCTATGCCTTTTTTTTTCTTTTCCTTTTTTTTTTTTTTT    -641
 -640 TTTGCCCCCCCGATAATTCTCCCCACACATAGGACATACTTTTTTTTTTTTTTCCTTCCACT    -581
 -580 CCCTTCAAGGTCTCCGATTCCGATAACCCCCTCTACCAGTTCGCCCTGCCTTTTTCTCTC    -521
 -520 CCCTCCCCCCGAAGCTCCATTTCTCTCTTCTTCCCCTCCATTCCTCATTCTTCCTCTTCCG    -461
 -460 TATTTCCTTTATATGCTCCTATCCCCAGACCATTTCTCCAGATTTCTCTCTCTTTCCCCT    -401
 -400 CTCTCCCTTTCGACAAATTGTTGCTTGACTACATCCATCTCGGGTTACCTACTTACAGTA    -341
 -340 CCAATTCCGGATATACTCTATCCCACCCATCACCACATTCCATAACAGCGCCCTTTCATT    -281
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-280 GGGAAAGTCACTCTTCCTTGAAATTGGTTACATCGCGGACCATCGTACCTTCTTTAATCG   -221
-220 CAAGGCTTGTGATACTCTTGCGGTGCTCGTTCATCAACTAGTACTTTGCCAAGAGCAAGT   -161
-160 CTCCGTCTTGTCGGGTGGTGATCGACTCTCCCCGATTTACCTACCCCTGTTGCGACGAAT   -101
-100 CCTGATTCGCCTCGGCTCGTCAGCCCTTCCGAGCTTCCCTTAAGTACAGGCTTCGTCCCC   - 41
 -40 TCTTTAGCTGCACTCCTCGGTGCTAGGTTAGGACGAGTCACATGCCACCACCGGCTTCTT    19
                                                MetProProProAlaSerS

  20 CAGTGGATTTCACCAATCTGCTGAACCCTCAGAATAACGAGACTGGTTCTGCACCTTCCA    79
     erValAspPheThrAsnLeuLeuAsnProGlnAsnAsnGluThrGlySerAlaProSerT

  80 CGCCAGTGGATAGCTCCAAGGCTCCCTCTACCCCGTCCAGTACTCAGTCCAACTCTACCA   139
     hrProValAspSerSerLysAlaProSerThrProSerSerThrGlnSerAsnSerThrM

 140 TGGCCTCGTCTGTTAGCTTACTACCGCCCCTCATGAAGGGTGCTCGTCCCGCAACGGAAG   199
     etAlaSerSerValSerLeuLeuProProLeuMetLysGlyAlaArgProAlaThrGluG

 200 AAGCGCGCCAGGATCTTCCCCGTCCATACAAGTGTCCCCTGTGTGATCGCGCCTTCCATC   259
     luAlaArgGlnAspLeuProArgProTyrLysCysProLeuCysAspArgAlaPheHisA

 260 GTTTGGAGCACCAGACCAGACATATTCGCACACATACGGGTGAAAAGCCACACGCTTGCC   319
     rgLeuGluHisGlnThrArgHisIleArgThrHisThrGlyGluLysProHisAlaCysG

 320 AGTTCCCGGGCTGCACAAAACGCTTTAGTCGCTCTGACGAGCTGACACGCCACTCAAGAA   379
     lnPheProGlyCysThrLysArgPheSerArgSerAspGluLeuThrArgHisSerArgI

 380 TTCACAACAACCCCAACTCCAGGCGGAGTAACAAGGCACATCTGGCCGCTGCCGCTGCCG   439
     leHisAsnAsnProAsnSerArgArgSerAsnLysAlaHisLeuAlaAlaAlaAlaAlaA

 440 CTGCCGCTGCCGGACAAGAGAATGCAATGGTAAATGTGACCAACGCGGGCTCGTTGATGC   499
     laAlaAlaAlaGlyGlnGluAsnAlaMetValAsnValThrAsnAlaGlySerLeuMetP

 500 CCCCGCCCACAAAGCCTATGACCCGCTCTGCGCCTGTCTCTCAGGTTGGATCTCCGGATG   559
     roProProThrLysProMetThrArgSerAlaProValSerGlnValGlySerProAspV

 560 TCTCCCCTCCGCACTCCTTCTCGAACTATGCCGGTCACATGCGTTCCAATCTGGGACCAT   619
     alSerProProHisSerPheSerAsnTyrAlaGlyHisMetArgSerAsnLeuGlyProT

 620 ATGCTCGCAACACCGAGCGGGCGTCCTCGGGAATGGATATCAATCTACTTGCCACCGCTG   679
     yrAlaArgAsnThrGluArgAlaSerSerGlyMetAspIleAsnLeuLeuAlaThrAlaA

 680 CATCTCAGGTTGAGCGTGATGAACAACATTTTGGGTTCCACGCTGGTCCACGTAATCACC   739
     laSerGlnValGluArgAspGluGlnHisPheGlyPheHisAlaGlyProArgAsnHisH

 740 ATTTGTTCGCCTCGCGTCACCACACCGGTCGTGGCCTGCCTTCCCTTTCAGCGTACGCCA   799
     isLeuPheAlaSerArgHisHisThrGlyArgGlyLeuProSerLeuSerAlaTyrAlaI
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800 TCTCGCACAGCATGAGCCGTTCTCACTTTCACGAGGACGAGGATGGTTACACTCATCGCG 859
leSerHisSerMetSerArgSerHisPheHisGluAspGluAspGlyTyrThrHisArgV

860 TCAAGCGCTCAAGGCCTAACTCACCAAACTCGACCGCTCCGTCCTCACCGACTTTCTCTC 919
alLysArgSerArgProAsnSerProAsnSerThrAlaProSerSerProThrPheSerH

920 ACGACTCTCTTTCCCCAACGCCAGACCACACTCCGTTGGCAACCCCTGCTCATTCGCCAC 979
isAspSerLeuSerProThrProAspHisThrProLeuAlaThrProAlaHisSerProA

980 GCTTGAGGTCATTGGGATCTAGCGAACTCCACCTTCCTTCGATTCGCCATCTGTCCCTCC 1039
rgLeuArgSerLeuGlySerSerGluLeuHisLeuProSerIleArgHisLeuSerLeuH

1040 ATCACACCCCTGCCCTTGCTCCAATGGAGCCCCAGCCGGAAGGCCCCAACTATTACAGTC 1099
isHisThrProAlaLeuAlaProMetGluProGlnProGluGlyProAsnTyrTyrSerP

1100 CCAGCCAGTCTCATGGTCCCACAATCAGCGATATCATGTCCAGACCCGACGGAACACAGC 1159
roSerGlnSerHisGlyProThrIleSerAspIleMetSerArgProAspGlyThrGlnA

1160 GTAAACTGCCCGTTCCACAGGTTCCCAAGGTCGCGGTGCAAGATATGCTGAACCCCAGCG 1219
rgLysLeuProValProGlnValProLysValAlaValGlnAspMetLeuAsnProSerA

1220 CTGGGTTTTCGTCGGTTTCCTCATCGACGAATAACTCTGTCGCAGGAAATGATTTGGCAG 1279
laGlyPheSerSerValSerSerSerThrAsnAsnSerValAlaGlyAsnAspLeuAlaG

1280 AACGTTTCTAGCCTGGTGCGGCTGCGAAACCCTTTCAATGTATAAAGTTTTGGGCTCAAA 1339
luArgPheEnd

1340 AAAAATTCTTGACTGTCATACGCGCTACGAAACGAATAGACTTTGTGCATTTACAGTGCG 1399
1400 TGGTTCATGGGCATCCTTGGTGTCGGCTGGCTTTCTTTGCTTACTTTGTTCGAGTATACT 1459
1460 TTTGCGAGGCGTCCATAGTGATAGACGGGTGGGATATTCTTGTGGCTTTTTCCGTGCTTG 1519
1520 TTCGATTCTCCCCTTTCGCTCTCCTTGAAAAATACCTTTCTTATCCTATAACCATTTGTT 1579
1580 TCATTATCCCAATGGGAATTGGCTCTACAGCTCTTATTCATTTTGTCTACTCCTCTCCTG 1639
1640 AGGCCCAGTCCCCTGATAATTCCGGGCTCTACCATATACATTTCATTTCGACTATGTCAG 1699
1700 TTTCCGCTTCGATTTAGACCTCGAGCAGGACGAGAGGGTTCCGAAAGAAAATACAAACAA 1759
1760 AAATTATAGTAATCTGCGTTTACTTTGGCATAATACAGTAGTCATTAGTTGAGGTAGGCA 1819
1820 TAATCTGGATGTCTAACCATCACTTGCCCTAACCTCCTACCATCTGCTGCTAGTATTTGT 1879
1880 CTTACCCGAAACCCAATTCAACGAGATAGATGGATTGACGAATAACAATTTGTTGTCCAG 1939
1940 CGACATGCATGATACATGCGTACGTACATACACTAATAGTAGTCACAGACCAGTTCATCA 1999
2000 CATCCTGGTCTCGGGTATTCAGATACGGAAATGCGTAAGATTGGAAGGGTCTAAGAAAAA 2059
2060 GCAAAGAAAAAGGAAAAGTTAACACTGGCTGGCGCTCTCTTTCCATCTCTGATCAATGTT 2119
2120 ATTGTTCGTCACTCAGCTGTGGACGTGGCTCCAGTCAAGTTGTGAATTATGATAGGGTAT 2179
2180 TGTTGACTTGACAAGTTGATCTTGATGGAATCAAATCTTCTCCCCGCCAGATTCTGACGC 2239
2240 TTGAGGCTCTCGGATCGAATGAACAACTTTTCGCACCACATCAACCGGTTGCCGCGTGAT 2299

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2300 GCTGGAGACAAACCGACCCAAACGTCACGGTCACACGGAGGATACGTTTGCTAGAGCCAG  2359
2360 CTGATACCCCAAGAGACAAGAAGGTAAAGGTCGCAAAAATCTTTTCAATAAGATGGCATC  2419
2420 TTCCCCCCACCAACCCTTAACCATTCTCCTTTCAAGCTGTGTTGCCCCGCTTTGGTGCAT  2479
2480 GGGCTTGGGTAGTGCGGTCGCAAAACTACTAATTTAATGACCGACTGCTGCTGCTTTTTC  2539
2540 ACTCGCCGCTCACGGACTAAGCATGTGGGAACAGGATCGCCCCGTCACTATTTCAGATCG  2599
2600 TGTCGTATCAAGGTGTTCGCCCGGTGCTGCTGGCACGAACGCCGGCCATCCAAGATCATT  2659
2660 GTTCTCATTCAAACCGGGCGGCTTACGTCTAGCCGCGGACGTAAGCACGAAGAGTGTGTG  2719
2720 TAGTGGTGGGAGTGAAGCCGTTGCCGAAACCATGCCGTCCTCCACGGCCGTCCCGTCGTT  2779
2780 ATCAAGCGACGCTGCCTCCGCTTCATCCTCATCAGCGGGTGTATCTCTGGAGACAAGATG  2839
2840 GGCGGAAGGTCTCACCGGCCAGGAGATATTAGAAGACGATGGAACGGGCGCGCTCGTCGT  2899
2900 CCCGCCGTCCCGCCCTGCTCGGCAATATCATCACCATACCTATATCTGTCTGTTCTATAT  2959
2960 CTTAGATTGTCACCACACCTTCGACGATGTCGAGCAATGGAAGACTCACGTTCTGAGCCA  3019
3020 CTTCCGAACCCACGAACCACCGCGAACAGCCCGATGCCCTCTATGTCCGGGTGAGCGGTT  3079
3080 CAGCGACACCCCCGAACAGAAAGGATGGGATCGCATGC                        3117
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Example 3

Genetic modification of the creA gene

In the DNA sequence stop codons were introduced at position +226-228 and +229-231, changing the sequence TACAAG encoding the dipeptide TyrLys into TAGTAG (StopStop).This mutation was introduced into pNFF212 by site directed mutagenesis using oligonucleotide CTTCCCCGTCCATAGTAGTGTCCCCTGTG and its complement CACAGGGGACACTACTATGGACGGGGAAG as described in the Quickchange protocol (Stratagene, Basel).

This mutation results in a truncation of the *cre*A gene product N-terminal of the DNA binding zinc finger domain, rendering it inactive. By introducing this construct into the A. oryzae NF2 (CNCM 1882, EP 97111378.2), *cre*A-*are*A double mutants could be selected directly on plating the microorganisms on MM plates containing 0.2% soy protein and 50 mM glucose solidified with 2% agar noble.

Example 4

Modification of the creA gene

Further, the creA gene was deleted from the molds genome as follows. pNFF212 was partially digested with EcoRI and the linear molecule was recovered from an agarose gel. After dephosphorylation and ligation to the 1843 bp *A. nidulans pyrG* fragment from pNFF38 (A. Doumas, P van den Broek, M. Affolter, M. Monod (1998) Characterisation of the Prolyl dipeptidyl peptidase gene (*dpp*IV) from the Koji mold *Aspergillus oryzae,* Applied and Environemental Microbiology **64**, 4809-4815), pNFF234 was generated. In pNFF234, the creA coding region is interrupted by a functional A. nidulans pyrG gene, truncating the gene product immediately downstream of the DNA binding zinc finger.

To obtain a creA mutant, pNFF234 was digested with *Bst*XI and introduced into *A. oryzae* NF1 by transformation. The primary transformants are selected on MM without uridine and screened for hypersensitivity towards allyl-alcohol and Fluor-acetate in the presence of 50 mM glucose. Sensitive transformants were then tested for the desired gene replacement by Southern analysis or PCR.

Example 5

Test for expression

In order to further prove a mutation in the creA gene several tests were performed.

1) Amylase test

The strains obtained in example 1 were grown on minimal medium (supra) containing 1% starch and 50 mM glucose as carbon source. Under these conditions wild type strains, in which the amylases are repressed by glucose, will not produce a halo when stained with a KI solution. In contrast thereto a creA mutant will produce a halo, since amylase expression is no longer repressed by glucose. All three colonies isolated in example 1 did produce a halo.

2) Acetamidase test

Strains can also be assayed for acetamidase activity when grown on a minimal medium (supra) containing acetamide and glucose as carbon source. Under these conditions wild type strains do not produce acetamidase activity, whereas a creA mutants do.

3) Halo production

On minimal medium plates containing 1-% skimmed milk and 50 mM glucose (initially turbid appearance of the plate) creA mutants exhibit a halo after 2 days at 30°C, whereas wild type strains do not.

Claims

1. A koji mold belonging to the genus Aspergillus, Rhizopus, Mucor or Penicillium, the proteolytic acitivity of which is not carbon repressed.

2. A koji mold according to claim 1, wherein the creA gene does not exert its inherent function.

3. A koji mold according to claim 2, wherein the creA gene is not transcribed to a mRNA capable to be translated to a functional polypeptide.

4. A koji mold according to any of the claims 1 to 3, wherein the creA gene has been mutated such that the gene product thereof is essentially non functional.

5. A koji mold according to claim 1, wherein the creA gene has been deleted.

6. A koji mold according to claim 1, which is Aspergillus oryzae I-2165 (NF14)

7. A koji mold according to claim 1 to 5, wherein the areA gene or a functional derivative thereof is overexpressed.

8. A method of producing proteolytic enzymes, comprising cultivating a koji mold according to claims 1-7 in a suitable growth medium in the presence of a carbon source under conditions that the mold expresses proteolytic enzymes, and optionally isolating the enzymes in the form of a concentrate.

9. Use of the koji mold according to claim 1-7 for the hydrolysis of protein-containing materials.

10. Use according to claim 8, in combination with an enzyme and/or a microorganism providing a prolidase activity.