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(54) Title: ENZYMES FOR STARCH PROCESSING

(57) Abstract: The present invention relates to a hybrid enzyme comprising carbohydrate-binding module amino acid sequence and a fungal alpha-amylase amino acid sequence and to a variant of a fungal wild type enzyme comprising a carbohydrate-binding module and an alpha-amylase catalytic module. The invention also relates to the use of the hybrid enzyme or the variant in starch liquefaction

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ENZYMES FOR STARCH PROCESSING**CROSS-REFERENCE TO RELATED APPLICATIONS**

This application claims the benefit of U.S. Provisional application nos. 60/569,862, filed May 10, 2004, 60,490,751, filed July 29, 2003, 60/511,044, filed 5 October 14, 2003, 60/482,589, filed June 25, 2003, and 60/514,854, filed October 27, 2003 and priority from Danish application nos. PA 2003 00949, filed June 25, 2003, and PA 2003 01568, filed October 24, 2003, the contents of which are fully incorporated herein by reference.

FIELD OF THE INVENTION

10 The present invention relates, inter alia, to an enzyme comprising a carbohydrate-binding module ("CBM") and an alpha-amylase catalytic domain. The enzyme may be a hybrid between a carbohydrate-binding module ("CBM") and an alpha-amylase or the enzyme may be a variant of a parent enzyme comprising a carbohydrate-binding module ("CBM") and an alpha-amylase catalytic domain. The invention also relates to the use of the enzyme in a 15 starch liquefaction process in which starch is degraded to smaller oligo- and/or polysaccharide fragments.

BACKGROUND OF THE INVENTION

A large number of enzymes and processes have been described for converting starch to starch hydrolysates, such as maltose, glucose or specialty syrups, either for use as 20 sweeteners or as precursors for other saccharides such as fructose. Glucose may also be fermented to ethanol or other fermentation products, such as citric acid, monosodium glutamate, gluconic acid, sodium gluconate, calcium gluconate, potassium gluconate, glucono delta lactone, or sodium erythorbate, itaconic acid, lactic acid, gluconic acid; ketones; amino acids, glutamic acid (sodium monoglutamate), penicillin, tetracyclin; 25 enzymes; vitamins, such as riboflavin, B12, beta-carotene or hormones.

Starch is a high molecular-weight polymer consisting of chains of glucose units. It usually consists of about 80% amylopectin and 20% amylose. Amylopectin is a branched polysaccharide in which linear chains of alpha-1,4 D-glucose residues are joined by alpha-1,6 glucosidic linkages.

30 Amylose is a linear polysaccharide built up of D-glucopyranose units linked together by alpha-1,4 glucosidic linkages. In the case of converting starch into a soluble starch hydrolysate, the starch is depolymerized. The conventional depolymerization process

consists of a gelatinization step and two consecutive process steps, namely a liquefaction process and a saccharification process.

Granular starch consists of microscopic granules, which are insoluble in water at room temperature. When an aqueous starch slurry is heated, the granules swell and eventually burst, dispersing the starch molecules into the solution. During this "gelatinization" process there is a dramatic increase in viscosity. As the solids level is 30-40% in a typical industrial process, the starch has to be thinned or "liquefied" so that it can be handled. This reduction in viscosity is today mostly obtained by enzymatic degradation. During the liquefaction step, the long-chained starch is degraded into smaller branched and linear units (maltodextrins) by an alpha-amylase. The liquefaction process is typically carried out at about 105-110°C for about 5 to 10 minutes followed by about 1-2 hours at about 95°C. The temperature is then lowered to 60°C, a glucoamylase or a beta-amylase and optionally a debranching enzyme, such as an isoamylase or a pullulanase are added, and the saccharification process proceeds for about 24 to 72 hours.

It will be apparent from the above discussion that the conventional starch conversion process is very energy consuming due to the different requirements in terms of temperature during the various steps. It is thus desirable to be able to select and/or design the enzymes used in the process so that the overall process can be performed without having to gelatinize the starch. Such processes are the subject for the patents US4591560, US4727026 and US4009074 and EP0171218, and Danish patent application PA 2003 00949. The present invention discloses a new hybrid enzyme and a genetical modification of a wild type enzyme designed for such processes and comprising an amino acid sequence of a CBM and an amino acid sequence of a fungal starch degrading enzyme. Hybrid enzymes are the subject of WO9814601, WO0077165 and Danish patent application PA 2003 00949.

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SUMMARY OF THE INVENTION

The invention provides in a first aspect a hybrid enzyme which comprises an amino acid sequence of a catalytic module having alpha-amylase activity and an amino acid sequence of a carbohydrate-binding module, wherein the catalytic module is of fungal origin.

The invention provides in a second aspect a variant of the fungal alpha-amylase shown as SEQ ID NO:41 or variants of an alpha-amylase having at least 60% homology, at least 70% homology, at least 80% homology, or even at least 90% homology to SEQ ID NO:41, which variant comprising an alteration at one or more of the positions: 13,15,18, 31, 32, 33, 34, 35, 36, 61, 63, 64, 68, 69, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 89, 117, 118, 119, 120, 121, 122, 123, 124, 125, 152, 153, 154, 155, 156, 157, 158,161,162, 165, 166, 167, 168, 169, 170, 171, 172, 173, 174, 175, 204, 205, 206, 207, 208, 209, 210, 211,

216, 229, 230, 231, 232, 233, 234, 235, 236, 237, 238, 239, 242, 245, 250, 252, 253, 255, 256, 257, 259, 260, 275, 292, 295, 296, 297, 298, 299, 304, 328, 339, 344, 348, 378, 383, 386, 387, 405, 448 and 480 wherein; (a) the alteration(s) are independently, i) an insertion of an amino acid downstream of the amino acid which occupies the position, ii) a deletion of the amino acid which occupies the position, or iii) a substitution of the amino acid which occupies the position with a different amino acid, (b) the variant has increased acid alpha-amylase activity and or improved enzyme stability relative to the parent fungal alpha-amylase and, (c) each position corresponds to a position of the amino acid sequence of the TAKA amylase shown in SEQ ID NO:43 and/or the *A.kawachii* alpha-amylase shown as SEQ ID NO:41.

In further aspects the invention provides an isolated DNA sequence encoding the hybrid enzyme of the first aspect or the variant of the second aspect, a DNA construct comprising the DNA sequence encoding the hybrid enzyme of the first aspect or the variant of the second aspect, an expression vector comprising the DNA sequence encoding the hybrid enzyme of the first aspect or the variant of the second aspect, and a host cell transformed with a vector; which host cell is capable of expressing the DNA sequence encoding the hybrid enzyme of the first aspect or the variant of the second aspect.

In an eighth aspect the invention provides a method for liquefying starch, wherein a gelatinized or granular starch substrate is treated in aqueous medium with the hybrid enzyme of the first aspect or the variant of the second aspect.

DETAILED DESCRIPTION OF THE INVENTION

The term "granular starch" is understood as raw uncooked starch, i.e. starch that has not been subjected to a gelatinization. Starch is formed in plants as tiny granules insoluble in water. These granules are preserved in starches at temperatures below the initial gelatinization temperature. When put in cold water, the grains may absorb a small amount of the liquid. Up to 50°C to 70°C the swelling is reversible, the degree of reversibility being dependent upon the particular starch. With higher temperatures an irreversible swelling called gelatinization begins.

The term "initial gelatinization temperature" is understood as the lowest temperature at which gelatinization of the starch commences. Starch heated in water begins to gelatinize between 50°C and 75°C; the exact temperature of gelatinization depends on the specific starch and can readily be determined by the skilled artisan. Thus, the initial gelatinization temperature may vary according to the plant species, to the particular variety of the plant species as well as with the growth conditions. In the context of this invention the initial gelatinization temperature of a given starch is the temperature at which birefringence is lost

in 5% of the starch granules using the method described by Gorinstein. S. and Lii. C., Starch/Stärke, Vol. 44 (12) pp. 461-466 (1992).

The term "soluble starch hydrolysate" is understood as the soluble products of the processes of the invention and may comprise mono- di-, and oligosaccharides, such as
5 glucose, maltose, maltodextrins, cyclodextrins and any mixture of these. Preferably at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97% or 98% of the dry solids of the granular starch is converted into a soluble starch hydrolysate.

The term polypeptide "homology" is understood as the degree of identity between two sequences indicating a derivation of the first sequence from the second. The homology may
10 suitably be determined by means of computer programs known in the art such as GAP provided in the GCG program package (Program Manual for the Wisconsin Package, Version 8, August 1994, Genetics Computer Group, 575 Science Drive, Madison, Wisconsin, USA 53711) (Needleman, S.B. and Wunsch, C.D., (1970), Journal of Molecular Biology, 48, 443-453. The following settings for amino acid sequence comparison are used: GAP
15 creation penalty of 3.0 and GAP extension penalty of 0.1. The relevant part of the amino acid sequence for the homology determination is the mature polypeptide, i.e. without the signal peptide.

Hybrid enzymes

Enzyme classification numbers (EC numbers) referred to in the present specification with
20 claims are in accordance with the Recommendations (1992) of the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology, Academic Press Inc, 1992.

Hybrid enzymes or a genetically modified wild type enzymes as referred to herein include
25 species comprising an amino acid sequence of an alpha-amylolytic enzyme (EC 3.2.1.1) linked (i.e. covalently bound) to an amino acid sequence comprising a carbohydrate-binding module (CBM).

CBM-containing hybrid enzymes, as well as detailed descriptions of the preparation and
purification thereof, are known in the art [see, e.g. WO 90/00609, WO 94/24158 and WO
95/16782, as well as Greenwood et al. Biotechnology and Bioengineering 44 (1994) pp. 1295-
30 1305]. They may, e.g. be prepared by transforming into a host cell a DNA construct comprising at least a fragment of DNA encoding the carbohydrate-binding module ligated, with or without a linker, to a DNA sequence encoding the enzyme of interest, and growing the transformed host cell to express the fused gene. The resulting recombinant product (hybrid enzyme) - often referred to in the art as a "fusion protein - may be described by the following general formula:

35 A-CBM-MR-X

In the latter formula, A-CBM is the N-terminal or the C-terminal region of an amino acid sequence comprising at least the carbohydrate-binding module (CBM) *per se*. MR is the middle region (the "linker"), and X is the sequence of amino acid residues of a polypeptide encoded by a DNA sequence encoding the enzyme (or other protein) to which the CBM is to be linked.

5 The moiety A may either be absent (such that A-CBM is a CBM *per se*, i.e. comprises no amino acid residues other than those constituting the CBM) or may be a sequence of one or more amino acid residues (functioning as a terminal extension of the CBM *per se*). The linker (MR) may be a bond, or a short linking group comprising from about 2 to about 100 carbon atoms, in particular of from 2 to 40 carbon atoms. However, MR is preferably a sequence of
10 from about 2 to about 100 amino acid residues, more preferably of from 2 to 40 amino acid residues, such as from 2 to 15 amino acid residues.

The moiety X may constitute either the N-terminal or the C-terminal region of the overall hybrid enzyme.

15 It will thus be apparent from the above that the CBM in a hybrid enzyme of the type in question may be positioned C-terminally, N-terminally or internally in the hybrid enzyme.

Linker sequence

The linker sequence may be any suitable linker sequence. In preferred embodiments the linker sequence is derived from the *Athelia rolfsii* AMG, the *A.niger* AMG or the *A. kawachii* alpha-amylase such as a linker sequence selected from the list consisting of *A. niger* AMG linker: T
20 G G T T T T A T P T G S G S V T S T S K T T A T A S K T S T S T S S T S A (SEQ ID NO:26), *A. kawachii* alpha-amylase linker: T T T T T T A A A T S T S K A T T S S S S S S A A A T T S S S (SEQ ID NO:27), *Athelia rolfsii* AMG linker: G A T S P G G S S G S (SEQ ID NO:28), and the PEPT linker: P E P T P E P T (SEQ ID NO:29). In another preferred
25 embodiment the hybrid enzymes has a linker sequence which differs from the amino acid sequence shown in SEQ ID NO:26, SEQ ID NO:27, SEQ ID NO:28, or SEQ ID NO:29 in no more than 10 positions, no more than 9 positions, no more than 8 positions, no more than 7 positions, no more than 6 positions, no more than 5 positions, no more than 4 positions, no more than 3 positions, no more than 2 positions, or even no more than 1 position.

30 Carbohydrate-binding modules

A carbohydrate-binding module (CBM), or as often referred to, a carbohydrate-binding domain (CBD), is a polypeptide amino acid sequence which binds preferentially to a poly- or oligosaccharide (carbohydrate), frequently - but not necessarily exclusively - to a water-insoluble (including crystalline) form thereof.

35 CBMs derived from starch degrading enzymes are often referred to as starch-binding modules or SBMs (CBMs which may occur in certain amylolytic enzymes, such as certain

glucoamylases, or in enzymes such as cyclodextrin glucanotransferases, or in alpha-amylases). Likewise, other sub-classes of CBMs would embrace, e.g. cellulose-binding modules (CBMs from cellulolytic enzymes), chitin-binding modules (CBMs which typically occur in chitinases), xylan-binding modules (CBMs which typically occur in xylanases),
5 mannan-binding modules (CBMs which typically occur in mannanases). SBMs are often referred to as SBDs (Starch Binding Domains)

CBMs are found as integral parts of large polypeptides or proteins consisting of two or more polypeptide amino acid sequence regions, especially in hydrolytic enzymes (hydrolases) which typically comprise a catalytic module containing the active site for
10 substrate hydrolysis and a carbohydrate-binding module (CBM) for binding to the carbohydrate substrate in question. Such enzymes can comprise more than one catalytic module and one, two or three CBMs, and optionally further comprise one or more polypeptide amino acid sequence regions linking the CBM(s) with the catalytic module(s), a region of the latter type usually being denoted a "linker". Examples of hydrolytic enzymes
15 comprising a CBM - some of which have already been mentioned above - are cellulases, xylanases, mannanases, arabinofuranosidases, acetylesterases and chitinases. CBMs have also been found in algae, e.g. in the red alga *Porphyra purpurea* in the form of a non-hydrolytic polysaccharide-binding protein.

In proteins/polypeptides in which CBMs occur (e.g. enzymes, typically hydrolytic
20 enzymes), a CBM may be located at the N or C terminus or at an internal position.

That part of a polypeptide or protein (e.g. hydrolytic enzyme) which constitutes a CBM *per se* typically consists of more than about 30 and less than about 250 amino acid residues. The "Carbohydrate-Binding Module of Family 20" or a CBM-20 module is in the context of this invention defined as a sequence of approximately 100 amino acids having at least 45%
25 homology to the Carbohydrate-Binding Module (CBM) of the polypeptide disclosed in figure 1 by Joergensen et al (1997) in *Biotechnol. Lett.* 19:1027-1031. The CBM comprises the last 102 amino acids of the polypeptide, i.e. the subsequence from amino acid 582 to amino acid 683. The numbering of Glycoside Hydrolase Families applied in this disclosure follows the concept of Coutinho, P.M. & Henrissat, B. (1999) *CAZy - Carbohydrate-Active Enzymes server* at URL: <http://afmb.cnrs-mrs.fr/~cazy/CAZY/index.html> or alternatively Coutinho, P.M. & Henrissat, B. 1999; The modular structure of cellulases and other carbohydrate-active enzymes: an integrated database approach. In "*Genetics, Biochemistry and Ecology of Cellulose Degradation*", K. Ohmiya, K. Hayashi, K. Sakka, Y. Kobayashi, S. Karita and T. Kimura eds., Uni Publishers Co., Tokyo, pp. 15-23, and Bourne, Y. & Henrissat, B. 2001;
30 Glycoside hydrolases and glycosyltransferases: families and functional modules, *Current Opinion in Structural Biology* 11:593-600.

Examples of enzymes which comprise a CBM suitable for use in the context of the invention are alpha-amylases, maltogenic alpha-amylases, cellulases, xylanases,

mannanases, arabinofuranosidases, acetylerases and chitinases. Further CBMs of interest in relation to the present invention include CBMs deriving from glucoamylases (EC 3.2.1.3) or from CGTases (EC 2.4.1.19).

5 CBMs deriving from fungal, bacterial or plant sources will generally be suitable for use in the context of the invention. Preferred are CBMs of fungal origin, more preferably from *Aspergillus* sp., *Bacillus* sp., *Klebsiella* sp., or *Rhizopus* sp. In this connection, techniques suitable for isolating the relevant genes are well known in the art.

Preferred for the invention is CBMs of Carbohydrate-Binding Module Family 20. CBMs of Carbohydrate-Binding Module Family 20 suitable for the invention may be derived from 10 glucoamylases of *Aspergillus awamori* (SWISSPROT Q12537), *Aspergillus kawachii* (SWISSPROT P23176), *Aspergillus niger* (SWISSPROT P04064), *Aspergillus oryzae* (SWISSPROT P36914), from alpha-amylases of *Aspergillus kawachii* (EMBL:#AB008370), *Aspergillus nidulans* (NCBI AAF17100.1), from beta-amylases of *Bacillus cereus* (SWISSPROT P36924), or from CGTases of *Bacillus circulans* (SWISSPROT P43379). 15 Preferred is a CBM from the alpha-amylase of *Aspergillus kawachii* (EMBL:#AB008370) as well as CBMs having at least 50%, 60%, 70%, 80% or even at least 90% homology to the CBM of the alpha-amylase of *Aspergillus kawachii* (EMBL:#AB008370), i.e. a CBM having at least 50%, 60%, 70%, 80% or even at least 90% homology to the amino acid sequence of SEQ ID NO:6. Also preferred for the invention are the CBMs of Carbohydrate-Binding 20 Module Family 20 having the amino acid sequences shown in SEQ ID NO:9, SEQ ID NO:10, and SEQ ID NO:11 and disclosed in Danish patent application PA 2003 00949 as SEQ ID NO:1, SEQ ID NO:2 and SEQ ID NO:3 respectively. Further preferred CBMs include the CBMs of the glucoamylase from *Hormoconis* sp. such as from *Hormoconis resinae* (Syn. Creosote fungus or *Amorphotheca resinae*) such as the CBM of SWISSPROT:Q03045 (SEQ 25 ID NO:12), from *Lentinula* sp. such as from *Lentinula edodes* (shiitake mushroom) such as the CBM of SPTREMBL:Q9P4C5 (SEQ ID NO:13), from *Neurospora* sp. such as from *Neurospora crassa* such as the CBM of SWISSPROT:P14804 (SEQ ID NO:14), from *Talaromyces* sp. such as from *Talaromyces byssochlamydioides* such as the CBM of NN005220 (SEQ ID NO:15), from *Geosmithia* sp. such as from *Geosmithia cylindrospora*, 30 such as the CBM of NN48286 (SEQ ID NO:16), from *Scorias* sp. such as from *Scorias spongiosa* such as the CBM of NN007096 (SEQ ID NO:17), from *Eupenicillium* sp. such as from *Eupenicillium ludwigii* such as the CBM of NN005968 (SEQ ID NO:18), from *Aspergillus* sp. such as from *Aspergillus japonicus* such as the CBM of NN001136 (SEQ ID NO:19), from *Penicillium* sp. such as from *Penicillium* cf. *miczynskii* such as the CBM of NN48691 (SEQ ID NO:20), from Mz1 *Penicillium* sp. such as the CBM of NN48690 (SEQ ID NO:21), 35 from *Thysanophora* sp. such as the CBM of NN48711 (SEQ ID NO:22), and from *Humicola* sp. such as from *Humicola grisea* var. *thermoidea* such as the CBM of SPTREMBL:Q12623 (SEQ ID NO:23). Most preferred CBMs include the CBMs of the glucoamylase from

Aspergillus sp. such as from *Aspergillus niger*, such as SEQ ID NO:24, and *Athelia* sp. such as from *Athelia rolfsii*, such as SEQ ID NO:25. Also preferred for the invention is any CBD having at least 50%, 60%, 70%, 80% or even at least 90% homology to any of the aforementioned CBD amino acid sequences.

5 Further suitable CBMs of Carbohydrate-Binding Module Family 20 may be found at URL: <http://afmb.cnrs-mrs.fr/~cazy/CAZY/index.html>.

Once a nucleotide sequence encoding the substrate-binding (carbohydrate-binding) region has been identified, either as cDNA or chromosomal DNA, it may then be manipulated in a variety of ways to fuse it to a DNA sequence encoding the enzyme of interest. The DNA
10 fragment encoding the carbohydrate-binding amino acid sequence, and the DNA encoding the enzyme of interest are then ligated with or without a linker. The resulting ligated DNA may then be manipulated in a variety of ways to achieve expression.

Alpha-amylolytic sequence

Alpha-amylases (in particular acid stable alpha-amylases) which are appropriate as the basis
15 for CBM/amylase hybrids of the types employed in the context of the present invention include those of fungal origin.

Preferably the alpha-amylase is a wild type enzyme. More preferably the alpha-amylase is a variant alpha-amylases comprising amino acid modifications leading to increased activity, increased protein stability at low pH, and/or at high pH, increased stability towards calcium
20 depletion, and/or increased stability at elevated temperature. Chemically or genetically modified mutants of such alpha-amylases are included in this connection.

Relevant alpha-amylases include, for example, alpha-amylases obtainable from *Aspergillus* species, in particular from *Aspergillus niger*, such as an acid stable alpha-amylase (SWISSPROT P56271), described in more detail in WO 8901969 (example 3) and having the
25 amino acid sequence shown in SEQ ID NO:8 and/or encode by the DNA sequence shown in SEQ ID NO:7. Also preferred are alpha-amylase sequences having more than 50%, such as 60%, or 70%, 80% or 90% homology to the amino acid sequence shown in SEQ ID NO:8 and/or encode by the DNA sequence shown in SEQ ID NO:7.

In another preferred embodiment the alpha-amylolytic sequence is derived from the
30 *A.oryzae* acid alpha-amylase (Fungamyl™). More preferably the alpha-amylolytic sequence has more than 50%, such as 60%, or 70%, 80% or 90% homology to the amino acid sequence shown in SEQ ID NO:30 and/or to the sequence shown as amino acids 21-498 of the amino acid sequence shown in SEQ ID NO:30.

Even more preferred is an embodiment wherein the hybrid enzyme comprises an alpha-
35 amylolytic sequence derived from the *A.oryzae* acid alpha-amylase (Fungamyl™, SEQ ID NO:30), and/or a linker sequence derived from the *A. kawachii* alpha-amylase or the *A. rolfsii* AMG, and/or a CBM derived from the *A. kawachii* alpha-amylase (SEQ ID NO:5) or the *A.*

rolfsii AMG (SEQ ID NO:25). In a particular such embodiment the hybrid enzyme has the amino acid sequence shown in SEQ ID NO:36 or in SEQ ID NO:40.

Also preferred is an embodiment wherein the hybrid enzyme comprises an alpha-amylolytic sequence derived from the *A.niger* acid alpha-amylase catalytic module having the sequence shown in SEQ ID NO:8, and/or a linker sequence derived from the *A. kawachii* alpha-amylase or the *A. rolfsii* AMG, and/or the CBM is derived from the *A. kawachii* alpha-amylase, the *A. rolfsii* AMG or the *A.niger* AMG. In a particularly preferred embodiment the hybrid enzyme comprises the *A.niger* acid alpha-amylase catalytic module having the sequence shown in SEQ ID NO:8 and the *A. kawachii* alpha-amylase linker and CBM (SEQ ID NO:6).

Preferably the hybrid enzyme comprises a CBD sequence having at least 50%, 60%, 70%, 80% or even at least 90% homology to any of the amino acid sequences shown in SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24 or SEQ ID NO:25. Even more preferred the hybrid enzyme comprises a CBD sequence having an amino acid sequence shown in SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24 or SEQ ID NO:25. In yet another preferred embodiment the CBM sequence has an amino acid sequence which differs from the amino acid sequence amino acid sequence shown in SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24 or SEQ ID NO:25 in no more than 10 amino acid positions, no more than 9 positions, no more than 8 positions, no more than 7 positions, no more than 6 positions, no more than 5 positions, no more than 4 positions, no more than 3 positions, no more than 2 positions, or even no more than 1 position. In a most preferred embodiment the hybrid enzyme comprises a CBM derived from an AMG from *A. rolfsii*, such as the AMG from *A. rolfsii* AHU 9627 described in US patent 4,727,026.

In a particular such embodiment the hybrid enzyme has the amino acid sequence shown in SEQ ID NO:32, SEQ ID NO:34 or SEQ ID NO:38 or the hybrid enzyme has an amino acid sequence having at least 50%, 60%, 70%, 80% or even at least 90% homology to any of the afore mentioned amino acid sequences.

In yet another preferred embodiment the hybrid enzymes has an amino acid sequence which differs from the amino acid sequence amino acid sequence shown in SEQ ID NO:32, SEQ ID NO:34, SEQ ID NO:36, SEQ ID NO:38 or SEQ ID NO:40 in no more than 10 positions, no more than 9 positions, no more than 8 positions, no more than 7 positions, no more than 6

positions, no more than 5 positions, no more than 4 positions, no more than 3 positions, no more than 2 positions, or even no more than 1 position.

In a preferred embodiment the hybrid enzymes is a variant comprises the catalytic domain shown as amino acids 21-498 of SEQ ID NO:40 (and/or amino acids 21-498 of SEQ ID NO:30) with one or more substitutions, more preferably a substitution in one or more positions selected from the list consisting of: 81, 158, 161, 163, 164, 175, 176, 177, 253, 264, 266, 466, 468, 470, and most preferably one or more substitutions selected from the list consisting of: Q81R, K158D, K158V, S161D, S161N, Q163S, Q163A, D164S, Y175W, E176D, D177N, D253N, N264K, N264E, M266L, G466D, D468S, and N470D. In an even more preferred embodiment the hybrid enzymes is one of the variants listed in Table 6. Also preferred are variants comprising the catalytic domain shown as amino acids 21-498 of SEQ ID NO:40 (and/or amino acids 21-498 of SEQ ID NO:30) with one or more substations or combination of substitutions selected from the list comprising: Y175W + E176D, 276W + P277Q, K380T + L381W, G62N, G62F, Y95D, D106K+ S109D, A140P, K158D + D164S, F171L+E182Q, K200E + V202A, K200E + V202A, K200E + V202A, T227S + K233P, L252D + D255N, N264K + M266L, K283E, N359K+ D360V, N390K + Y391L, G466D + N470D, Y484L + S498S, Y484L + S498G.and Y484L + S498R.

In a preferred embodiment the hybrid enzyme comprises the catalytic module according shown as amino acids 21-498 of SEQ ID NO:40 (and/or amino acids 21-498 of SEQ ID NO:30) with one or more substitutions selected from the list consisting of: K158V, S161N, Q163A, D164S, N264K, M266L, G466D, D468S and N470D.

In another preferred embodiment the hybrid enzyme comprises the catalytic module shown as amino acids 21-498 of SEQ ID NO:40 (and/or amino acids 21-498 of SEQ ID NO:30) with one or more substitutions selected from the list consisting of: Q81R, K158V, S161N, Q163A, D164S, Y175W, E176D, D177N, N264K, M266L, G466D, D468S and N470D.

Expression vectors

The present invention also relates to recombinant expression vectors which may comprise a DNA sequence encoding the hybrid enzyme or a genetically modified wild type enzyme, a promoter, a signal peptide sequence, and transcriptional and translational stop signals. The various DNA and control sequences described above may be joined together to produce a recombinant expression vector which may include one or more convenient restriction sites to allow for insertion or substitution of the DNA sequence encoding the polypeptide at such sites. Alternatively, the DNA sequence of the present invention may be expressed by inserting the DNA sequence or a DNA construct comprising the sequence into an appropriate vector for expression. In creating the expression vector, the coding sequence is

located in the vector so that the coding sequence is operably linked with the appropriate control sequences for expression, and possibly secretion.

The recombinant expression vector may be any vector (e.g. a plasmid or virus), which can be conveniently subjected to recombinant DNA procedures and can bring about the expression of the DNA sequence. The choice of the vector will typically depend on the compatibility of the vector with the host cell into which the vector is to be introduced. The vectors may be linear or closed circular plasmids. The vector may be an autonomously replicating vector, i.e. a vector which exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication, e.g. a plasmid, an extrachromosomal element, a minichromosome, a cosmid or an artificial chromosome. The vector may contain any means for assuring self-replication. Alternatively, the vector may be one which, when introduced into the host cell, is integrated into the genome and replicated together with the chromosome(s) into which it has been integrated. The vector system may be a single vector or plasmid or two or more vectors or plasmids which together contain the total DNA to be introduced into the genome of the host cell, or a transposon.

Markers

The vectors of the present invention preferably contain one or more selectable markers, which permit easy selection of transformed cells. A selectable marker is a gene the product of which provides for biocide or viral resistance, resistance to heavy metals, prototrophy to auxotrophs, and the like.

Examples of selectable markers for use in a filamentous fungus host cell may be selected from the group including, but not limited to, *amdS* (acetamidase), *argB* (ornithine carbamoyltransferase), *bar* (phosphinothricin acetyltransferase), *hygB* (hygromycin phosphotransferase), *niaD* (nitrate reductase), *pyrG* (orotidine-5'-phosphate decarboxylase), *sC* (sulfate adenylyltransferase), *trpC* (anthranilate synthase), and glufosinate resistance markers, as well as equivalents from other species. Preferred for use in an *Aspergillus* cell are the *amdS* and *pyrG* markers of *Aspergillus nidulans* or *Aspergillus oryzae* and the *bar* marker of *Streptomyces hygroscopicus*. Furthermore, selection may be accomplished by co-transformation, e.g. as described in WO 91/17243, where the selectable marker is on a separate vector.

The vectors of the present invention preferably contain an element(s) that permits stable integration of the vector into the host cell genome or autonomous replication of the vector in the cell independent of the genome of the cell.

The vectors of the present invention may be integrated into the host cell genome when introduced into a host cell. For integration, the vector may rely on the DNA sequence encoding the polypeptide of interest or any other element of the vector for stable integration

of the vector into the genome by homologous or none homologous recombination. Alternatively, the vector may contain additional DNA sequences for directing integration by homologous recombination into the genome of the host cell. The additional DNA sequences enable the vector to be integrated into the host cell genome at a precise location(s) in the chromosome(s). To increase the likelihood of integration at a precise location, the integrational elements should preferably contain a sufficient number of DNAs, such as 100 to 1,500 base pairs, preferably 400 to 1,500 base pairs, and most preferably 800 to 1,500 base pairs, which are highly homologous with the corresponding target sequence to enhance the probability of homologous recombination. The integrational elements may be any sequence that is homologous with the target sequence in the genome of the host cell. Furthermore, the integrational elements may be non-encoding or encoding DNA sequences. On the other hand, the vector may be integrated into the genome of the host cell by non-homologous recombination. These DNA sequences may be any sequence that is homologous with a target sequence in the genome of the host cell, and, furthermore, may be non-encoding or encoding sequences.

For autonomous replication, the vector may further comprise an origin of replication enabling the vector to replicate autonomously in the host cell in question.

The episomal replicating the AMA1 plasmid vector disclosed in WO 00/24883 may be used.

More than one copy of a DNA sequence encoding a polypeptide of interest may be inserted into the host cell to amplify expression of the DNA sequence. Stable amplification of the DNA sequence can be obtained by integrating at least one additional copy of the sequence into the host cell genome using methods well known in the art and selecting for transformants.

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

30 **Host cells**

The host cell of the invention, either comprising a DNA construct or an expression vector comprising the DNA sequence encoding the hybrid enzyme or a genetically modified wild type enzyme, is advantageously used as a host cell in the recombinant production of the hybrid enzyme or a genetically modified wild type enzyme. The cell may be transformed with an expression vector. Alternatively, the cell may be transformed with the DNA construct of the invention encoding the hybrid enzyme or a genetically modified wild type enzyme,

conveniently by integrating the DNA construct (in one or more copies) in the host chromosome. Integration of the DNA construct into the host chromosome may be performed according to conventional methods, e.g. by homologous or heterologous recombination.

5 The host cell may be any appropriate prokaryotic or eukaryotic cell, e.g. a bacterial cell, a filamentous fungus cell, a plant cell or a mammalian cell.

In a preferred embodiment, the host cell is a filamentous fungus represented by the following groups of *Ascomycota*, include, e.g. *Neurospora*, *Eupenicillium* (= *Penicillium*), *Emericella* (= *Aspergillus*), *Eurotium* (= *Aspergillus*).

10 In a more preferred embodiment, the filamentous fungus include all filamentous forms of the subdivision *Eumycota* and *Oomycota* (as defined by Hawksworth *et al.* In, *Ainsworth and Bisby's Dictionary of The Fungi*, 8th edition, 1995, CAB International, University Press, Cambridge, UK. The filamentous fungi are characterized by a vegetative mycelium composed of chitin, cellulose, glucan, chitosan, mannan, and other complex polysaccharides. Vegetative growth is by hyphal elongation and carbon catabolism is
15 obligately aerobic.

In an even more preferred embodiment, the filamentous fungus host cell is a cell of a species of, but not limited to a cell selected from the group consisting of a strain belonging to a species of *Aspergillus*, preferably *Aspergillus oryzae*, *Aspergillus niger*, *Aspergillus awamori*, *Aspergillus kawachii*, or a strain of *Bacillus*, or a strain of *Fusarium*, such as a
20 strain of *Fusarium oxysporium*, *Fusarium graminearum* (in the perfect state named *Gibberella zeae*, previously *Sphaeria zeae*, synonym with *Gibberella roseum* and *Gibberella roseum* f. sp. *cerealis*), or *Fusarium sulphureum* (in the perfect state named *Gibberella puricaris*, synonym with *Fusarium trichothecioides*, *Fusarium bactridioides*, *Fusarium sambucium*, *Fusarium roseum*, and *Fusarium roseum* var. *graminearum*), *Fusarium cerealis*
25 (synonym with *Fusarium crookwellense*), or *Fusarium venenatum*,

In a most preferred embodiment, the filamentous fungus host cell is a cell of a strain belonging to a species of *Aspergillus*, preferably *Aspergillus oryzae*, or *Aspergillus niger*.

The host cell may be a wild type filamentous fungus host cell or a variant, a mutant or a genetically modified filamentous fungus host cell. In a preferred embodiment of the
30 invention the host cell is a protease deficient or protease minus strain. Also specifically contemplated is *Aspergillus* strains, such as *Aspergillus niger* strains, genetically modified to disrupt or reduce expression of glucoamylase, acid-stable alpha-amylase, alpha-1,6 transglucosidase, and protease activities.

Transformation of filamentous fungus host cells

35 Filamentous fungus host cells may be transformed by a process involving protoplast formation, transformation of the protoplasts, and regeneration of the cell wall in a manner

known in the art. Suitable procedures for transformation of *Aspergillus* host cells are described in EP 238 023, EP 184 438, and Yelton *et al.* 1984, *Proceedings of the National Academy of Sciences USA* 81:1470-1474. A suitable method of transforming *Fusarium* species is described by Malardier *et al.* 1989, *Gene* 78:147-156 or US 6,060,305.

5 **Isolating and cloning a DNA sequence encoding a parent alpha-amylase**

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

15 The DNA sequence encoding a parent alpha-amylase may be isolated from any cell or microorganism producing the alpha-amylase in question, using various methods well known in the art. First, a genomic DNA and/or cDNA library should be constructed using chromosomal DNA or messenger RNA from the organism that produces the alpha-amylase to be studied. Then, if the amino acid sequence of the alpha-amylase is known, labeled oligonucleotide
20 probes may be synthesized and used to identify alpha-amylase-encoding clones from a genomic library prepared from the organism in question. Alternatively, a labelled oligonucleotide probe containing sequences homologous to another known alpha-amylase gene could be used as a probe to identify alpha-amylase-encoding clones, using hybridization and washing conditions of very low to very high stringency.

25 Yet another method for identifying alpha-amylase-encoding clones would involve inserting fragments of genomic DNA into an expression vector, such as a plasmid, transforming alpha-amylase-negative bacteria with the resulting genomic DNA library, and then plating the transformed bacteria onto agar containing a substrate for alpha-amylase (*i.e.* maltose), thereby allowing clones expressing the alpha-amylase to be identified.

30 Alternatively, the DNA sequence encoding the enzyme may be prepared synthetically by established standard methods, *e.g.* the phosphoroamidite method described S.L. Beaucage and M.H. Caruthers, (1981), *Tetrahedron Letters* 22, p. 1859-1869, or the method described by Matthes *et al.* (1984), *EMBO J.* 3, p. 801-805. In the phosphoroamidite method, oligonucleotides are synthesized, *e.g.* in an automatic DNA synthesizer, purified, annealed, ligated and
35 cloned in appropriate vectors.

Finally, the DNA sequence may be of mixed genomic and synthetic origin, mixed synthetic and cDNA origin or mixed genomic and cDNA origin, prepared by ligating fragments of

synthetic, genomic or cDNA origin (as appropriate, the fragments corresponding to various parts of the entire DNA sequence), in accordance with standard techniques. The DNA sequence may also be prepared by polymerase chain reaction (PCR) using specific primers, for instance as described in US 4,683,202 or R.K. Saiki et al. (1988), Science 239, 1988, pp. 487-491.

Isolated DNA sequence

The present invention relates, inter alia, to an isolated DNA sequence comprising a DNA sequence encoding a hybrid enzyme or a genetically modified wild type enzyme comprising an amino acid sequence of a catalytic module having alpha-amylase activity and an amino acid sequence of a carbohydrate-binding module, wherein the catalytic module is of fungal origin.

The term "isolated DNA sequence" as used herein refers to a DNA sequence, which is essentially free of other DNA sequences, e.g. at least about 20% pure, preferably at least about 40% pure, more preferably at least about 60% pure, even more preferably at least about 80% pure, and most preferably at least about 90% pure as determined by agarose electrophoresis.

For example, an isolated DNA sequence can be obtained by standard cloning procedures used in genetic engineering to relocate the DNA sequence from its natural location to a different site where it will be reproduced. The cloning procedures may involve excision and isolation of a desired DNA fragment comprising the DNA sequence encoding the polypeptide of interest, insertion of the fragment into a vector molecule, and incorporation of the recombinant vector into a host cell where multiple copies or clones of the DNA sequence will be replicated. An isolated DNA sequence may be manipulated in a variety of ways to provide for expression of the polypeptide of interest. Manipulation of the DNA sequence prior to its insertion into a vector may be desirable or necessary depending on the expression vector. The techniques for modifying DNA sequences utilizing recombinant DNA methods are well known in the art.

DNA construct

The present invention relates, inter alia, to a DNA construct comprising a DNA sequence encoding a hybrid enzyme or a genetically modified wild type enzyme comprising an amino acid sequence of a catalytic module having alpha-amylase activity and an amino acid sequence of a carbohydrate-binding module, wherein the catalytic module is of fungal origin. "DNA construct" is defined herein as a DNA molecule, either single- or double-stranded, which is isolated from a naturally occurring gene or which has been modified to contain

segments of DNA, which are combined and juxtaposed in a manner, which would not otherwise exist in nature. The term DNA construct is synonymous with the term expression cassette when the DNA construct contains all the control sequences required for expression of a coding sequence of the present invention.

5 **Site-directed mutagenesis**

Once a parent alpha-amylase-encoding DNA sequence has been isolated, and desirable sites for mutation identified, mutations may be introduced using synthetic oligonucleotides. These oligonucleotides contain nucleotide sequences flanking the desired mutation sites. In a specific method, a single-stranded gap of DNA, the alpha-amylase-encoding sequence, is created in a vector carrying the alpha-amylase gene. Then the synthetic nucleotide, bearing the desired mutation, is annealed to a homologous portion of the single-stranded DNA. The remaining gap is then filled in with DNA polymerase I (Klenow fragment) and the construct is ligated using T4 ligase. A specific example of this method is described in Morinaga et al. (1984), *Biotechnology* 2, p. 646-639. US 4,760,025 disclose the introduction of oligonucleotides encoding multiple mutations by performing minor alterations of the cassette. However, an even greater variety of mutations can be introduced at any one time by the Morinaga method, because a multitude of oligonucleotides, of various lengths, can be introduced.

Another method for introducing mutations into alpha-amylase-encoding DNA sequences is described in Nelson and Long, (1989), *Analytical Biochemistry* 180, p. 147-151. It involves the 3-step generation of a PCR fragment containing the desired mutation introduced by using a chemically synthesized DNA strand as one of the primers in the PCR reactions. From the PCR-generated fragment, a DNA fragment carrying the mutation may be isolated by cleavage with restriction endonucleases and reinserted into an expression plasmid.

25 **Localized random mutagenesis**

The random mutagenesis may be advantageously localized to a part of the parent alpha-amylase in question. This may, *e.g.* be advantageous when certain regions of the enzyme have been identified to be of particular importance for a given property of the enzyme, and when modified are expected to result in a variant having improved properties. Such regions may normally be identified when the tertiary structure of the parent enzyme has been elucidated and related to the function of the enzyme.

The localized or region-specific, random mutagenesis is conveniently performed by use of PCR generated mutagenesis techniques as described above or any other suitable technique known in the art. Alternatively, the DNA sequence encoding the part of the DNA sequence to be modified may be isolated, *e.g.* by insertion into a suitable vector, and said part

may be subsequently subjected to mutagenesis by use of any of the mutagenesis methods discussed above.

Variants of hybrid or wild type enzymes

5 The performance in a starch degradation process of a wild type or hybrid enzyme comprising a carbohydrate-binding module ("CBM") and an alpha-amylase catalytic module may be improved through protein engineering, such as by site directed mutagenesis, by localized random mutagenesis, by synthetically preparing a new variant of the parent wild type enzyme or parent hybrid enzyme, or by any other suitable protein engineering techniques.

10 Parent alpha-amylase contemplated for the invention include wild-type fungal alpha-amylase having a CBM, in particular fungal alpha-amylase obtainable from an *Aspergillus* strain, such as an *Aspergillus kawachii* acid alpha-amylase and variants or mutants thereof, homologous alpha-amylases, and further wild type or artificial alpha-amylases being structurally and/or functionally similar to the acid alpha-amylase from *Aspergillus kawachii*
15 shown in SEQ ID NO:41.

While the acid alpha-amylase from *Aspergillus kawachii* is an example of a wild type alpha-amylase comprising a carbohydrate-binding module ("CBM"), the *Aspergillus kawachii* acid alpha-amylase catalytic module has a limited specific activity, e.g. compared to the catalytic activity of the acid alpha-amylase from *A. niger*. Furthermore, the CBM of the
20 *Aspergillus kawachii* alpha-amylase is easily cleaved off from the catalytic module during a conventional fermentation process thereby reducing the suitability of the enzyme for industrial application. Thus the *Aspergillus kawachii* alpha-amylase has several serious drawbacks as an industrial enzyme for raw starch hydrolysis. Protein engineering techniques can be applied to yield more suitable variants of a parent acid alpha-amylase wherein the
25 catalytic activity and/or the enzyme stability has been improved.

Preferred variants are variants of the fungal alpha-amylase shown as SEQ ID NO:41 or variants of an alpha-amylase having at least 60% homology, at least 70% homology, at least 80% homology, or even at least 90% homology to SEQ ID NO:41, which variant comprising an alteration at one or more of the positions: 13,15,18, 31, 32, 33, 34, 35, 36, 61,
30 63, 64, 68, 69, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 89, 117, 118, 119, 120, 121, 122, 123, 124, 125, 152, 153, 154, 155, 156, 157, 158,161,162, 165, 166, 167, 168, 169, 170, 171, 172, 173, 174, 175, 204, 205, 206, 207, 208, 209, 210, 211, 216, 229, 230, 231, 232, 233, 234, 235, 236, 237, 238, 239, 242, 245, 250, 252, 253, 255, 256, 257,259, 260, 275, 292, 295, 296, 297, 298, 299, 304, 328, 339, 344, 348, 378, 383, 386, 387, 405, 448
35 and 480 and more preferred at one or more of the positions 31, 33, 36, 74, 75, 77, 84, 120, 153, 154, 155, 156, 157, 158, 162, 166, 169, 170, 199, 232, 233, 235, 238, 239, 245, 256, 257, 331, 336, 339, 340, 342, 348, 378, 383, 386, 387, 405, 448 and 480 wherein (a) the

alteration(s) are independently (i) an insertion of an amino acid downstream of the amino acid which occupies the position, (ii) a deletion of the amino acid which occupies the position, or iii) a substitution of the amino acid which occupies the position with a different amino acid, (b) the variant has increased acid alpha-amylase activity and or improved enzyme stability relative to the parent fungal alpha-amylase and (c) each position corresponds to a position of the amino acid sequence of the TAKA amylase shown in SEQ ID NO:43 and/or the *A.kawachii* alpha-amylase shown as SEQ ID NO:41.

More preferred variants are variants of the *A.kawachii* alpha-amylase shown as SEQ ID NO:41 or variants of an alpha-amylase having at least 60% homology, at least 70% homology, at least 80% homology, or even at least 90% homology to SEQ ID NO:41 and comprising alterations in one or more of the following positions: 13,15,18, 31, 32, 33, 34, 35, 36, 61, 63, 64, 68, 69, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 89, 117, 118, 119, 120, 121, 122, 123, 124, 125, 152, 153, 154, 155, 156, 157, 158,161,162, 165, 166, 167, 168, 169, 170, 171, 172, 173, 174, 175, 204, 205, 206, 207, 208, 209, 210, 211, 216, 229, 230, 231, 232, 233, 234, 235, 236, 237, 238, 239, 242, 245, 250, 252, 253, 255, 256, 257,259, 260, 275, 292, 295, 296, 297, 298, 299, 304, 328, 339, 344, 348, 378, 383, 386, 387, 405, 448 and 480 wherein (a) the alteration(s) are independently (i) an insertion of an amino acid downstream of the amino acid which occupies the position, (ii) a deletion of the amino acid which occupies the position, or iii) a substitution of the amino acid which occupies the position with a different amino acid, (b) the variant has increased acid alpha-amylase activity and or improved enzyme stability relative to the parent fungal alpha-amylase and (c) each position corresponds to a position of the amino acid sequence of the TAKA amylase shown in SEQ ID NO:43 and/or the *A.kawachii* alpha-amylase shown as SEQ ID NO:41. Even more preferred variants of the *A.kawachii* alpha-amylase shown as SEQ ID NO:41 or variants of an alpha-amylase having at least 60% homology, at least 70% homology, at least 80% homology, or even at least 90% homology to SEQ ID NO:41 and comprising alterations in one or more of the following positions: 31, 33, 36, 74, 75, 77, 84, 120, 153, 154, 155, 156, 157, 158, 162, 166, 169, 170, 199, 232, 233, 235, 238, 239, 245, 256, 257, 331, 336, 339, 340, 342, 348, 378, 383, 386, 387, 405, 448 and 480.

Yet more preferred are variants of the *A.kawachii* alpha-amylase shown as SEQ ID NO:41 or variants of an alpha-amylase having at least 60% homology, at least 70% homology, at least 80% homology, or even at least 90% homology to SEQ ID NO:41 comprising one or more of the following amino acid substitution: G33A, I36K, S74A, D75Y, E77D, P120A, I153D, D154N, W155Y, D156E, N157D, L158Q, Q162E, E166L, T169N, I170T, E199K, E199L, D232L, N233D, N235D, L238Y, D239T, W256Y, Q257P, E331Q, S336A, D339K, D339N, V340D, and Y342A, and most preferred variants comprises one or more of the following amino acid substitution S74A, E166L, E199L, D339K, and D156E, such as the variant comprising the multiple amino acid substitution: S74A/E166L/E199L,

wherein the variant has improve activity and/or enzyme stability relative to the parent alpha-amylase shown as SEQ ID NO:41.

The variants may also be a variants of the *A. kawachii* alpha-amylase shown as SEQ ID NO:41 or variants of an alpha-amylase having at least 60% homology, at least 70% homology, at least 80% homology, or even at least 90% homology to SEQ ID NO:41 and comprising alterations in one or more of the following positions: 31, 74, 89, 209, 245, 348, 378, 383, 386, 387, 405, 448, and 480, wherein (a) the alteration(s) are independently (i) an insertion of an amino acid downstream of the amino acid which occupies the position, (ii) a deletion of the amino acid which occupies the position, or iii) a substitution of the amino acid which occupies the position with a different amino acid, (b) the variant has increased acid alpha-amylase activity and or improved enzyme stability relative to the parent fungal alpha-amylase.

Preferably the variants is a variants of the *A.kawachii* alpha-amylase shown as SEQ ID NO:41 or variants of an alpha-amylase having at least 60% homology, at least 70% homology, at least 80% homology, or even at least 90% homology to SEQ ID NO:41 and comprising one or more of the following substitutions: N31D, S74A, Y89D, E209L, Y245V, D348K, D378A, K383A, P386A, I387F, I405V, N448S, and N480R, wherein the variant has increased acid alpha-amylase activity and or improved enzyme stability relative to the parent fungal alpha-amylase shown as SEQ ID NO:41.

Most preferred is a variants of the *A.kawachii* alpha-amylase shown as SEQ ID NO:41 and comprising the following substitutions: N31D, S74A, Y89D, E209L, Y245V, D348K, D378A, K383A, P386A, I387F, I405V, N448S, and N480R.

The variants may be produced using conventional protein engineering techniques

Expression of the enzymes in plants

A DNA sequence encoding an enzyme of interest, such as a hybrid enzyme or a variant of a wild type enzyme or a hybrid of the present invention, may be transformed and expressed in transgenic plants as described below.

The transgenic plant can be dicotyledonous or monocotyledonous, for short a dicot or a monocot. Examples of monocot plants are grasses, such as meadow grass (blue grass, *Poa*), forage grass such as *Festuca*, *Lolium*, temperate grass, such as *Agrostis*, and cereals, e.g. wheat, oats, rye, barley, rice, sorghum and maize (corn).

Examples of dicot plants are tobacco, legumes, such as lupins, potato, sugar beet, pea, bean and soybean, and cruciferous plants (family *Brassicaceae*), such as cauliflower, oil seed rape and the closely related model organism *Arabidopsis thaliana*.

Examples of plant parts are stem, callus, leaves, root, fruits, seeds, and tubers as well as the individual tissues comprising these parts, e.g. epidermis, mesophyll, parenchyme, vascular tissues, meristems. In the present context, also specific plant cell

compartments, such as chloroplast, apoplast, mitochondria, vacuole, peroxisomes and cytoplasm are considered to be a plant part. Furthermore, any plant cell, whatever the tissue origin, is considered to be a plant part. Likewise, plant parts such as specific tissues and cells isolated to facilitate the utilisation of the invention are also considered plant parts e.g. embryos, endosperms, aleurone and seeds coats.

Also included within the scope of the invention are the progeny of such plants, plant parts and plant cells.

The transgenic plant or plant cell expressing the enzyme of interest may be constructed in accordance with methods known in the art. In short the plant or plant cell is constructed by incorporating one or more expression constructs encoding the enzyme of interest into the plant host genome and propagating the resulting modified plant or plant cell into a transgenic plant or plant cell.

Conveniently, the expression construct is a DNA construct which comprises a gene encoding the enzyme of interest in operable association with appropriate regulatory sequences required for expression of the gene in the plant or plant part of choice. Furthermore, the expression construct may comprise a selectable marker useful for identifying host cells into which the expression construct has been integrated and DNA sequences necessary for introduction of the construct into the plant in question (the latter depends on the DNA introduction method to be used).

The choice of regulatory sequences, such as promoter and terminator sequences and optionally signal or transit sequences is determined, e.g. on the basis of when, where and how the enzyme is desired to be expressed. For instance, the expression of the gene encoding the enzyme of the invention may be constitutive or inducible, or may be developmental, stage or tissue specific, and the gene product may be targeted to a specific cell compartment, tissue or plant part such as seeds or leaves. Regulatory sequences are, e.g. described by Tague et al, Plant, Phys., 86, 506, 1988.

For constitutive expression the 35S-CaMV, the maize ubiquitin 1 and the rice actin 1 promoter may be used (Franck et al. 1980. Cell 21: 285-294, Christensen AH, Sharrock RA and Quail 1992. Maize polyubiquitin genes: structure, thermal perturbation of expression and transcript splicing, and promoter activity following transfer to protoplasts by electroporation. Plant Mo. Biol. 18, 675-689.; Zhang W, McElroy D. and Wu R 1991, Analysis of rice *Act1 5'* region activity in transgenic rice plants. Plant Cell 3, 1155-1165). Organ-specific promoters may, e.g. be a promoter from storage sink tissues such as seeds, potato tubers, and fruits (Edwards & Coruzzi, 1990. Annu. Rev. Genet. 24: 275-303), or from metabolic sink tissues such as meristems (Ito et al., 1994. Plant Mol. Biol. 24: 863-878), a seed specific promoter such as the glutelin, prolamin, globulin or albumin promoter from rice (Wu et al., Plant and Cell Physiology Vol. 39, No. 8 pp. 885-889 (1998)), a *Vicia faba* promoter from the legumin B4 and the unknown seed protein gene from *Vicia faba* described by Conrad U. et al,

Journal of Plant Physiology Vol. 152, No. 6 pp. 708-711 (1998), a promoter from a seed oil body protein (Chen et al., Plant and cell physiology vol. 39, No. 9 pp. 935-941 (1998), the storage protein napA promoter from *Brassica napus*, or any other seed specific promoter known in the art, e.g. as described in WO 91/14772. Furthermore, the promoter may be a
5 leaf specific promoter such as the rbcS promoter from rice or tomato (Kyoizuka et al., Plant Physiology Vol. 102, No. 3 pp. 991-1000 (1993), the chlorella virus adenine methyltransferase gene promoter (Mitra, A. and Higgins, DW, Plant Molecular Biology Vol. 26, No. 1 pp. 85-93 (1994), or the aldP gene promoter from rice (Kagaya et al., Molecular and General Genetics Vol. 248, No. 6 pp. 668-674 (1995), or a wound inducible promoter
10 such as the potato pin2 promoter (Xu et al, Plant Molecular Biology Vol. 22, No. 4 pp. 573-588 (1993). Likewise, the promoter may inducible by abiotic treatments such as temperature, drought or alterations in salinity or induced by exogenously applied substances that activate the promoter e.g. ethanol, oestrogens, plant hormones like ethylene, abscisic acid and gibberellic acid and heavy metals.

15 A promoter enhancer element may be used to achieve higher expression of the enzyme in the plant. For instance, the promoter enhancer element may be an intron which is placed between the promoter and the nucleotide sequence encoding the enzyme. For instance, Xu et al. *op cit* disclose the use of the first intron of the rice actin 1 gene to enhance expression.

20 The selectable marker gene and any other parts of the expression construct may be chosen from those available in the art.

The DNA construct is incorporated into the plant genome according to conventional techniques known in the art, including *Agrobacterium*-mediated transformation, virus-mediated transformation, micro injection, particle bombardment, biolistic transformation, and
25 electroporation (Gasser et al, Science, 244, 1293; Potrykus, Bio/Techn. 8, 535, 1990; Shimamoto et al, Nature, 338, 274, 1989).

Presently, *Agrobacterium tumefaciens* mediated gene transfer is the method of choice for generating transgenic dicots (for review Hooykas & Schilperoort, 1992. Plant Mol. Biol. 19: 15-38), and can also be used for transforming monocots, although other
30 transformation methods often are used for these plants. Presently, the method of choice for generating transgenic monocots supplementing the *Agrobacterium* approach is particle bombardment (microscopic gold or tungsten particles coated with the transforming DNA) of embryonic calli or developing embryos (Christou, 1992. Plant J. 2: 275-281; Shimamoto, 1994. Curr. Opin. Biotechnol. 5: 158-162; Vasil et al., 1992. Bio/Technology 10: 667-674).
35 An alternative method for transformation of monocots is based on protoplast transformation as described by Omirulleh S, et al., Plant Molecular biology Vol. 21, No. 3 pp. 415-428 (1993).

Following transformation, the transformants having incorporated the expression construct are selected and regenerated into whole plants according to methods well-known in the art. Often the transformation procedure is designed for the selective elimination of selection genes either during regeneration or in the following generations by using e.g. co-
5 transformation with two separate T-DNA constructs or site specific excision of the selection gene by a specific recombinase.

Starch processing

The hybrid enzyme of the first aspect of the invention or the variant of the second aspect of
10 the invention may in an eighth aspect be used in a process for liquefying starch, wherein a gelatinized or granular starch substrate is treated in aqueous medium with the hybrid enzyme. Preferably the process comprising hydrolysis of a slurry of gelatinized or granular starch, in particular hydrolysis of granular starch into a soluble starch hydrolysate at a temperature below the initial gelatinization temperature of said granular starch.

15 In a preferred embodiment the starch slurry in addition to being contacted with the hybrid enzyme of the first aspect or the variant of the second aspect of the invention is contacted with an enzyme selected from the list consisting of; a fungal alpha-amylase (EC 3.2.1.1), a beta-amylase (E.C. 3.2.1.2), and a glucoamylase (E.C.3.2.1.3). In an embodiment further a Termamyl-like alpha-amylase or a debranching enzyme, such as an isoamylase
20 (E.C. 3.2.1.68) or a pullulanases (E.C. 3.2.1.41) is added. In the context of the present invention a Termamyl-like alpha-amylase is an alpha-amylase as defined in WO99/19467 on page 3, line 18 to page 6, line 27.

The starch slurry to be subjected to the process of the seventh aspect of the invention may have 20-55% dry solids granular starch, preferably 25-40% dry solids granular
25 starch, more preferably 30-35% dry solids granular starch.

After being subjected to the process of the seventh aspect of the invention at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%,
30 or preferably at least 99% of the dry solids of the granular starch is converted into a soluble starch hydrolysate.

According to the invention the process of the seventh aspect is conducted at a temperature below the initial gelatinization temperature. Preferably the temperature at which the processes are conducted is at least 30°C, at least 31°C, at least 32°C, at least 33°C, at least 34°C, at least 35°C, at least 36°C, at least 37°C, at least 38°C, at least 39°C, at least
35 40°C, at least 41°C, at least 42°C, at least 43°C, at least 44°C, at least 45°C, at least 46°C, at least 47°C, at least 48°C, at least 49°C, at least 50°C, at least 51°C, at least 52°C, at least

53°C, at least 54°C, at least 55°C, at least 56°C, at least 57°C, at least 58°C, at least 59°C, or preferably at least 60°C.

The pH at which the process of the seventh aspect of the invention is conducted may in be in the range of 3.0 to 7.0, preferably from 3.5 to 6.0, or more preferably from 4.0-5.0.

5 The granular starch to be processed in the process of the invention may in particular be obtained from tubers, roots, stems, legumes, cereals or whole grain. More specifically the granular starch may be obtained from corns, cobs, wheat, barley, rye, milo, sago, cassava, tapioca, sorghum, rice, peas, bean, banana or potatoes. Specially contemplated are both waxy and non-waxy types of corn and barley. The granular starch to be processed may be a
10 highly refined starch quality, preferably at least 90%, at least 95%, at least 97% or at least 99.5 % pure or it may be a more crude starch containing material comprising milled whole grain including non-starch fractions such as germ residues and fibres. The raw material, such as whole grain, is milled in order to open up the structure and allowing for further processing. Two milling processes are preferred according to the invention: wet and dry
15 milling. In dry milling the whole kernel is milled and used. Wet milling gives a good separation of germ and meal (starch granules and protein) and is with a few exceptions applied at locations where the starch hydrolysate is used in production of syrups. Both dry and wet milling is well known in the art of starch processing and are equally contemplated for the process of the invention. Also corn grits, and preferably milled corn grits may be applied.

20 In an embodiment of the process of the seventh aspect of the invention the hybrid enzyme is used in a process for production of fuel or potable ethanol comprising contacting the treated starch with a yeast. Preferably the process comprises fermentation with a yeast carried out simultaneously or separately/sequential to the hydrolysis of the granular starch slurry. When the fermentation is performed simultaneous to the hydrolysis the temperature is
25 preferably between 30°C and 35°C, and more preferably between 31°C and 34°C.

In another embodiment the granular starch slurry is being contacted with a polypeptide comprising a CBM, but no catalytic module, i.e. application of loose CBMs. The loose CBMs may be starch binding modules, cellulose-binding modules, chitin-binding modules, xylan-binding modules, mannan-binding modules, and other binding modules. Preferred CBMs in
30 the present context are microbial CBMs, particularly bacterial or fungal CBMs. Particularly preferred are the starch binding modules disclosed in Danish patent application PA 2003 01568 as the polypeptide sequences SEQ ID NO:1, SEQ ID NO:2, and SEQ ID NO:3 or the starch binding modules shown in the present disclosure as SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:15; SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18; SEQ ID
35 NO:19, SEQ ID NO:20, SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23. Most preferred CBMs include the CBMs disclosed in the present disclosure as SEQ ID NO:24 and as SEQ ID NO:25. Also preferred for the invention is the application of any CBM having at least 50%, 60%, 70%, 80% or even at least 90% homology to any of the afore mentioned CBM amino

acid sequences. The loose CBMs may be applied to the granular starch slurry in effective amounts.

The glucose may also be fermented in to other fermentation products, such as citric acid, itaconic acid, lactic acid, gluconic acid; ketones; amino acids, such as glutamic acid (sodium monoglutamate), but also more complex compounds such as antibiotics, such as penicillin, tetracyclin; enzymes; vitamins, such as riboflavin, B12, beta-carotene; hormones, which are difficult to produce synthetically.

Dough-based products

10 The hybrid enzyme may be used for the preparation of a dough-based edible product, particularly a hybrid enzyme comprising an amylolytic module derived from *Aspergillus oryzae* such as the amino acid sequence shown in SEQ ID NO: 30. The hybrid enzyme used for the preparation of a dough-based edible product is in particular a hybrid enzyme comprising the sequence shown as SEQ ID NO: 30 and/or as amino acids 21-498 of SEQ ID
15 NO: 30 or any variant thereof such as a variant comprising one or more substitutions selected from the list consisting of: Q81R, K158D, K158V, S161D, S161N, Q163S, Q163A, D164S, Y175W, E176D, D177N, N264K, N264E, M266L, G466D, D468S, and N470D. The hybrid enzyme used for the preparation of a dough-based edible product is in particular a hybrid enzyme comprising an amino acid sequence which has at least 50% homology, preferably at
20 least 60%, 70%, 80%, 85% or at least 90%, e.g. at least 95%, 97%, 98%, or at least 99%, such as 100% homology to the sequences set forth in SEQ ID NO: 30 herein.

The dough generally comprises flour (particularly wheat flour) and water. The dough is leavened e.g. by adding chemical leavening agents or yeast, usually *Saccharomyces cerevisiae* (baker's yeast).

25 The dough-based product is made by leavening and heating the dough, e.g. by baking or steaming. Examples are steamed or baked bread (in particular white, whole-meal or rye bread), typically in the form of loaves or rolls,

The dough may comprise one or more additional enzymes, e.g. a second amylase (e.g. a maltogenic alpha-amylase), a cyclodextrin glucanotransferase, a protease or
30 peptidase, in particular an exopeptidase, a transglutaminase, a lipase, a phospholipase, a cellulase, a hemicellulase (e.g. a pentosanase or xylanase), a glycosyltransferase, a branching enzyme or an oxidase such as glucose oxidase or an oxidase with higher activity on maltose than on glucose.

The hybrid enzyme may be used at a lower dosage than the catalytic module with
35 alpha-amylase activity used alone (compared on a weight basis).

MATERIALS AND METHODS

Purchased material

5 Enzymes for DNA manipulations (e.g. restriction endonucleases, ligases etc.) are obtainable from New England Biolabs, Inc. and were used according to the manufacturer's instructions. Amplified plasmids were recovered with Qiagen® Plasmid Kit (Qiagen). Polymerase Chain Reaction (PCR) was carried out with Expand™ PCR system (Roche). QIAquick™ Gel Extraction Kit (Qiagen) was used for purification of PCR fragments and DNA fragments excised from agarose gels.

10 Strains and plasmids

Aspergillus kawachii strain IFO4308 was used as donor of CBM and linker sequences. *Aspergillus niger* strain DSM 2761 donated the amyolytic sequence (WO89/01969). *Aspergillus niger* strain MBin120 described in US provisional patent application No. 60/459902 (10345.000-US) was used as host strain. The *Aspergillus* expression plasmid pMT2188 described in the patent WO200295014 was used as vector. The *pyrF* defective *Escherichia coli* strain DB6507 (ATCC 35673) was used for propagation of pHUda381 and pHUda387

Plasmid pMT2188 consists of an expression cassette based on the *Aspergillus niger* neutral amylase II promoter fused to the *Aspergillus nidulans* triose phosphate isomerase non translated leader sequence (Pna2/tpi) and the *Aspergillus niger* amyloglycosidase terminator (Tamg). Also present on the plasmid is the *Aspergillus* selective marker *amdS* from *Aspergillus nidulans* enabling growth on acetamide as sole nitrogen source and the URA3 marker from *Saccharomyces cerevisiae* enabling growth of the *pyrF* defective *Escherichia coli* strain DB6507 (ATCC 35673), which propagates pHUda381 and pHUda387.

25 Transformation into *E. coli* DB6507 using the *S. cerevisiae* URA 3 gene as selective marker was done in the following way:

E. coli DB6507 was made competent by the method of Mandel and Higa (Mandel, M. and A. Higa (1970) J. Mol. Biol. 45, 154). Transformants were selected on solid M9 medium (Sambrook et. al (1989) Molecular cloning, a laboratory manual, 2. edition, Cold Spring Harbor Laboratory Press) supplemented with 1 g/l casaminoacids, 500 µg/l thiamine and 10 mg/l kanamycin.

30

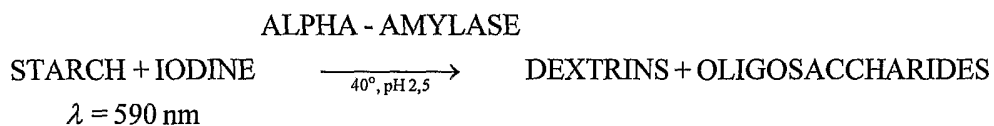
Media and substrates

Cove was composed of 342.3 g/L Sucrose, 20 ml/L COVE salt solution, 10mM Acetamide and 30 g/L noble agar. Cove-2 was composed of 30 g/L Sucrose, 20 ml/L COVE salt solution, 10mM, Acetamide and 30 g/L noble agar. COVE salt solution was composed of 26
 5 g/L KCl, 26 g/L MgSO₄·7H₂O, 76 g/L KH₂PO₄ and 50ml/L Cove trace metals. COVE trace metals was composed of 0.04 g/L Na₂B₄O₇·10 H₂O, 0.4 g/L CuSO₄·5H₂O, 1.2 g/L FeSO₄·7H₂O, 1.0 g/L MnSO₄·H₂O, 0.8 g/L Na₂MoO₂·2H₂O and 10 g/L ZnSO₄·7H₂O. YPG was composed of 4 g/L yeast extract, 1 g/L K₂HPO₄, 0.5 g/L MgSO₄·7H₂O and 15 g/L glucose, pH 6.0. STC was composed of 0.8 M Sorbitol, 25 mM Tris pH 8 and 25 mM CaCl₂.
 10 STPC was composed of 40 % PEG4000 in STC buffer. Cove top agarose was composed of 342.3 g/L Sucrose, 20 ml/L COVE salt solution, 10mM Acetamide and 10 g/L low melt agarose. MLC was composed of 40 g/L Glucose, 50 g/L Soybean powder, 4 g/L Citric acid, pH 5.0. GO-50 was composed of glucose 50 g/L, KH₂PO₄ 2 g/L, MgSO₄-7aq 2 g/L, K₂SO₄ 3 g, citric acid 3 g/L, oxalic acid 50 g/L, AMG trace metal solution 0.5 ml and urea 3 g/L, pH
 15 5.0. AMG trace metal solution was composed of 6.8 g/L ZnCl₂·7H₂O, 2.5 g/L CuSO₄·5H₂O, 0.24 g/L NiCl₂·6H₂O, 13.9 g/L FeSO₄·7H₂O, 13.5 g/L MnSO₄·H₂O and 3 g/L citric acid.

Acid stable alpha-amylase activity

When used according to the present invention the activity of any acid stable alpha-amylase may be measured in FAU (Acid Fungal Alpha-amylase Units), which are determined
 20 relative to an enzyme standard. 1 FAU is defined as the amount of enzyme which degrades 5.260 mg starch dry matter per hour under the below mentioned standard conditions.

Acid stable alpha-amylase, an endo-alpha-amylase (1,4-alpha-D-glucan-glucano-
 hydrolase, E.C. 3.2.1.1) hydrolyzes alpha-1,4-glucosidic bonds in the inner regions of the
 starch molecule to form dextrans and oligosaccharides with different chain lengths. The
 25 intensity of color formed with iodine is directly proportional to the concentration of starch. Amylase activity is determined using reverse colorimetry as a reduction in the concentration of starch under the specified analytical conditions.



30 blue/violet t = 23 sec. decoloration

Standard conditions/reaction conditions:

Substrate:	Soluble starch, approx. 0.17 g/L
Buffer:	Citrate, approx. 0.03 M
Iodine (I ₂):	0.03 g/L
CaCl ₂ :	1.85 mM
pH:	2.50 ± 0.05
Incubation temperature:	40°C
Reaction time:	23 seconds
Wavelength:	590nm
Enzyme concentration:	0.025 AFAU/mL
Enzyme working range:	0.01-0.04 AFAU/mL

A folder EB-SM-0259.02/01 describing this analytical method in more detail is available upon request to Novozymes A/S, Denmark, which folder is hereby included by reference.

5

Glucoamylase activity

Glucoamylase activity may be measured in AmyloGlucosidase Units (AGU). The AGU is defined as the amount of enzyme, which hydrolyzes 1 micromole maltose per minute under the standard conditions 37°C, pH 4.3, substrate: maltose 23.2 mM, buffer: acetate 0.1 M, reaction time 5 minutes.

An autoanalyzer system may be used. Mutarotase is added to the glucose dehydrogenase reagent so that any alpha-D-glucose present is turned into beta-D-glucose. Glucose dehydrogenase reacts specifically with beta-D-glucose in the reaction mentioned above, forming NADH which is determined using a photometer at 340 nm as a measure of the original glucose concentration.

15

AMG incubation:

Substrate:	maltose 23.2 mM
Buffer:	acetate 0.1 M
pH:	4.30 ± 0.05
Incubation temperature:	37°C ± 1
Reaction time:	5 minutes
Enzyme working range:	0.5-4.0 AGU/mL

Color reaction:

GlucDH:	430 U/L
Mutarotase:	9 U/L
NAD:	0.21 mM
Buffer:	phosphate 0.12 M; 0.15 M NaCl
pH:	7.60 ± 0.05
Incubation temperature:	37°C ± 1
Reaction time:	5 minutes
Wavelength:	340 nm

5 A folder ([EB-SM-0131.02/01](#)) describing this analytical method in more detail is available on request from Novozymes A/S, Denmark, which folder is hereby included by reference.

DNA manipulations

Unless otherwise stated, DNA manipulations and transformations were performed using standard methods of molecular biology as described in Sambrook et al. (1989) Molecular cloning: A laboratory manual, Cold Spring Harbor lab. Cold Spring Harbor, NY; Ausubel, F. M. et al. (eds.) "Current protocols in Molecular Biology", John Wiley and Sons, 1995; 10 Harwood, C. R. and Cutting, S. M. (eds.)

Example 1: Cloning of carbohydrate binding module (CBM) from *Aspergillus kawachii*

15 In order to clone the carbohydrate binding module (CBM) with linker from *A.kawachii*, the primers CBM1 (SEQ ID NO:1) and CBM2 (SEQ ID NO:2) were designed based on the nucleotide sequences of *Aspergillus kawachii* acid stable alpha-amylase in the EMBL

database (EMBL:#AB008370). CBM1 and CBM2 comprise a BamHI site and a Sall site, respectively.

5 CBM1: 5'- gaaggatccgattttactagtagtacatccaaagccaccac- 3'
 CBM2: 5'- ttgtcgacctacctccacgtatcaaccaccgtctcc- 3'

10 PCR reaction was carried out with the primers CBM1 and CBM2 using genomic DNA from *A. kawachii* (IFO4308) as template. Reaction components (1 ng /microL of genomic DNA, 250 mM dNTP each, primer 250 nM each, 0.1 U/ microL in Taq polymerase in 1X buffer (Roche Diagnostics, Japan)) were mixed and submitted for PCR under the following conditions.

Step	Temperature	Time
1	94°C	2 min
2	92°C	1 min
3	55°C	1 min
4	72°C	1 min
5	72°C	10 min
6	4°C	forever

Steps 2 to 4 were repeated 30 times.

15 A 0.4 kb fragment comprising the CBM with linker region was amplified. The amplified DNA was cut by Sal I and BamH I and ligated into pMT2188 digested by Xho I and BamH I to create pHUda381 having CBM with linker region from *Aspergillus kawachii*, *Aspergillus niger* neutral amylase promoter, *Aspergillus nidulans* TPI leader sequences, *Aspergillus niger* glucoamylase terminator and *Aspergillus nidulans* amdS gene as a marker. The pHUda381 was sequenced to confirm presence of correct CBM with linker sequence.
 20 The CBM with linker sequence is shown in SEQ ID NO:5.

Example 2: Expression of the hybrid enzyme in *Aspergillus niger*

25 The production of the cDNA sequence of *Aspergillus niger* acid stable alpha-amylase gene and the cDNA clone of *Aspergillus niger* acid stable alpha-amylase are described in WO 8901969 (example 1 and 3). A PCR reaction with cDNA clone of *Aspergillus niger* acid stable alpha-amylase as template was performed using the primers (SEQ ID NO:3) and (SEQ ID NO:4) to introduce a BamHI site and a SpeI site, respectively.

30 (SEQ ID NO:3): 5'- ttggatccaccatgagattatcgacttcgagtctcttc- 3'

The acid stable alpha-amylase activity in the supernatant was determined as decrease of blue color of starch-iodine complex measured in OD590nm. 25 microL of enzyme samples dissolved in sample buffer (51.4 mM calcium chloride in 2 mM citrate buffer [pH 2.5]) was mixed with 135 microL of substrate solution (0.6 g/L of starch [Merck 1253] and 12 g/L of sodium acetate in 100 mM citrate buffer [pH 2.5]) and incubated at 37 °C for 325 sec. After 325 sec, 90 microL of iodine solution (1.2 g/L of potassium iodine [Merck 5043] and 0.12 g/L of iodine [Merck 4761]) was added to the reaction mixture and incubated at 37 °C for 25 sec. Activity was measured at 590 nm on a spectrophotometer. 25 microL of distilled water was used instead of enzyme samples in blank experiments.

10

Example 3: Performance of the hybrid enzyme in SSF of non-gelatinized starch

The relative performance of *Aspergillus niger* acid stable alpha-amylase and *Aspergillus niger* acid stable alpha-amylase with an attached carbohydrate-binding module was evaluated via mini-scale SSF (Simultaneous Saccharification and Fermentation) fermentations. Dosages used were 0.3, 0.5 and 1.0 AFAU/g DS of the respective alpha-amylase complemented with a 0.5 AGU/g DS dose of purified *Aspergillus niger* glucoamylase. Briefly, approximately 1.9 g of ground corn (yellow #2 dent corn ground in a pilot scale hammer mill and passed through a 2 mm screen, 11.2% moisture content) was added to 16 ml polystyrene tubes (Falcon 352025). A solution of 0.02 N H₂SO₄ with 3 mg/ml penicillin was added in an amount appropriate to bring the dry solids level (DS) to 34% and the pH to 5.0. Treatments were made in six replicates.

15

20

After dosing the enzymes, the tubes were inoculated with 4.74×10^8 yeast cells/ml. Tubes were capped with a screw on lid which had been punctured with a very small needle to allow gas release and vortexed briefly before weighing and incubation at 32°C. Fermentation progress was followed by weighing the tubes over time. Tubes were vortexed briefly before weighing. The relationship used between amount of CO₂ loss and the weight of ethanol was: $CO_2 \text{ loss (g)} \times 1.045 = EtOH \text{ (g)}$. The results are shown in table 2.

Table 2. Average ethanol yield as g ethanol/g DS after 50 hrs.

Dose (AFAU/g DS)	<i>Aspergillus niger</i> alpha-amylase	<i>Aspergillus niger</i> alpha-amylase+CBM
0.3	0.342	0.366
0.5	0.348	0.383
1.0	0.365	0.390

95% confidence limits: + / - 0.008

30

On an equal activity basis, *Aspergillus niger* acid stable alpha-amylase with an attached carbohydrate-binding module (hybrid) significantly outperformed *Aspergillus niger*

acid stable alpha-amylase (native) at all dosages tested. The hybrid enzyme was approximately three times more effective than the native enzyme (i.e. performance of *Aspergillus niger* acid stable alpha-amylase could be matched with about three times less *Aspergillus niger* acid stable alpha-amylase with an attached carbohydrate-binding module).

5

Example 4: Performance of hybrid enzymes with different combinations of CBM, linker sequence and catalytic module

Hybrid enzyme variants comprising the *Aspergillus niger* acid stable alpha-amylase (AA) and a CBM from *A. kawachii* alpha-amylase, *A. niger* AMG, *T. emersonii* AMG, *Athelia rolfsii* AMG, or *Bacillus* sp. maltogenic alpha-amylase (Novamyl) were constructed. The linker sequence from *A. kawachii* alpha-amylase (SEQ ID NO:27) was used in all the variants.

10

The performance of the variants was assessed using corn starch (SIGMA S-9679) as substrate, *Aspergillus niger* glucoamylase G2 0.5 AGU/g DS, variant 1.0 AFAU/g DS and quantification of the liberated glucose with the Glucose B-test kit (Wako Pure Chemicals 271-3141). The results are shown in table 3.

15

Table 3. Performance of hybrid enzymes with different CBMs. Glucose mg/ml after 20, 48, and 68 hrs (1.0 AFAU/gDS)						
Variant	Catalytic module	Linker	SBD	20 hrs	48 hrs	68 hrs
JA001	<i>Aspergillus niger</i>	<i>kawachii</i> AA	<i>kawachii</i> AA	0.18	0.51	1.07
JA002	<i>Aspergillus niger</i>	<i>kawachii</i> AA	<i>niger</i> AMG	0.38	1.08	1.88
JA003	<i>Aspergillus niger</i>	<i>kawachii</i> AA	<i>emersonii</i> AMG	0.04	0.19	0.43
JA004	<i>Aspergillus niger</i>	<i>kawachii</i> AA	<i>rolfsii</i> AMG	0.48	1.43	2.48
JA005	<i>Aspergillus niger</i>	<i>kawachii</i> AA	<i>bacillus</i> MA	-0.03	-0.04	0.10

Hybrid enzyme variants comprising the *Aspergillus niger* acid stable alpha-amylase and a CBM from *A. kawachii* alpha-amylase, *A. niger* AMG, or *Athelia rolfsii* AMG, were constructed. Also variants with different linker sequence were constructed. The linker sequences used were *A. niger* AMG linker (SEQ ID NO:26), *A. kawachii* alpha-amylase linker (SEQ ID NO:27), *Athelia rolfsii* AMG linker (SEQ ID NO:28), and the PEPT linker (SEQ ID NO:29). A variant having two CBDs in tandem was also constructed (JA012).

20

The performance of the variants was assessed as above, only with *Aspergillus niger* glucoamylase G1 and the variant was dosed as 0.3 AFAU/g DS. The results are shown in table 4.

25

Table 4. Performance of hybrid enzymes with different linker sequences and catalytic modules. Glucose mg/ml after 18, 24, and 42 hrs.

Variant	Catalytic module	Linker	SBD	18 hrs	24 hrs	42 hrs	68 hrs
JA001	<i>Aspergillus niger</i>	kawachii AA	Kawachii AA	2.73	4.40	7.01	8.93
JA002	<i>Aspergillus niger</i>	kawachii AA	niger AMG	2.75	4.30	7.03	9.23
JA004	<i>Aspergillus niger</i>	kawachii AA	rolfsii AMG	2.91	4.35	7.16	9.11
JA008	<i>Aspergillus niger</i>	niger AMG	niger AMG	3.01	4.26	7.31	8.76
JA009	<i>Aspergillus niger</i>	rolfsii AMG	niger AMG	3.02	4.69	7.60	9.11
JA010	<i>Aspergillus niger</i>	PEPT	niger AMG	3.18	4.66	7.82	9.38
JA011	<i>Aspergillus niger</i>	rolfsii AMG	rolfsii AMG	3.31	4.62	7.49	9.60
JA012	<i>Aspergillus niger</i>	kawachii AA	niger AMG +rolfsii AMG	2.99	4.10	6.97	8.85

Hybrid enzyme variants comprising the linker and CBM from *A. kawachii* alpha-amylase with different catalytic modules were constructed. The catalytic modules applied were from *Aspergillus niger* acid stable alpha-amylase, *A. kawachii* alpha-amylase or *Aspergillus oryzae* alpha-amylase (Fungamyl™). The performance of the variants was assessed as above, with *Aspergillus niger* glucoamylase G1 and the variant dosed as 0.3 AFAU/g DS. The results are shown in table 5.

Table 5. Performance of hybrid enzymes with different catalytic modules. Glucose mg/ml after 18, 24, 42 and 68 hrs.

Variant	catalytic module	Linker	SBD	18 hrs	24 hrs	42 hrs	68 hrs
JA001	A. niger AA	kawachii AA	kawachii AA	2.73	4.40	7.01	8.93
JA006	A. oryzae AA	kawachii AA	kawachii AA	3.39	5.10	7.99	9.77
JA007	A. kawachii AA	kawachii AA	kawachii AA	2.91	3.99	6.82	8.57

Hybrid enzyme variants comprising the linker and CBM from *A. kawachii* alpha-amylase or *A. rolfsii* AMG with different catalytic modules from *Aspergillus niger* acid stable alpha-amylase or *Aspergillus oryzae* alpha-amylase (Fungamyl™). The performance of the variants was assessed as above with *Aspergillus niger* glucoamylase G1 and the variant dosed as 0.3 AFAU/g DS. The results are shown in table 6.

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Variant	catalytic module	Linker	SBD	18 hrs	24 hrs	42 hrs
JA001	<i>A. niger</i> AA	kawachii AA	kawachii AA	4.64	5.61	8.38
JA006	<i>A. oryzae</i> AA	kawachii AA	kawachii AA	6.27	7.93	9.40
JA017	<i>A. oryzae</i> AA	rolfsii AMG	rolfsii AMG	6.68	8.87	9.80

Example 5: Variants of JA017 with one or more substitutions in the catalytic module.

5 Variants of the hybrid enzyme JA017 (amino acid sequence shown in SEQ ID NO:40) comprising the *A. rolfsii* AMG CBM, *A. rolfsii* linker sequence and the *A. oryzae* alpha amylase catalytic module with one or more substitutions in the catalytic module were constructed using conventional protein engineering techniques. Table 7 lists the substitutions in the variants.

Table 7. Variants of JA017 (SEQ ID NO:40) with one or more substitutions in the catalytic module.

No.	Substitution
JA019	Y175W E176D
JA050	N264K M266L
JA056	D253N
JA057	K158D S161D Q163S D164S G466D D468S N470D
JA059	G60N N264K M266L
JA060	D177N
JA061	K158D S161D Q163S D164S Y175W E176D G466D D468S N470D
JA062	K158D S161D Q163S D164S Y175W E176D N264K M266L G466D D468S N470D
JA063	K158V S161D Q163S D164S G466D D468S N470D
JA069	K158D S161N Q163A D164S G466D D468S N470D
JA074	K158V S161N Q163A D164S N264K M266L G466D D468S N470D
JA076	Q81R K158V S161N Q163A D164S Y175W E176D G466D D468S N470D
JA083	K158D S161D Q163S D164S Y175W E176D N264E M266L G466D D468S N470D
JA085	Q81R K158V S161N Q163A D164S Y175W E176D D177N N264K M266L G466D D468S N470D
JA093	Q81R K158V S161N Q163A D164S Y175W E176D D177N N264E M266L G466D D468S N470D
JA094	K158V S161N Q163A D164S Y175W E176D D177N N264K M266L G466D D468S N470D
JA095	K158V S161N Q163A D164S Y175W E176D D177N N264E M266L G466D D468S N470D
JA096	K158V S161N Q163A D164S D177N N264K M266L G466D D468S N470D
JA097	K158V S161N Q163A D164S D177N N264E M266L G466D D468S N470D

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The variants were expressed in *Aspergillus oryzae* and characterized for pH stability, thermostability and the performance toward raw starch by glucose releasing test.

JA085 showed at least by 5 °C higher thermostability than JA017 at pH 4.5 and was stable even at pH 3.0.

The new variants JA085 and JA074 showed a high specific activity in the glucose release test. JA085 continued to release glucose even at pH 3.8 where JA017 was quickly inactivated. JA074 had the highest glucose yields at pH 4.0 but was inactivated at pH 3.8.

Tables 8-10 list the temperature stability as residual activity after 30 min incubation at 55°C and 60°C at pH 4.5, the specific activity, the pH stability and the activity in the glucose releasing test of a number of variants.

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Table 8. Temperature stability and specific activity of selected PE variants.

	Temperature stability (%)			Specific activity (mFAU/OD280)
	In 1mM CaCl ₂		No CaCl ₂	
	55°C	60°C	55°C	
JA017	2	0	0	3157
JA057	12	0	0	
JA061	51	0	16	
JA063	18	0	0	
JA069	16	0	0	
JA074	19	0	0	
JA085	68	20	12	3653
JA095	59	7	2	4240
JA096	53	0	0	
JA097	40	0	0	

Table 9a. Temperature stability (pH4.5, 1 hour incubation)

Temp. °C	JA017A	JA085A
35	101	107
40	109	102
45	98	96
50	64	95
55	0	56
60	0	27
65	0	0

Table 9b. pH stability (32°C, 1 hour incubation)

pH	JA017A	JA085A
2,5	0	20
3,0	23	76
3,5	74	83
4,0	88	92
4,5	86	95
5,0	96	99
5,5	100	100

Table 10a. Performance in glucose releasing test with purified *A.niger* AMG G2 at pH 4.0. Glucose mg/ml.

	0 min	19 min	43 min	67 min	91 min
JA001	0.48	3.48	5.67	8.26	10.79
JA017	0.46	6.45	7.97	8.71	8.98
JA074	0.44	8.85	14.69	18.24	20.29
JA085	0.41	5.87	10.62	13.92	17.34

Table 10b. Performance in glucose releasing test with purified *A.niger* AMG G2 at pH 3.8. Glucose mg/ml.

	0 min	19 min	43 min	67 min	91 min
JA001	0.48	3.19	5.28	7.63	10.32
JA017	0.46	4.28	4.71	4.98	5.13
JA074	0.43	7.75	11.58	13.55	13.93
JA085	0.41	5.71	9.73	12.99	15.76

Table 11a. Performance in glucose releasing test with purified *A.niger* AMG G2 at pH 3.9. Glucose mg/ml.

	0 min	18 min	90 min
JA074	0.41	6.83	16.87
JA085	0.40	4.50	14.08
JA094	0.40	4.48	14.00
JA095	0.40	4.49	13.82

Table 11b. Performance in glucose releasing test with purified *A.niger* AMG G2 at pH 3.6. Glucose mg/ml.

	0 min	18 min	90 min
JA074	0.43	5.01	7.57
JA085	0.43	4.18	10.61
JA094	0.42	4.18	11.18
JA095	0.42	4.33	11.52

5 Example 6: Variants of the *Aspergillus kawachii* acid alpha-amylase

A variant of the *Aspergillus kawachii* acid alpha-amylase suitable for raw starch hydrolysis may be produced by conventional protein engineered of the sequence comprising the catalytic module. Specific position wherein alterations can improve specific activity and/or stability was predicted based on similarity to one of the following fungal alpha-amylases having the indicated amino acid sequence and a three-dimensional structure found under the indicated identifier in the RCSB Protein Data Bank (www.rcsb.org): the *A. niger* acid alpha-amylase (2aaa, SEQ ID NO:42 herein) and the alpha-amylase (Taka amylase) from *Aspergillus oryzae* (6taa or 7taa, SEQ ID NO:43 herein). The two 3D models were superimposed by aligning the amino acid residues of each catalytic triad. This was done by methods known in the art based on the deviations of the three pairs of C-alpha atoms, e.g. by minimizing the sum of squares of the three deviations or by aligning so as to keep each deviation below 0.8 Å, e.g. below 0.6 Å, below 0.4 Å, below 0.3 Å or below 0.2 Å.

Alternatively, the superimposition may be done by aligning the two amino acid sequences by a conventional method and minimizing the sum of squares of the deviations of all corresponding pairs of amino acid residues. The sequence alignment may be done by conventional methods, e.g. by use the software GAP from UWGCG Version 8.

5 On the alignment of the three alpha-amylases from *A. kawachii*, *A. niger* (aaa_new) and *A. oryzae* (7taa) shown below are indicated the residues which are within 10Å from the acarbose substrate in 7taa.pdb structure. These residues are targets for alterations conferring improved specific activity.

10	kawachi	MRVSTSSIAL	AVSLFGKLAL	GLSAAEWRTQ	SIYFLLTDRF	GRTDNSTTAT	50
	aaa_new	MRLSTSSLFL	SVSLLGKLAL	GLSAAEWRTQ	SIYFLLTDRF	GRTDNSTTAT	
	7taaATPADWRSQ	SIYFLLTDRF	ARTDGSTTAT	29
					* * *		
15	kawachi	CNTGDQIYCG	GSWQGIINHL	DYIQGMGFTA	IWISPITEQL	PQDTSDEGAY	100
	aaa_new	CDTGDQIYCG	GSWQGIINHL	DYIQGMGFTA	IWISPITEQL	PQDTADGEAY	
	7taa	CNTADQKYCG	GTWQGIIDKL	DYIQGMGFTA	IWITPVTAQL	PQTTAYGDAY	79
		*****			* ** **	*****	
20	kawachi	HGYWQQKIYY	VNSNFGTADD	LKSLSDALHA	RGMYLMVDVV	PNHMGYAGNG	150
	aaa_new	HGYWQQKIYD	VNSNFGTADD	LKSLSDALHA	RGMYLMVDVV	PNHMGYAGNG	
	7taa	HGYWQQDIYS	LNENYGTADD	LKALSSALHE	RGMYLMVDVV	ANHMGYDGAG	129
		*****			***	*****	
25	kawachi	NDVDYSVFDP	FDSSSYFHPY	CLITDWDNLT	MVQDCWEGDT	IVSLPDLNNT	200
	aaa_new	NDVDYSVFDP	FDSSSYFHPY	CLITDWDNLT	MVQDCWEGDT	IVSLPDLNNT	
	7taa	SSVDYSVFKP	FSSQDYFHPF	CFIQNYEDQT	QVEDCWLGDN	TVSLPDLDTT	179
				*****	**	*****	*****
30	kawachi	ETAVRTIWYD	WVADLVSNYS	VDGLRIDSVE	EVEPDDFFPGY	QEAAGVYCVG	250
	aaa_new	ETAVRTIWYD	WVADLVSNYS	VDGLRIDSVL	EVEPDDFFPGY	QEAAGVYCVG	
	7taa	KDVVKNEWYD	WVGLSVSNYS	IDGLRIDTVK	HVQKDFWPGY	NKAAGVYCVG	229
				*****	**	*	*
35	kawachi	EVDNGNPALD	CPYQKYLDGV	LNYPYIWQLL	YAFESSSGSI	SNLYNMIKSV	300
	aaa_new	EVDNGNPALD	CPYQKVLGVD	LNYPYIWQLL	YAFESSSGSI	SNLYNMIKSV	
	7taa	EVLDGDPAYT	CPYQNVMDGV	LNYPYIYPLL	NAFKSTSGSM	DDLNMINTV	279
		*****	*	* ** * * *		*	
40	kawachi	ASDCSDPTLL	GNFIENHDNP	RFASYTSDYS	QAKNVLSYIF	LSDGIPIVYA	350
	aaa_new	ASDCSDPTLL	GNFIENHDNP	RFASYTSDYS	QAKNVLSYIF	LSDGIPIVYA	
	7taa	KSDCPDSTLL	GTFVENHDNP	RFASYTNDIA	LAKNVAAFII	LNDGIPIIYA	329
			* *****	*		*	
45	kawachi	GEEQHYSGGD	VPYNREATWL	SGYDTSAEELY	TWIATTNAIR	KLAISADSDY	400
	aaa_new	GEEQHYSGGK	VPYNREATWL	SGYDTSAEELY	TWIATTNAIR	KLAISADSAY	
	7taa	GQEQHYAGGN	DPANREATWL	SGYPTDSELY	KLIASANAIR	NYAISKDTGF	379
			* *****				
50	kawachi	ITYKNDFIYT	DSNTIAMRKG	TSGSQIITVL	SNKGSSGSSY	TTLTSLSGSYT	450
	aaa_new	ITYANDAFYT	DSNTIAMRKG	TSGSQIVITVL	SNKGSSGSSY	TTLTSLSGSYT	
	7taa	VTYKNWPIYK	DDTTIAMRKG	TDGSQIVTIL	SNKGASGDSY	TLSLGSAGYT	429

	kawachi	SGTKLIEAYT	CTSVTVDSNG	DIPVPMASGL	PRVLLPASVV	DSSSLCGGSG	500
	aaa_new	SGTKLIEAYT	CTSVTVDSNG	DIPVPMASGL	PRVLLPASVV	DSSSLCGGSG	
	7taa	AGQQLTEVIG	CTTVTVGSDG	NVPVPMAGGL	PRVLYPTEKL	AGSKICS...	474
5	kawachi	NTTTTTTAAT	STSKATTSSS	SSSAAATTSS	SCTATSTTLP	ITFEELVTTT	550
	aaa_new	RLYVE.....	505
	7taa	
10	kawachi	YGEEVYLSGS	ISQLGEWHTS	DAVKLSADDY	TSSNPEWSVT	VSLPVGTTFE	600
	aaa_new	
	7taa	
		601			640		
15	kawachi	YKFIKVDEGG	SVTWESDPNR	EYTVPECGSG	SGETVVDTWR		640
	aaa_new		
	7taa		

The specific alterations for increased specific activity is one substitution or a combination of substitutions in the following positions within the "10Å distance of the substrate": 13,15,18,32-36 (i.e. 32, 33, 34, 35, 36), 61,63-64, 68-69,73-84 (e.g. 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84),117-125 (e.g. 117, 118, 119, 120, 121, 122, 123, 124, 125),152-158 (e.g. 152, 153, 154, 155, 156, 157, 158),161-162, 165-175 (e.g.165, 166, 167, 168, 169, 170, 171, 172, 173, 174, 175), 204-211 (e.g. 204, 205, 206, 207, 208, 209, 210, 211), 216,229-239 (229, 230, 231, 232, 233, 234, 235, 236, 237, 238, 239), 242, 250, 252-253, 255-257 (e.g. 255, 256, 257),259-260, 275, 292, 295-299 (e.g. 295, 296, 297, 298, 299), 304, 328, 339-344 according to the 7taa numbering in the alignment above where the N-terminal is ATPAD in 7taa (TAKA amylase). Most interesting positions are: 33, 36, 74, 75, 77, 120, 153, 154, 155, 156, 157, 158, 162, 166, 169, 170, 199, 232, 233, 235, 238, 239, 256, 257, 331, 336, 339, 340, and 342. The substitutions may be to any amino acid residue.

Variants comprising one of the following substitutions or combinations thereof may be produced: G33A, I36K, S74A, D75Y, E77D, P120A, I153D, D154N, W155Y, D156E, N157D, L158Q, Q162E, E166L, T169N, I170T, E199K, E199L, D232L, N233D, N235D, L238Y, D239T, W256Y, Q257P, E331Q, S336A, D339K, D339N, V340D, and Y342A. The most interesting variants comprise one or more of the following substitutions; S74A, E166L, E199L, D339K, and D156E. Yet more interesting is the variant having the multiple substitutions S74A/E166L/E199L.

Based on similarity between *Aspergillus kawachii* and the *A. niger* acid alpha-amylases the following substitutions is predicted to improve the specific activity; N31D, S74A, Y89D, E209L, Y245V, D348K, D378A, K383A, P386A, I387F, I405V, N448S, and N480R, and a variant named WA01 comprising these substitutions can be produced.

Example 7: Performance of a variant of the *Aspergillus kawachii* acid alpha-amylase

The sequences of the hybrids JA007 and JA001 described in example 4 are identical to respectively the sequence of the *A. kawachii* acid alpha-amylase (SEQ ID NO:41) and the sequence of the variant WA01 described in the previous example. As the two hybrids JA001 and JA007 have been compared in several tests the improvement in specific activity conferred by the substitutions to the variant WA01 can be very accurately predicted. Specific activity of the variant WA01 will be 1.7 to 1.8 times higher than the specific activity of the *A. kawachii* wild type amylase. The prediction is based on the data shown in table 12.

Table 12. Performance of the hybrid JA007 (identical to *A. kawachii* acid alpha-amylase) and the hybrid JA001 (identical to WA01, a variant of the *A. kawachii* acid alpha-amylase comprising the substitutions N31D, S74A, Y89D, E209L, Y245V, D348K, D378A, K383A, P386A, I387F, I405V, N448S, and N480R)

	AFAU/ml	mg/ml	AFAU/mg	A280	AFAU/A280
JA001	1.41	0.67	2.11	1.22	1.15
JA007	0.56	0.46	1.22	0.89	0.63

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Example 8: Raw starch hydrolysis with an acid fungal alpha-amylase with or without CBD

This example illustrates the conversion of granular wheat starch or corn grits into glucose using acid fungal amylase without a CBM (SEQ ID NO:8) or the corresponding hybrid with a CBM (JA001). A slurry with 33% dry solids (DS) granular starch was prepared by adding 247.5 g of wheat starch or maize grits under stirring to 502.5 ml of water. The pH was adjusted with HCl to 4.5. The granular starch slurry was distributed to 100 ml blue cap flasks with 75 g in each flask. The flasks were incubated with magnetic stirring in a 60°C water bath. At zero hours the enzyme activities were dosed to the flasks; glucoamylase (200 AGU/kg DS), an acid fungal amylase (50 AFAU/kg DS) and the wild type *Aspergillus niger* acid alpha-amylase (SEQ ID NO:8) or the *Aspergillus niger* acid alpha-amylase but fused to a linker and CBM derived from *A. kawachii* (SEQ ID NO:8 fused to SEQ ID NO:6). The *Aspergillus niger* acid alpha-amylase w/wo CBM was dosed as 100 KNU/kg DS. Samples were withdrawn after 24, 48, 72, and 96 hours.

Total dry solids starch was determined using the following method. The starch was completely hydrolyzed by adding an excess amount of alpha-amylase (300 KNU/Kg dry solids) and placing the sample in an oil bath at 95 °C for 45 minutes. Subsequently the samples were cooled to 60°C and an excess amount of glucoamylase (600 AGU/kg DS) was added followed by incubation for 2 hours at 60°C.

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Soluble dry solids in the starch hydrolysate were determined by refractive index measurement on samples after filtering through a 0.22 microM filter. The sugar profiles were determined by HPLC. The amount of glucose was calculated as DX.

The results are shown in tables 13-14 (wheat starch) and 15-16 (corn grits).

5

Table 13. Soluble dry solids as percentage of total dry substance from wheat starch. Enzymes: glucoamylase, acid fungal alpha-amylase and more acid fungal alpha-amylase with the CBM or without the CBM.

	24 hours	46 hours	70 hours	90 hours
Without CBM	81,1	88,6	89,4	90,7
With CBM	86,2	92,6	93,7	95,1

Table 14. The DX of the soluble hydrolysate from wheat starch: Enzymes: glucoamylase, acid fungal amylase and more acid fungal alpha-amylase with the CBM or without the CBM.

	24 hours	46 hours	70 hours	90 hours
Without CBM	78,1	84,9	85,6	86,7
With CBM	82,3	88,4	89,5	90,5

Table 15. Soluble dry solids as percentage of total dry substance from corn grits. Enzymes: glucoamylase, acid fungal alpha-amylase and more acid fungal alpha-amylase with the CBM or without the CBM.

	24 hours	46 hours	70 hours	90 hours
Without CBM	44,0	49,6	56,8	62,2
With CBM	53,6	59,7	63,8	68,4

Table 16. The DX of the soluble hydrolysate from corn grits: Enzymes: glucoamylase, acid fungal amylase and more acid fungal alpha-amylase with the CBM or without the CBM.

	24 hours	46 hours	70 hours	90 hours
Without CBM	42,1	47,3	53,9	58,8
With CBM	51,2	56,8	60,4	64,5

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CLAIMS

- 1) A hybrid enzyme which comprises an amino acid sequence of a catalytic module and an amino acid sequence of a carbohydrate binding module,
- 5 a) wherein the catalytic module is an amino acid sequence which has at least 70% homology to the amino acid sequence of SEQ ID NO:8 or an amino acid sequence which has at least 70% homology to the amino acid sequence of SEQ ID NO:30;
- 10 b) wherein the carbohydrate binding module is an amino acid sequence having at least 50% homology to an amino acid sequence selected from the group consisting of SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24 or SEQ ID NO:25.
- 15 2) The hybrid enzyme of claim 1, wherein the hybrid enzyme further comprises a linker amino acid sequence which connects the amino acid sequence of the catalytic module and the amino acid sequence of the carbohydrate-binding module.
- 20 3) The hybrid enzyme of claim 2, wherein the linker sequence is a linker sequence selected from the group consisting of the linker sequence of SEQ ID NO:26, SEQ ID NO:27, SEQ ID NO:28, and SEQ ID NO:29 or a linker sequence which differs from the amino acid sequence shown in SEQ ID NO:26, SEQ ID NO:27, SEQ ID NO:28, or SEQ ID NO:29 by no more than 10 positions, no more than 9 positions, no more than 8 positions, no more than 7 positions, no more than 6 positions, no more than 5 positions, no more than 4 positions, no more than 3 positions, no more than 2 positions, or no more than 1 position.
- 25 4) The hybrid enzyme of claim 1, wherein the catalytic module is an amino acid sequence which has at least 70% homology to the amino acid sequence of SEQ ID NO:8.
- 5) The hybrid enzyme of claim 1, wherein the catalytic module is an amino acid sequence which has at least 80% homology to the amino acid sequence of SEQ ID NO:8.
- 6) The hybrid enzyme of claim 1, wherein the catalytic module is an amino acid sequence which has at least 90% homology to the amino acid sequence of SEQ ID NO:8.

- 7) The hybrid enzyme of claim 1, wherein the catalytic module is an amino acid sequence which has at least 95% homology to the amino acid sequence of SEQ ID NO:8.
- 8) The hybrid enzyme of claim 1, wherein the catalytic module is an amino acid sequence which has at least 70% homology to the amino acid sequence of SEQ ID NO:30.
- 5 9) The hybrid enzyme of claim 1, wherein the catalytic module is an amino acid sequence which has at least 80% homology to the amino acid sequence of SEQ ID NO:30.
- 10) The hybrid enzyme of claim 1, wherein the catalytic module is an amino acid sequence which has at least 90% homology to the amino acid sequence of SEQ ID NO:30.
- 11) The hybrid enzyme of claim 1, wherein the catalytic module is an amino acid sequence
10 which has at least 95% homology to the amino acid sequence of SEQ ID NO:30.
- 12) The hybrid enzyme of claim 1, wherein the carbohydrate binding module is an amino acid sequence having at least 60% homology to any of the amino acid sequences shown in SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:19,
15 SEQ ID NO:20, SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24 or SEQ ID NO:25
- 13) The hybrid enzyme of claim 1, wherein the carbohydrate binding module is an amino acid sequence having at least 70% homology to any of the amino acid sequences shown in SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:19,
20 SEQ ID NO:20, SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24 or SEQ ID NO:25
- 14) The hybrid enzyme of claim 1, wherein the carbohydrate binding module is an amino acid sequence having at least 80% homology to any of the amino acid sequences shown in SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24 or SEQ ID NO:25
25
- 15) The hybrid enzyme of claim 1, wherein the carbohydrate binding module is an amino

acid sequence having at least 90% homology to any of the amino acid sequences shown in SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24 or SEQ ID NO:25

5 16) The hybrid enzyme comprising the catalytic domain of the *A. oryzae* alpha amylase shown as amino acids 21-498 of the sequence SEQ ID NO:40 and an amino acid sequence having at least 70% homology amino acid sequence shown as amino acids 21-498 of the sequence SEQ ID NO:40.

10 17) A variant alpha-amylase having at least 80% homology to SEQ ID NO:41, which variant comprises an alteration at one or more of the positions selected from the group consisting of: 13,15,18, 31, 32, 33, 34, 35, 36, 61, 63, 64, 68, 69, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 89, 117, 118, 119, 120, 121, 122, 123, 124, 125, 152, 153, 154, 155, 156, 157, 158,161,162, 165, 166, 167, 168, 169, 170, 171, 172, 173, 174, 175, 204, 205, 206, 15 207, 208, 209, 210, 211, 216, 229, 230, 231,,232, 233, 234, 235, 236, 237, 238, 239, 242, 245, 250, 252, 253, 255, 256, 257,259, 260, 275, 292, 295, 296, 297, 298, 299, 304, 328, 339, 344, 348, 378, 383, 386, 387, 405, 448 and 480 wherein;

a) the alteration(s) are independently,
i) an insertion of an amino acid downstream of the amino acid which occupies the
20 position,
ii) a deletion of the amino acid which occupies the position, or
iii) a substitution of the amino acid which occupies the position with a different amino acid,
b) the variant has alpha-amylase activity.

25 18) The variant according to claim 17, comprising one or more of the following substitutions: G33A, I36K, S74A, D75Y, E77D, P120A, I153D, D154N, W155Y, D156E, N157D, L158Q, Q162E, E166L, T169N, I170T, E199K, E199L, D232L, N233D, N235D, L238Y, D239T, W256Y, Q257P, E331Q, S336A, D339K, D339N, V340D, and Y342A.

19) The variant according to claim 17, comprising an alteration at one or more of the
30 following positions: 31, 74, 89, 209, 245, 348, 378, 383, 386, 387, 405, 448 and 480.

20) The variant according to claim 17, comprising one or more of the following substitutions:

N31D, S74A, Y89D, E209L, Y245V, D348K, D378A, K383A, P386A, I387F, I405V, N448S, N480R.

- 21) The variant according to claim 17, comprising the following substitutions: N31D, S74A, Y89D, E209L, Y245V, D348K, D378A, K383A, P386A, I387F, I405V, N448S, N480R.
- 5 22) An isolated DNA sequence encoding the hybrid enzyme or the variant according to any of the preceding claims.
- 23) A DNA construct comprising the DNA sequence according to claim 22.
- 24) An expression vector comprising the DNA sequence according to claim 22.
- 25) A host cell transformed with a vector according to claim 24 which host cell is capable of
10 expressing the isolated DNA sequence of claim 18.
- 26) The host cell according to claim 25, which host cell is from a fungus, a bacteria, a plant or a mammal.
- 27) A method for liquefying starch, wherein a gelatinized or granular starch substrate is treated in aqueous medium with the hybrid enzyme or the variant according to any of
15 claims 1-21.
- 28) The method according to claim 27 comprising contacting the treated starch with a yeast to produce fuel or potable ethanol.
- 29) The method according to any of claims 27-28 comprising fermenting the treated starch into a fermentation product, such as citric acid, monosodium glutamate, gluconic acid,
20 sodium gluconate, calcium gluconate, potassium gluconate, glucono delta lactone, sodium erythorbate, itaconic acid, lactic acid, gluconic acid; ketones; amino acids, glutamic acid (sodium monoglutamate), penicillin, tetracyclin; enzymes; vitamins, such as riboflavin, B12, beta-carotene or hormones.
- 30) The method according to any of claims 27-28 wherein the starch slurry is contacted with
25 a polypeptide comprising a carbohydrate binding modcule, but no catalytic module.
- 31) A process for preparing a dough-based product, comprising adding the hybrid enzyme or

the variant of any of claims 1-21 to a dough.

- 5 32) A hybrid enzyme comprising the catalytic domain of the *A. oryzae* alpha amylase shown as amino acids 21-498 of the sequence SEQ ID NO:40 or a sequence having at least 50 % homology amino acid sequence shown as amino acids 21-498 of the sequence SEQ ID NO:40, and a carbohydrate binding domain.
- 33) The hybrid enzyme according to claim 32 comprising a substitution in one or more positions selected from the list comprising: of: 81, 158, 161, 163, 164, 175, 176, 177, 264, 266, 466, 468, and 470.
- 10 34) The hybrid enzyme according to claim 32 comprising one or more substitutions selected from the list consisting of: Q81R, K158D, K158V, S161D, S161N, Q163S, Q163A, D164S, Y175W, E176D, D177N, N264K, N264E, M266L, G466D, D468S, and N470D.
- 35) The hybrid enzyme according claim 32 comprising one or more substitutions selected from the list consisting of: K158V S161N Q163A D164S N264K M266L G466D D468S N470D
- 15 36) The hybrid enzyme according to claim 32 comprising one or more substitutions selected from the list consisting of: Q81R K158V S161N Q163A D164S Y175W E176D D177N N264K M266L G466D D468S N470D.

SEQUENCE LISTING

<110> Udagawa, Hiroaki
Taira, Rikako
Tkagi, Shinobu
Allain, Eric
Hjort, Carsten
Vikso-Nielsen, Anders

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 35 40 45

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 50 55 60

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 Lys Leu Ser Ala Asp Asp Tyr Thr Ser Ser Asn Pro Glu Trp Ser Val
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 Val Asp Glu Gly Gly Ser Val Thr Trp Glu Ser Asp Pro Asn Arg Glu
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 35 40 45

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Lys Leu Ser Ala Asp Asp Tyr Thr Ser Ser Asn Pro Glu Trp Ser Val
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 35 40 45

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Arg Ile Asp Ser Val Leu Glu Val Glu Pro Asp Phe Phe Pro Gly Tyr	
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Pro Ala Leu Asp Cys Pro Tyr Gln Lys Val Leu Asp Gly Val Leu Asn	
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Tyr Phe Leu Leu Thr Asp Arg Phe Gly Arg Thr Asp Asn Ser Thr Thr
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Ala Thr Cys Asp Thr Gly Asp Gln Ile Tyr Cys Gly Gly Ser Trp Gln
 50 55 60

Gly Ile Ile Asn His Leu Asp Tyr Ile Gln Gly Met Gly Phe Thr Ala
 65 70 75 80

Ile Trp Ile Ser Pro Ile Thr Glu Gln Leu Pro Gln Asp Thr Ala Asp
 85 90 95

Gly Glu Ala Tyr His Gly Tyr Trp Gln Gln Lys Ile Tyr Asp Val Asn
 100 105 110

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Gly Tyr Ala Gly Asn Gly Asn Asp Val Asp Tyr Ser Val Phe Asp Pro
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Phe Asp Ser Ser Ser Tyr Phe His Pro Tyr Cys Leu Ile Thr Asp Trp
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Arg Ile Asp Ser Val Leu Glu Val Glu Pro Asp Phe Phe Pro Gly Tyr
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Lys Gly Ser Ser Gly Ser Ser Tyr Thr Leu Thr Leu Ser Gly Ser Gly
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Tyr Thr Ser Gly Thr Lys Leu Ile Glu Ala Tyr Thr Cys Thr Ser Val
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 35 40 45

Thr Trp Thr Val Thr Ile Pro Leu Leu Gln Gly Gln Asn Ile Gln Phe
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Lys Phe Ile Lys Lys Asp Ser Ala Gly Asn Val Ile Trp Glu Asp Ile
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 35 40 45

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 35 40 45

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 35 40 45

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35 40 45

Gln Leu Gly Asp Trp Asp Thr Ser Asn Ala Val Ala Leu Ser Ala Ala
50 55 60

Asp Tyr Thr Ser Ser Asp His Leu Trp Phe Val Asp Ile Asp Leu Pro
65 70 75 80

Ala Gly Thr Val Phe Glu Tyr Lys Tyr Ile Arg Ile Glu Ser Asp Gly
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 35 40 45

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 50 55 60

Ala Thr Val His Leu Pro Ala Gly Thr Thr Phe Gln Tyr Lys Tyr Ile
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 35 40 45

Gln Leu Ala Gly Ser Ile Ser Ala Leu Gly Ser Trp Asp Thr Ser Ser
 50 55 60

Ala Ile Arg Leu Ser Ala Ser Gln Tyr Thr Ser Ser Asn His Leu Trp
 65 70 75 80

Tyr Val Ala Val Ser Leu Pro Ala Gly Gln Val Phe Gln Tyr Lys Tyr
 85 90 95

Ile Arg Val Ala Ser Ser Gly Thr Ile Thr Trp Glu Ser Asp Pro Asn
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Ser Asp Thr Trp Arg
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 <213> Mz1 Penicillium sp.

<400> 21

Thr Lys Thr Ser Thr Ser Thr Ser Cys Thr Thr Pro Thr Ala Val Ala
 1 5 10 15

Val Thr Phe Asp Leu Ile Ala Thr Thr Thr Tyr Gly Glu Asn Ile Lys
 20 25 30

Ile Ala Gly Ser Ile Ala Ala Leu Gly Ala Trp Asp Thr Asp Asp Ala
 35 40 45

Val Ala Leu Ser Ala Ala Asp Tyr Thr Asp Ser Asp His Leu Trp Phe
 50 55 60

Val Thr Gln Ser Ile Pro Ala Gly Thr Val Phe Glu Tyr Lys Tyr Ile
 65 70 75 80

Arg Val Glu Ser Asp Gly Thr Ile Glu Trp Glu Ser Asp Pro Asn Arg
 85 90 95

Ser Tyr Thr Val Pro Ala Ala Cys Ala Thr Thr Ala Val Thr Glu Ser
 100 105 110

Asp Thr Trp Arg

115

<210> 22
 <211> 114
 <212> PRT
 <213> Thysanophora sp

<400> 22

Phe Thr Ser Thr Thr Lys Thr Ser Cys Thr Thr Pro Thr Ser Val Ala
 1 5 10 15

Val Thr Phe Asp Leu Ile Ala Thr Thr Thr Tyr Gly Glu Ser Ile Arg
 20 25 30

Leu Val Gly Ser Ile Ser Glu Leu Gly Asp Trp Asp Thr Gly Ser Ala
 35 40 45

Ile Ala Leu His Ala Thr Asp Tyr Thr Asp Ser Asp His Leu Trp Phe
 50 55 60

Val Thr Val Gly Leu Pro Ala Gly Ala Ser Phe Glu Tyr Lys Tyr Ile
 65 70 75 80

Arg Val Glu Ser Ser Gly Thr Ile Glu Trp Glu Ser Asp Pro Asn Arg
 85 90 95

Ser Tyr Thr Val Pro Ala Ala Cys Ala Thr Thr Ala Val Thr Glu Ser
 100 105 110

Asp Thr

<210> 23
 <211> 111
 <212> PRT
 <213> Humicola grisea var. thermoidea

<400> 23

Ala Asp Ala Ser Glu Val Tyr Val Thr Phe Asn Glu Arg Val Ser Thr
 1 5 10 15

Ala Trp Gly Glu Thr Ile Lys Val Val Gly Asn Val Pro Ala Leu Gly
 20 25 30

Asn Trp Asp Thr Ser Lys Ala Val Thr Leu Ser Ala Ser Gly Tyr Lys
 35 40 45

Ser Asn Asp Pro Leu Trp Ser Ile Thr Val Pro Ile Lys Ala Thr Gly
 50 55 60

Ser Ala Val Gln Tyr Lys Tyr Ile Lys Val Gly Thr Asn Gly Lys Ile
 65 70 75 80

Thr Trp Glu Ser Asp Pro Asn Arg Ser Ile Thr Leu Gln Thr Ala Ser
 85 90 95

Ser Ala Gly Lys Cys Ala Ala Gln Thr Val Asn Asp Ser Trp Arg
 100 105 110

<210> 24
 <211> 108
 <212> PRT
 <213> *Aspergillus niger*

<400> 24

Cys Thr Thr Pro Thr Ala Val Ala Val Thr Phe Asp Leu Thr Ala Thr
 1 5 10 15

Thr Thr Tyr Gly Glu Asn Ile Tyr Leu Val Gly Ser Ile Ser Gln Leu
 20 25 30

Gly Asp Trp Glu Thr Ser Asp Gly Ile Ala Leu Ser Ala Asp Lys Tyr
 35 40 45

Thr Ser Ser Asp Pro Leu Trp Tyr Val Thr Val Thr Leu Pro Ala Gly
 50 55 60

Glu Ser Phe Glu Tyr Lys Phe Ile Arg Ile Glu Ser Asp Asp Ser Val
 65 70 75 80

Glu Trp Glu Ser Asp Pro Asn Arg Glu Tyr Thr Val Pro Gln Ala Cys
 85 90 95

Gly Thr Ser Thr Ala Thr Val Thr Asp Thr Trp Arg
 100 105

<210> 25
 <211> 97
 <212> PRT
 <213> *Aspergillus rolfsii*

<400> 25

Val Glu Val Thr Phe Asp Val Tyr Ala Thr Thr Val Tyr Gly Gln Asn
 1 5 10 15

Ile Tyr Ile Thr Gly Asp Val Ser Glu Leu Gly Asn Trp Thr Pro Ala
 20 25 30

Asn Gly Val Ala Leu Ser Ser Ala Asn Tyr Pro Thr Trp Ser Ala Thr
 35 40 45

Ile Ala Leu Pro Ala Asp Thr Thr Ile Gln Tyr Lys Tyr Val Asn Ile
 50 55 60

Asp Gly Ser Thr Val Ile Trp Glu Asp Ala Ile Ser Asn Arg Glu Ile
 65 70 75 80

Thr Thr Pro Ala Ser Gly Thr Tyr Thr Glu Lys Asp Thr Trp Asp Glu
 85 90 95

Ser

<210> 26
 <211> 38
 <212> PRT
 <213> *Aspergillus niger*

<400> 26

Thr Gly Gly Thr Thr Thr Thr Ala Thr Pro Thr Gly Ser Gly Ser Val
 1 5 10 15

Thr Ser Thr Ser Lys Thr Thr Ala Thr Ala Ser Lys Thr Ser Thr Ser
 20 25 30

Thr Ser Ser Thr Ser Ala
 35

<210> 27
 <211> 31
 <212> PRT
 <213> *Aspergillus kawachii*

<400> 27

Thr Thr Thr Thr Thr Thr Ala Ala Ala Thr Ser Thr Ser Lys Ala Thr
 1 5 10 15

Thr Ser Ser Ser Ser Ser Ser Ala Ala Ala Thr Thr Ser Ser Ser
 20 25 30

<210> 28
 <211> 11

<212> PRT
 <213> Athelia rolfsii

<400> 28

Gly Ala Thr Ser Pro Gly Gly Ser Ser Gly Ser
 1 5 10

<210> 29
 <211> 8
 <212> PRT
 <213> Artificial

<220>
 <223> Artificial

<400> 29

Pro Glu Pro Thr Pro Glu Pro Thr
 1 5

<210> 30
 <211> 498
 <212> PRT
 <213> Aspergillus oryzae

<400> 30

Met Val Ala Trp Trp Ser Leu Phe Leu Tyr Gly Leu Gln Val Ala Ala
 1 5 10 15

Pro Ala Leu Ala Ala Thr Pro Ala Asp Trp Arg Ser Gln Ser Ile Tyr
 20 25 30

Phe Leu Leu Thr Asp Arg Phe Ala Arg Thr Asp Gly Ser Thr Thr Ala
 35 40 45

Thr Cys Asn Thr Ala Asp Gln Lys Tyr Cys Gly Gly Thr Trp Gln Gly
 50 55 60

Ile Ile Asp Lys Leu Asp Tyr Ile Gln Gly Met Gly Phe Thr Ala Ile
 65 70 75 80

Trp Ile Thr Pro Val Thr Ala Gln Leu Pro Gln Thr Thr Ala Tyr Gly
 85 90 95

Asp Ala Tyr His Gly Tyr Trp Gln Gln Asp Ile Tyr Ser Leu Asn Glu
 100 105 110

Asn Tyr Gly Thr Ala Asp Asp Leu Lys Ala Leu Ser Ser Ala Leu His
 115 120 125

Glu Arg Gly Met Tyr Leu Met Val Asp Val Val Ala Asn His Met Gly
 130 135 140

Tyr Asp Gly Ala Gly Ser Ser Val Asp Tyr Ser Val Phe Lys Pro Phe
 145 150 155 160

Ser Ser Gln Asp Tyr Phe His Pro Phe Cys Phe Ile Gln Asn Tyr Glu
 165 170 175

Asp Gln Thr Gln Val Glu Asp Cys Trp Leu Gly Asp Asn Thr Val Ser
 180 185 190

Leu Pro Asp Leu Asp Thr Thr Lys Asp Val Val Lys Asn Glu Trp Tyr
 195 200 205

Asp Trp Val Gly Ser Leu Val Ser Asn Tyr Ser Ile Asp Gly Leu Arg
 210 215 220

Ile Asp Thr Val Lys His Val Gln Lys Asp Phe Trp Pro Gly Tyr Asn
 225 230 235 240

Lys Ala Ala Gly Val Tyr Cys Ile Gly Glu Val Leu Asp Gly Asp Pro
 245 250 255

Ala Tyr Thr Cys Pro Tyr Gln Asn Val Met Asp Gly Val Leu Asn Tyr
 260 265 270

Pro Ile Tyr Tyr Pro Leu Leu Asn Ala Phe Lys Ser Thr Ser Gly Ser
 275 280 285

Met Asp Asp Leu Tyr Asn Met Ile Asn Thr Val Lys Ser Asp Cys Pro
 290 295 300

Asp Ser Thr Leu Leu Gly Thr Phe Val Glu Asn His Asp Asn Pro Arg
 305 310 315 320

Phe Ala Ser Tyr Thr Asn Asp Ile Ala Leu Ala Lys Asn Val Ala Ala
 325 330 335

Phe Ile Ile Leu Asn Asp Gly Ile Pro Ile Ile Tyr Ala Gly Gln Glu
 340 345 350

Gln His Tyr Ala Gly Gly Asn Asp Pro Ala Asn Arg Glu Ala Thr Trp
 355 360 365

Leu Ser Gly Tyr Pro Thr Asp Ser Glu Leu Tyr Lys Leu Ile Ala Ser
 370 375 380

Ala Asn Ala Ile Arg Asn Tyr Ala Ile Ser Lys Asp Thr Gly Phe Val
 385 390 395 400

Thr Tyr Lys Asn Trp Pro Ile Tyr Lys Asp Asp Thr Thr Ile Ala Met
 405 410 415

Arg Lys Gly Thr Asp Gly Ser Gln Ile Val Thr Ile Leu Ser Asn Lys
 420 425 430

Gly Ala Ser Gly Asp Ser Tyr Thr Leu Ser Leu Ser Gly Ala Gly Tyr
 435 440 445

Thr Ala Gly Gln Gln Leu Thr Glu Val Ile Gly Cys Thr Thr Val Thr
 450 455 460

Val Gly Ser Asp Gly Asn Val Pro Val Pro Met Ala Gly Gly Leu Pro
 465 470 475 480

Arg Val Leu Tyr Pro Thr Glu Lys Leu Ala Gly Ser Lys Ile Cys Ser
 485 490 495

Ser Ser

- <210> 31
- <211> 1923
- <212> DNA
- <213> Artificial
- <220>
- <223> Artificial
- <220>
- <221> CDS
- <222> (1)..(1923)

<400> 31
 atg aga tta tog act tcg agt ctc ttc ctt tcc gtg tct ctg ctg ggg 48
 Met Arg Leu Ser Thr Ser Ser Leu Phe Leu Ser Val Ser Leu Leu Gly
 1 5 10 15

aag ctg gcc ctc ggg ctg tog gct gca gaa tgg cgc act cag tcg att 96
 Lys Leu Ala Leu Gly Leu Ser Ala Ala Glu Trp Arg Thr Gln Ser Ile
 20 25 30

tac ttc cta ttg acg gat cgg ttc ggt agg acg gac aat tcg acg aca 144
 Tyr Phe Leu Leu Thr Asp Arg Phe Gly Arg Thr Asp Asn Ser Thr Thr
 35 40 45

gct aca tgc gat acg ggt gac caa atc tat tgt ggt ggc agt tgg caa Ala Thr Cys Asp Thr Gly Asp Gln Ile Tyr Cys Gly Gly Ser Trp Gln 50 55 60	192
gga atc atc aac cat ctg gat tat atc cag ggc atg gga ttc acg gcc Gly Ile Ile Asn His Leu Asp Tyr Ile Gln Gly Met Gly Phe Thr Ala 65 70 75 80	240
atc tgg atc tgc cct atc act gaa cag ctg ccc cag gat act gct gat Ile Trp Ile Ser Pro Ile Thr Glu Gln Leu Pro Gln Asp Thr Ala Asp 85 90 95	288
ggt gaa gct tac cat gga tat tgg cag cag aag ata tac gac gtg aac Gly Glu Ala Tyr His Gly Tyr Trp Gln Gln Lys Ile Tyr Asp Val Asn 100 105 110	336
tcc aac ttc ggc act gca gat gac ctc aag tcc ctc tca gat gcg ctt Ser Asn Phe Gly Thr Ala Asp Asp Leu Lys Ser Leu Ser Asp Ala Leu 115 120 125	384
cat gcc cgc gga atg tac ctc atg gtg gac gtc gtc cct aac cac atg His Ala Arg Gly Met Tyr Leu Met Val Asp Val Val Pro Asn His Met 130 135 140	432
ggc tac gcc ggc aac ggc aac gat gta gac tac agc gtc ttc gac ccc Gly Tyr Ala Gly Asn Gly Asn Asp Val Asp Tyr Ser Val Phe Asp Pro 145 150 155 160	480
ttc gat tcc tcc tcc tac ttc cac cca tac tgc ctg atc aca gat tgg Phe Asp Ser Ser Ser Tyr Phe His Pro Tyr Cys Leu Ile Thr Asp Trp 165 170 175	528
gac aac ttg acc atg gtc caa gat tgt tgg gag ggt gac acc atc gta Asp Asn Leu Thr Met Val Gln Asp Cys Trp Glu Gly Asp Thr Ile Val 180 185 190	576
tct ctg cca gac cta aac acc acc gaa act gcc gtg aga aca atc tgg Ser Leu Pro Asp Leu Asn Thr Thr Glu Thr Ala Val Arg Thr Ile Trp 195 200 205	624
tat gac tgg gta gcc gac ctg gta tcc aat tat tca gtc gac gga ctc Tyr Asp Trp Val Ala Asp Leu Val Ser Asn Tyr Ser Val Asp Gly Leu 210 215 220	672
cgc atc gac agt gtc ctc gaa gtc gaa cca gac ttc ttc ccg ggc tac Arg Ile Asp Ser Val Leu Glu Val Glu Pro Asp Phe Phe Pro Gly Tyr 225 230 235 240	720
cag gaa gca gca ggt gtc tac tgc gtc ggc gaa gtc gac aac ggc aac Gln Glu Ala Ala Gly Val Tyr Cys Val Gly Glu Val Asp Asn Gly Asn 245 250 255	768
cct gcc ctc gac tgc cca tac cag aag gtc ctg gac ggc gtc ctc aac Pro Ala Leu Asp Cys Pro Tyr Gln Lys Val Leu Asp Gly Val Leu Asn 260 265 270	816
tat ccg atc tac tgg caa ctc ctc tac gcc ttc gaa tcc tcc agc ggc Tyr Pro Ile Tyr Trp Gln Leu Leu Tyr Ala Phe Glu Ser Ser Ser Gly 275 280 285	864

agc atc agc aat ctc tac aac atg atc aaa tcc gtc gca agc gac tgc Ser Ile Ser Asn Leu Tyr Asn Met Ile Lys Ser Val Ala Ser Asp Cys 290 295 300	912
tcc gat cgg aca cta ctc ggc aac ttc atc gaa aac cac gac aat ccc Ser Asp Pro Thr Leu Leu Gly Asn Phe Ile Glu Asn His Asp Asn Pro 305 310 315 320	960
cgt ttc gcc tcc tac acc tcc gac tac tcg caa gcc aaa aac gtc ctc Arg Phe Ala Ser Tyr Thr Ser Asp Tyr Ser Gln Ala Lys Asn Val Leu 325 330 335	1008
agc tac atc ttc ctc tcc gac ggc atc ccc atc gtc tac gcc ggc gaa Ser Tyr Ile Phe Leu Ser Asp Gly Ile Pro Ile Val Tyr Ala Gly Glu 340 345 350	1056
gaa cag cac tac tcc ggc ggc aag gtg ccc tac aac cgc gaa gcg acc Glu Gln His Tyr Ser Gly Gly Lys Val Pro Tyr Asn Arg Glu Ala Thr 355 360 365	1104
tgg ctt tca ggc tac gac acc tcc gca gag ctg tac acc tgg ata gcc Trp Leu Ser Gly Tyr Asp Thr Ser Ala Glu Leu Tyr Thr Trp Ile Ala 370 375 380	1152
acc acg aac gcg atc cgc aaa cta gcc atc tca gct gac tcg gcc tac Thr Thr Asn Ala Ile Arg Lys Leu Ala Ile Ser Ala Asp Ser Ala Tyr 385 390 395 400	1200
att acc tac gcg aat gat gca ttc tac act gac agc aac acc atc gca Ile Thr Tyr Ala Asn Asp Ala Phe Tyr Thr Asp Ser Asn Thr Ile Ala 405 410 415	1248
atg cgc aaa ggc acc tca ggg agc caa gtc atc acc gtc ctc tcc aac Met Arg Lys Gly Thr Ser Gly Ser Gln Val Ile Thr Val Leu Ser Asn 420 425 430	1296
aaa ggc tcc tca gga agc agc tac acc ctg acc ctc agc gga agc ggc Lys Gly Ser Ser Gly Ser Ser Tyr Thr Leu Thr Leu Ser Gly Ser Gly 435 440 445	1344
tac aca tcc ggc acg aag ctg atc gaa gcg tac aca tgc aca tcc gtg Tyr Thr Ser Gly Thr Lys Leu Ile Glu Ala Tyr Thr Cys Thr Ser Val 450 455 460	1392
acc gtg gac tcg agc ggc gat att ccc gtg ccg atg gcg tcg gga tta Thr Val Asp Ser Ser Gly Asp Ile Pro Val Pro Met Ala Ser Gly Leu 465 470 475 480	1440
ccg aga gtt ctt ctg ccc gcg tcc gtc gtc gat agc tct tcg ctc tgt Pro Arg Val Leu Leu Pro Ala Ser Val Val Asp Ser Ser Ser Leu Cys 485 490 495	1488
ggc ggg agc gga aga aca acc acg acc aca act gct gct gct act agt Gly Gly Ser Gly Arg Thr Thr Thr Thr Thr Ala Ala Ala Thr Ser 500 505 510	1536
aca tcc aaa gcc acc acc tcc tct tct tct tct tct gct gct gct act Thr Ser Lys Ala Thr Thr Ser Ser Ser Ser Ser Ser Ala Ala Ala Thr 515 520 525	1584
act tct tca tca tgt acc act ccc acc gcc gtg gct gtg act ttc gat	1632

Thr Ser Ser Ser Cys Thr Thr Pro Thr Ala Val Ala Val Thr Phe Asp
 530 535 540

ctg aca gct acc acc acc tac ggc gag aac atc tac ctg gtc gga tcg 1680
 Leu Thr Ala Thr Thr Thr Tyr Gly Glu Asn Ile Tyr Leu Val Gly Ser
 545 550 555 560

atc tct cag ctg ggt gac tgg gaa acc agc gac ggc ata gct ctg agt 1728
 Ile Ser Gln Leu Gly Asp Trp Glu Thr Ser Asp Gly Ile Ala Leu Ser
 565 570 575

gct gac aag tac act tcc agc gac ccg ctc tgg tat gtc act gtg act 1776
 Ala Asp Lys Tyr Thr Ser Ser Asp Pro Leu Trp Tyr Val Thr Val Thr
 580 585 590

ctg ccg gct ggt gag tcg ttt gag tac aag ttt atc cgc att gag agc 1824
 Leu Pro Ala Gly Glu Ser Phe Glu Tyr Lys Phe Ile Arg Ile Glu Ser
 595 600 605

gat gac tcc gtg gag tgg gag agt gat ccc aac cga gaa tac acc gtt 1872
 Asp Asp Ser Val Glu Trp Glu Ser Asp Pro Asn Arg Glu Tyr Thr Val
 610 615 620

cct cag gcg tgc gga acg tcg acc gcg acg gtg act gac acc tgg cgg 1920
 Pro Gln Ala Cys Gly Thr Ser Thr Ala Thr Val Thr Asp Thr Trp Arg
 625 630 635 640

tag 1923

<210> 32
 <211> 640
 <212> PRT
 <213> Artificial

<220>
 <223> Artificial

<400> 32

Met Arg Leu Ser Thr Ser Ser Leu Phe Leu Ser Val Ser Leu Leu Gly
 1 5 10 15

Lys Leu Ala Leu Gly Leu Ser Ala Ala Glu Trp Arg Thr Gln Ser Ile
 20 25 30

Tyr Phe Leu Leu Thr Asp Arg Phe Gly Arg Thr Asp Asn Ser Thr Thr
 35 40 45

Ala Thr Cys Asp Thr Gly Asp Gln Ile Tyr Cys Gly Gly Ser Trp Gln
 50 55 60

Gly Ile Ile Asn His Leu Asp Tyr Ile Gln Gly Met Gly Phe Thr Ala
 65 70 75 80

Ile Trp Ile Ser Pro Ile Thr Glu Gln Leu Pro Gln Asp Thr Ala Asp

85 90 95

Gly Glu Ala Tyr His Gly Tyr Trp Gln Gln Lys Ile Tyr Asp Val Asn
100 105 110

Ser Asn Phe Gly Thr Ala Asp Asp Leu Lys Ser Leu Ser Asp Ala Leu
115 120 125

His Ala Arg Gly Met Tyr Leu Met Val Asp Val Val Pro Asn His Met
130 135 140

Gly Tyr Ala Gly Asn Gly Asn Asp Val Asp Tyr Ser Val Phe Asp Pro
145 150 155 160

Phe Asp Ser Ser Ser Tyr Phe His Pro Tyr Cys Leu Ile Thr Asp Trp
165 170 175

Asp Asn Leu Thr Met Val Gln Asp Cys Trp Glu Gly Asp Thr Ile Val
180 185 190

Ser Leu Pro Asp Leu Asn Thr Thr Glu Thr Ala Val Arg Thr Ile Trp
195 200 205

Tyr Asp Trp Val Ala Asp Leu Val Ser Asn Tyr Ser Val Asp Gly Leu
210 215 220

Arg Ile Asp Ser Val Leu Glu Val Glu Pro Asp Phe Phe Pro Gly Tyr
225 230 235 240

Gln Glu Ala Ala Gly Val Tyr Cys Val Gly Glu Val Asp Asn Gly Asn
245 250 255

Pro Ala Leu Asp Cys Pro Tyr Gln Lys Val Leu Asp Gly Val Leu Asn
260 265 270

Tyr Pro Ile Tyr Trp Gln Leu Leu Tyr Ala Phe Glu Ser Ser Ser Gly
275 280 285

Ser Ile Ser Asn Leu Tyr Asn Met Ile Lys Ser Val Ala Ser Asp Cys
290 295 300

Ser Asp Pro Thr Leu Leu Gly Asn Phe Ile Glu Asn His Asp Asn Pro
305 310 315 320

Arg Phe Ala Ser Tyr Thr Ser Asp Tyr Ser Gln Ala Lys Asn Val Leu
325 330 335

Ser Tyr Ile Phe Leu Ser Asp Gly Ile Pro Ile Val Tyr Ala Gly Glu
 340 345 350

Glu Gln His Tyr Ser Gly Gly Lys Val Pro Tyr Asn Arg Glu Ala Thr
 355 360 365

Trp Leu Ser Gly Tyr Asp Thr Ser Ala Glu Leu Tyr Thr Trp Ile Ala
 370 375 380

Thr Thr Asn Ala Ile Arg Lys Leu Ala Ile Ser Ala Asp Ser Ala Tyr
 385 390 395 400

Ile Thr Tyr Ala Asn Asp Ala Phe Tyr Thr Asp Ser Asn Thr Ile Ala
 405 410 415

Met Arg Lys Gly Thr Ser Gly Ser Gln Val Ile Thr Val Leu Ser Asn
 420 425 430

Lys Gly Ser Ser Gly Ser Ser Tyr Thr Leu Thr Leu Ser Gly Ser Gly
 435 440 445

Tyr Thr Ser Gly Thr Lys Leu Ile Glu Ala Tyr Thr Cys Thr Ser Val
 450 455 460

Thr Val Asp Ser Ser Gly Asp Ile Pro Val Pro Met Ala Ser Gly Leu
 465 470 475 480

Pro Arg Val Leu Leu Pro Ala Ser Val Val Asp Ser Ser Ser Leu Cys
 485 490 495

Gly Gly Ser Gly Arg Thr Thr Thr Thr Thr Ala Ala Ala Thr Ser
 500 505 510

Thr Ser Lys Ala Thr Thr Ser Ser Ser Ser Ser Ala Ala Ala Thr
 515 520 525

Thr Ser Ser Ser Cys Thr Thr Pro Thr Ala Val Ala Val Thr Phe Asp
 530 535 540

Leu Thr Ala Thr Thr Thr Tyr Gly Glu Asn Ile Tyr Leu Val Gly Ser
 545 550 555 560

Ile Ser Gln Leu Gly Asp Trp Glu Thr Ser Asp Gly Ile Ala Leu Ser
 565 570 575

Ala Asp Lys Tyr Thr Ser Ser Asp Pro Leu Trp Tyr Val Thr Val Thr
 580 585 590

Leu Pro Ala Gly Glu Ser Phe Glu Tyr Lys Phe Ile Arg Ile Glu Ser
 595 600 605

Asp Asp Ser Val Glu Trp Glu Ser Asp Pro Asn Arg Glu Tyr Thr Val
 610 615 620

Pro Gln Ala Cys Gly Thr Ser Thr Ala Thr Val Thr Asp Thr Trp Arg
 625 630 635 640

<210> 33

<211> 1890

<212> DNA

<213> Artificial

<220>

<223> Artificial

<220>

<221> CDS

<222> (1)..(1890)

<400> 33

atg aga tta tcg act tcg agt ctc ttc ctt tcc gtg tct ctg ctg ggg 48
 Met Arg Leu Ser Thr Ser Ser Leu Phe Leu Ser Val Ser Leu Leu Gly
 1 5 10 15

aag ctg gcc ctc ggg ctg tcg gct gca gaa tgg cgc act cag tcg att 96
 Lys Leu Ala Leu Gly Leu Ser Ala Ala Glu Trp Arg Thr Gln Ser Ile
 20 25 30

tac ttc cta ttg acg gat cgg ttc ggt agg acg gac aat tcg acg aca 144
 Tyr Phe Leu Leu Thr Asp Arg Phe Gly Arg Thr Asp Asn Ser Thr Thr
 35 40 45

gct aca tgc gat acg ggt gac caa atc tat tgt ggt ggc agt tgg caa 192
 Ala Thr Cys Asp Thr Gly Asp Gln Ile Tyr Cys Gly Gly Ser Trp Gln
 50 55 60

gga atc atc aac cat ctg gat tat atc cag ggc atg gga ttc acg gcc 240
 Gly Ile Ile Asn His Leu Asp Tyr Ile Gln Gly Met Gly Phe Thr Ala
 65 70 75 80

atc tgg atc tcg cct atc act gaa cag ctg ccc cag gat act gct gat 288
 Ile Trp Ile Ser Pro Ile Thr Glu Gln Leu Pro Gln Asp Thr Ala Asp
 85 90 95

ggt gaa gct tac cat gga tat tgg cag cag aag ata tac gac gtg aac 336
 Gly Glu Ala Tyr His Gly Tyr Trp Gln Gln Lys Ile Tyr Asp Val Asn
 100 105 110

tcc aac ttc ggc act gca gat gac ctc aag tcc ctc tca gat gcg ctt 384
 Ser Asn Phe Gly Thr Ala Asp Asp Leu Lys Ser Leu Ser Asp Ala Leu

115	120	125	
cat gcc cgc gga atg tac ctc atg gtg gac gtc gtc cct aac cac atg His Ala Arg Gly Met Tyr Leu Met Val Asp Val Val Pro Asn His Met 130 135 140			432
ggc tac gcc ggc aac ggc aac gat gta gac tac agc gtc ttc gac ccc Gly Tyr Ala Gly Asn Gly Asn Asp Val Asp Tyr Ser Val Phe Asp Pro 145 150 155 160			480
ttc gat tcc tcc tcc tac ttc cac cca tac tgc ctg atc aca gat tgg Phe Asp Ser Ser Ser Tyr Phe His Pro Tyr Cys Leu Ile Thr Asp Trp 165 170 175			528
gac aac ttg acc atg gtc caa gat tgt tgg gag ggt gac acc atc gta Asp Asn Leu Thr Met Val Gln Asp Cys Trp Glu Gly Asp Thr Ile Val 180 185 190			576
tct ctg cca gac cta aac acc acc gaa act gcc gtg aga aca atc tgg Ser Leu Pro Asp Leu Asn Thr Thr Glu Thr Ala Val Arg Thr Ile Trp 195 200 205			624
tat gac tgg gta gcc gac ctg gta tcc aat tat tca gtc gac gga ctc Tyr Asp Trp Val Ala Asp Leu Val Ser Asn Tyr Ser Val Asp Gly Leu 210 215 220			672
cgc atc gac agt gtc ctc gaa gtc gaa cca gac ttc ttc ccg ggc tac Arg Ile Asp Ser Val Leu Glu Val Glu Pro Asp Phe Phe Pro Gly Tyr 225 230 235 240			720
cag gaa gca gca ggt gtc tac tgc gtc ggc gaa gtc gac aac ggc aac Gln Glu Ala Ala Gly Val Tyr Cys Val Gly Glu Val Asp Asn Gly Asn 245 250 255			768
cct gcc ctc gac tgc cca tac cag aag gtc ctg gac ggc gtc ctc aac Pro Ala Leu Asp Cys Pro Tyr Gln Lys Val Leu Asp Gly Val Leu Asn 260 265 270			816
tat ccg atc tac tgg caa ctc ctc tac gcc ttc gaa tcc tcc agc ggc Tyr Pro Ile Tyr Trp Gln Leu Leu Tyr Ala Phe Glu Ser Ser Ser Gly 275 280 285			864
agc atc agc aat ctc tac aac atg atc aaa tcc gtc gca agc gac tgc Ser Ile Ser Asn Leu Tyr Asn Met Ile Lys Ser Val Ala Ser Asp Cys 290 295 300			912
tcc gat ccg aca cta ctc ggc aac ttc atc gaa aac cac gac aat ccc Ser Asp Pro Thr Leu Leu Gly Asn Phe Ile Glu Asn His Asp Asn Pro 305 310 315 320			960
cgt ttc gcc tcc tac acc tcc gac tac tcg caa gcc aaa aac gtc ctc Arg Phe Ala Ser Tyr Thr Ser Asp Tyr Ser Gln Ala Lys Asn Val Leu 325 330 335			1008
agc tac atc ttc ctc tcc gac ggc atc ccc atc gtc tac gcc ggc gaa Ser Tyr Ile Phe Leu Ser Asp Gly Ile Pro Ile Val Tyr Ala Gly Glu 340 345 350			1056
gaa cag cac tac tcc ggc ggc aag gtg ccc tac aac cgc gaa gcg acc Glu Gln His Tyr Ser Gly Gly Lys Val Pro Tyr Asn Arg Glu Ala Thr 355 360 365			1104

tgg ctt tca ggc tac gac acc tcc gca gag ctg tac acc tgg ata gcc	1152
Trp Leu Ser Gly Tyr Asp Thr Ser Ala Glu Leu Tyr Thr Trp Ile Ala	
370 375 380	
acc acg aac gcg atc cgc aaa cta gcc atc tca gct gac tcg gcc tac	1200
Thr Thr Asn Ala Ile Arg Lys Leu Ala Ile Ser Ala Asp Ser Ala Tyr	
385 390 395 400	
att acc tac gcg aat gat gca ttc tac act gac agc aac acc atc gca	1248
Ile Thr Tyr Ala Asn Asp Ala Phe Tyr Thr Asp Ser Asn Thr Ile Ala	
405 410 415	
atg cgc aaa ggc acc tca ggg agc caa gtc atc acc gtc ctc tcc aac	1296
Met Arg Lys Gly Thr Ser Gly Ser Gln Val Ile Thr Val Leu Ser Asn	
420 425 430	
aaa ggc tcc tca gga agc agc tac acc ctg acc ctc agc gga agc ggc	1344
Lys Gly Ser Ser Gly Ser Ser Tyr Thr Leu Thr Leu Ser Gly Ser Gly	
435 440 445	
tac aca tcc ggc acg aag ctg atc gaa gcg tac aca tgc aca tcc gtg	1392
Tyr Thr Ser Gly Thr Lys Leu Ile Glu Ala Tyr Thr Cys Thr Ser Val	
450 455 460	
acc gtg gac tcg agc ggc gat att ccc gtg ccg atg gcg tcg gga tta	1440
Thr Val Asp Ser Ser Gly Asp Ile Pro Val Pro Met Ala Ser Gly Leu	
465 470 475 480	
ccg aga gtt ctt ctg ccc gcg tcc gtc gtc gat agc tct tcg ctc tgt	1488
Pro Arg Val Leu Leu Pro Ala Ser Val Val Asp Ser Ser Ser Leu Cys	
485 490 495	
ggc ggg agc gga aga aca acc acg acc aca act gct gct gct act agt	1536
Gly Gly Ser Gly Arg Thr Thr Thr Thr Thr Ala Ala Ala Thr Ser	
500 505 510	
aca tcc aaa gcc acc acc tcc tct tct tct tct tct gct gct gct act	1584
Thr Ser Lys Ala Thr Thr Ser Ser Ser Ser Ser Ser Ala Ala Ala Thr	
515 520 525	
act tct tca tca gtc gag gtc act ttc gac gtt tac gct acc aca gta	1632
Thr Ser Ser Ser Val Glu Val Thr Phe Asp Val Tyr Ala Thr Thr Val	
530 535 540	
tat ggc cag aac atc tat atc acc ggt gat gtg agt gag ctc ggc aac	1680
Tyr Gly Gln Asn Ile Tyr Ile Thr Gly Asp Val Ser Glu Leu Gly Asn	
545 550 555 560	
tgg aca ccc gcc aat ggt gtt gca ctc tct tct gct aac tac ccc acc	1728
Trp Thr Pro Ala Asn Gly Val Ala Leu Ser Ser Ala Asn Tyr Pro Thr	
565 570 575	
tgg agt gcc acg atc gct ctc ccc gct gac acg aca atc cag tac aag	1776
Trp Ser Ala Thr Ile Ala Leu Pro Ala Asp Thr Thr Ile Gln Tyr Lys	
580 585 590	
tat gtc aac att gac ggc agc acc gtc atc tgg gag gat gct atc agc	1824
Tyr Val Asn Ile Asp Gly Ser Thr Val Ile Trp Glu Asp Ala Ile Ser	
595 600 605	

aat cgc gag atc acg acg ccc gcc agc ggc aca tac acc gaa aaa gac 1872
 Asn Arg Glu Ile Thr Thr Pro Ala Ser Gly Thr Tyr Thr Glu Lys Asp
 610 615 620

act tgg gat gaa tct tag 1890
 Thr Trp Asp Glu Ser
 625

<210> 34
 <211> 629
 <212> PRT
 <213> Artificial

<220>
 <223> Artificial

<400> 34

Met Arg Leu Ser Thr Ser Ser Leu Phe Leu Ser Val Ser Leu Leu Gly
 1 5 10 15

Lys Leu Ala Leu Gly Leu Ser Ala Ala Glu Trp Arg Thr Gln Ser Ile
 20 25 30

Tyr Phe Leu Leu Thr Asp Arg Phe Gly Arg Thr Asp Asn Ser Thr Thr
 35 40 45

Ala Thr Cys Asp Thr Gly Asp Gln Ile Tyr Cys Gly Gly Ser Trp Gln
 50 55 60

Gly Ile Ile Asn His Leu Asp Tyr Ile Gln Gly Met Gly Phe Thr Ala
 65 70 75 80

Ile Trp Ile Ser Pro Ile Thr Glu Gln Leu Pro Gln Asp Thr Ala Asp
 85 90 95

Gly Glu Ala Tyr His Gly Tyr Trp Gln Gln Lys Ile Tyr Asp Val Asn
 100 105 110

Ser Asn Phe Gly Thr Ala Asp Asp Leu Lys Ser Leu Ser Asp Ala Leu
 115 120 125

His Ala Arg Gly Met Tyr Leu Met Val Asp Val Val Pro Asn His Met
 130 135 140

Gly Tyr Ala Gly Asn Gly Asn Asp Val Asp Tyr Ser Val Phe Asp Pro
 145 150 155 160

Phe Asp Ser Ser Ser Tyr Phe His Pro Tyr Cys Leu Ile Thr Asp Trp
 165 170 175

Asp Asn Leu Thr Met Val Gln Asp Cys Trp Glu Gly Asp Thr Ile Val
 180 185 190

Ser Leu Pro Asp Leu Asn Thr Thr Glu Thr Ala Val Arg Thr Ile Trp
 195 200 205

Tyr Asp Trp Val Ala Asp Leu Val Ser Asn Tyr Ser Val Asp Gly Leu
 210 215 220

Arg Ile Asp Ser Val Leu Glu Val Glu Pro Asp Phe Phe Pro Gly Tyr
 225 230 235 240

Gln Glu Ala Ala Gly Val Tyr Cys Val Gly Glu Val Asp Asn Gly Asn
 245 250 255

Pro Ala Leu Asp Cys Pro Tyr Gln Lys Val Leu Asp Gly Val Leu Asn
 260 265 270

Tyr Pro Ile Tyr Trp Gln Leu Leu Tyr Ala Phe Glu Ser Ser Ser Gly
 275 280 285

Ser Ile Ser Asn Leu Tyr Asn Met Ile Lys Ser Val Ala Ser Asp Cys
 290 295 300

Ser Asp Pro Thr Leu Leu Gly Asn Phe Ile Glu Asn His Asp Asn Pro
 305 310 315 320

Arg Phe Ala Ser Tyr Thr Ser Asp Tyr Ser Gln Ala Lys Asn Val Leu
 325 330 335

Ser Tyr Ile Phe Leu Ser Asp Gly Ile Pro Ile Val Tyr Ala Gly Glu
 340 345 350

Glu Gln His Tyr Ser Gly Gly Lys Val Pro Tyr Asn Arg Glu Ala Thr
 355 360 365

Trp Leu Ser Gly Tyr Asp Thr Ser Ala Glu Leu Tyr Thr Trp Ile Ala
 370 375 380

Thr Thr Asn Ala Ile Arg Lys Leu Ala Ile Ser Ala Asp Ser Ala Tyr
 385 390 395 400

Ile Thr Tyr Ala Asn Asp Ala Phe Tyr Thr Asp Ser Asn Thr Ile Ala
 405 410 415

Met Arg Lys Gly Thr Ser Gly Ser Gln Val Ile Thr Val Leu Ser Asn
420 425 430

Lys Gly Ser Ser Gly Ser Ser Tyr Thr Leu Thr Leu Ser Gly Ser Gly
435 440 445

Tyr Thr Ser Gly Thr Lys Leu Ile Glu Ala Tyr Thr Cys Thr Ser Val
450 455 460

Thr Val Asp Ser Ser Gly Asp Ile Pro Val Pro Met Ala Ser Gly Leu
465 470 475 480

Pro Arg Val Leu Leu Pro Ala Ser Val Val Asp Ser Ser Ser Leu Cys
485 490 495

Gly Gly Ser Gly Arg Thr Thr Thr Thr Thr Thr Ala Ala Ala Thr Ser
500 505 510

Thr Ser Lys Ala Thr Thr Ser Ser Ser Ser Ser Ala Ala Ala Thr
515 520 525

Thr Ser Ser Ser Val Glu Val Thr Phe Asp Val Tyr Ala Thr Thr Val
530 535 540

Tyr Gly Gln Asn Ile Tyr Ile Thr Gly Asp Val Ser Glu Leu Gly Asn
545 550 555 560

Trp Thr Pro Ala Asn Gly Val Ala Leu Ser Ser Ala Asn Tyr Pro Thr
565 570 575

Trp Ser Ala Thr Ile Ala Leu Pro Ala Asp Thr Thr Ile Gln Tyr Lys
580 585 590

Tyr Val Asn Ile Asp Gly Ser Thr Val Ile Trp Glu Asp Ala Ile Ser
595 600 605

Asn Arg Glu Ile Thr Thr Pro Ala Ser Gly Thr Tyr Thr Glu Lys Asp
610 615 620

Thr Trp Asp Glu Ser
625

<210> 35
<211> 1923
<212> DNA
<213> Artificial

<220>

<223> Artificial

<220>

<221> CDS

<222> (1)..(1923)

<400> 35

atg gtc gcg tgg tgg tct cta ttt ctg tac ggc ctt cag gtc gcg gca 48
 Met Val Ala Trp Trp Ser Leu Phe Leu Tyr Gly Leu Gln Val Ala Ala
 1 5 10 15

cct gct ttg gct gca acg cct gcg gac tgg cga tcg caa tcc att tat 96
 Pro Ala Leu Ala Ala Thr Pro Ala Asp Trp Arg Ser Gln Ser Ile Tyr
 20 25 30

ttc ctt ctc acg gat cga ttt gca agg acg gat ggg tcg acg act gcg 144
 Phe Leu Leu Thr Asp Arg Phe Ala Arg Thr Asp Gly Ser Thr Thr Ala
 35 40 45

act tgt aat act gcg gat cag aaa tac tgt ggt gga aca tgg cag ggc 192
 Thr Cys Asn Thr Ala Asp Gln Lys Tyr Cys Gly Gly Thr Trp Gln Gly
 50 55 60

atc atc gac aag ttg gac tat atc cag gga atg ggc ttc aca gcc atc 240
 Ile Ile Asp Lys Leu Asp Tyr Ile Gln Gly Met Gly Phe Thr Ala Ile
 65 70 75 80

tgg atc acc ccc gtt aca gcc cag ctg ccc cag acc acc gca tat gga 288
 Trp Ile Thr Pro Val Thr Ala Gln Leu Pro Gln Thr Thr Ala Tyr Gly
 85 90 95

gat gcc tac cat ggc tac tgg cag cag gat ata tac tct ctg aac gaa 336
 Asp Ala Tyr His Gly Tyr Trp Gln Gln Asp Ile Tyr Ser Leu Asn Glu
 100 105 110

aac tac ggc act gca gat gac ttg aag gcg ctc tct tog gcc ctt cat 384
 Asn Tyr Gly Thr Ala Asp Asp Leu Lys Ala Leu Ser Ser Ala Leu His
 115 120 125

gag agg ggg atg tat ctt atg gtc gat gtg gtt gct aac cat atg ggc 432
 Glu Arg Gly Met Tyr Leu Met Val Asp Val Val Ala Asn His Met Gly
 130 135 140

tat gat gga gcg ggt agc tca gtc gat tac agt gtg ttt aaa ccg ttc 480
 Tyr Asp Gly Ala Gly Ser Ser Val Asp Tyr Ser Val Phe Lys Pro Phe
 145 150 155 160

agt tcc caa gac tac ttc cac ccg ttc tgt ttc att caa aac tat gaa 528
 Ser Ser Gln Asp Tyr Phe His Pro Phe Cys Phe Ile Gln Asn Tyr Glu
 165 170 175

gat cag act cag gtt gag gat tgc tgg cta gga gat aac act gtc tcc 576
 Asp Gln Thr Gln Val Glu Asp Cys Trp Leu Gly Asp Asn Thr Val Ser
 180 185 190

ttg cct gat ctc gat acc acc aag gat gtg gtc aag aat gaa tgg tac 624
 Leu Pro Asp Leu Asp Thr Thr Lys Asp Val Val Lys Asn Glu Trp Tyr
 195 200 205

gac tgg gtg gga tca ttg gta tcg aac tac tcc att gac ggc ctc cgt Asp Trp Val Gly Ser Leu Val Ser Asn Tyr Ser Ile Asp Gly Leu Arg 210 215 220	672
atc gac aca gta aaa cac gtc cag aag gac ttc tgg ccc ggg tac aac Ile Asp Thr Val Lys His Val Gln Lys Asp Phe Trp Pro Gly Tyr Asn 225 230 235 240	720
aaa gcc gca ggc gtg tac tgt atc ggc gag gtg ctc gac ggt gat ccg Lys Ala Ala Gly Val Tyr Cys Ile Gly Glu Val Leu Asp Gly Asp Pro 245 250 255	768
gcc tac act tgt ccc tac cag aac gtc atg gac ggc gta ctg aac tat Ala Tyr Thr Cys Pro Tyr Gln Asn Val Met Asp Gly Val Leu Asn Tyr 260 265 270	816
ccc att tac tat cca ctc ctc aac gcc ttc aag tca acc tcc ggc agc Pro Ile Tyr Tyr Pro Leu Leu Asn Ala Phe Lys Ser Thr Ser Gly Ser 275 280 285	864
atg gac gac ctc tac aac atg atc aac acc gtc aaa tcc gac tgt cca Met Asp Asp Leu Tyr Asn Met Ile Asn Thr Val Lys Ser Asp Cys Pro 290 295 300	912
gac tca aca ctc ctg ggc aca ttc gtc gag aac cac gac aac cca cgg Asp Ser Thr Leu Leu Gly Thr Phe Val Glu Asn His Asp Asn Pro Arg 305 310 315 320	960
ttc gct tct tac acc aac gac ata gcc ctc gcc aag aac gtc gca gca Phe Ala Ser Tyr Thr Asn Asp Ile Ala Leu Ala Lys Asn Val Ala Ala 325 330 335	1008
ttc atc atc ctc aac gac gga atc ccc atc atc tac gcc ggc caa gaa Phe Ile Ile Leu Asn Asp Gly Ile Pro Ile Ile Tyr Ala Gly Gln Glu 340 345 350	1056
cag cac tac gcc ggc gga aac gac ccc gcg aac cgc gaa gca acc tgg Gln His Tyr Ala Gly Gly Asn Asp Pro Ala Asn Arg Glu Ala Thr Trp 355 360 365	1104
ctc tcg ggc tac ccg acc gac agc gag ctg tac aag tta att gcc tcc Leu Ser Gly Tyr Pro Thr Asp Ser Glu Leu Tyr Lys Leu Ile Ala Ser 370 375 380	1152
gcg aac gca atc cgg aac tat gcc att agc aaa gat aca gga ttc gtg Ala Asn Ala Ile Arg Asn Tyr Ala Ile Ser Lys Asp Thr Gly Phe Val 385 390 395 400	1200
acc tac aag aac tgg ccc atc tac aaa gac gac aca acg atc gcc atg Thr Tyr Lys Asn Trp Pro Ile Tyr Lys Asp Asp Thr Thr Ile Ala Met 405 410 415	1248
cgc aag ggc aca gat ggg tcg cag atc gtg act atc ttg tcc aac aag Arg Lys Gly Thr Asp Gly Ser Gln Ile Val Thr Ile Leu Ser Asn Lys 420 425 430	1296
ggt gct tcg ggt gat tcg tat acc ctc tcc ttg agt ggt gcg ggt tac Gly Ala Ser Gly Asp Ser Tyr Thr Leu Ser Leu Ser Gly Ala Gly Tyr 435 440 445	1344

aca gcc ggc cag caa ttg acg gag gtc att ggc tgc acg acc gtg acg 1392
 Thr Ala Gly Gln Gln Leu Thr Glu Val Ile Gly Cys Thr Thr Val Thr
 450 455 460

ggt ggt tgc gat gga aat gtg cct gtt cct atg gca ggt ggg cta cct 1440
 Val Gly Ser Asp Gly Asn Val Pro Val Pro Met Ala Gly Gly Leu Pro
 465 470 475 480

agg gta ttg tat ccg act gag aag ttg gca ggt agc aag atc tgt agt 1488
 Arg Val Leu Tyr Pro Thr Glu Lys Leu Ala Gly Ser Lys Ile Cys Ser
 485 490 495

agc tcg gga aga aca acc acg acc aca act gct gct gct act agt aca 1536
 Ser Ser Gly Arg Thr Thr Thr Thr Thr Ala Ala Ala Thr Ser Thr
 500 505 510

tcc aaa gcc acc acc tcc tct tct tct tct tct gct gct gct act act 1584
 Ser Lys Ala Thr Thr Ser Ser Ser Ser Ser Ala Ala Ala Thr Thr
 515 520 525

tct tca tca tgc acc gca aca agc acc acc ctc ccc atc acc ttc gaa 1632
 Ser Ser Ser Cys Thr Ala Thr Ser Thr Thr Leu Pro Ile Thr Phe Glu
 530 535 540

gaa ctc gtc acc act acc tac ggg gaa gaa gtc tac ctc agc gga tct 1680
 Glu Leu Val Thr Thr Thr Tyr Gly Glu Glu Val Tyr Leu Ser Gly Ser
 545 550 555 560

atc tcc cag ctc gga gag tgg gat acg agt gac gcg gtg aag ttg tcc 1728
 Ile Ser Gln Leu Gly Glu Trp Asp Thr Ser Asp Ala Val Lys Leu Ser
 565 570 575

gcg gat gat tat acc tcg agt aac ccc gag tgg tct gtt act gtg tcg 1776
 Ala Asp Asp Tyr Thr Ser Ser Asn Pro Glu Trp Ser Val Thr Val Ser
 580 585 590

ttg ccg gtg ggg acg acc ttc gag tat aag ttt att aag gtc gat gag 1824
 Leu Pro Val Gly Thr Thr Phe Glu Tyr Lys Phe Ile Lys Val Asp Glu
 595 600 605

ggt gga agt gtg act tgg gaa agt gat ccg aat agg gag tat act gtg 1872
 Gly Gly Ser Val Thr Trp Glu Ser Asp Pro Asn Arg Glu Tyr Thr Val
 610 615 620

cct gaa tgt ggg aat ggg agt ggg gag acg gtg gtt gat acg tgg agg 1920
 Pro Glu Cys Gly Asn Gly Ser Gly Glu Thr Val Val Asp Thr Trp Arg
 625 630 635 640

tag 1923

<210> 36
 <211> 640
 <212> PRT
 <213> Artificial

<220>
 <223> Artificial

<400> 36

Met Val Ala Trp Trp Ser Leu Phe Leu Tyr Gly Leu Gln Val Ala Ala
 1 5 10 15
 Pro Ala Leu Ala Ala Thr Pro Ala Asp Trp Arg Ser Gln Ser Ile Tyr
 20 25 30
 Phe Leu Leu Thr Asp Arg Phe Ala Arg Thr Asp Gly Ser Thr Thr Ala
 35 40 45
 Thr Cys Asn Thr Ala Asp Gln Lys Tyr Cys Gly Gly Thr Trp Gln Gly
 50 55 60
 Ile Ile Asp Lys Leu Asp Tyr Ile Gln Gly Met Gly Phe Thr Ala Ile
 65 70 75 80
 Trp Ile Thr Pro Val Thr Ala Gln Leu Pro Gln Thr Thr Ala Tyr Gly
 85 90 95
 Asp Ala Tyr His Gly Tyr Trp Gln Gln Asp Ile Tyr Ser Leu Asn Glu
 100 105 110
 Asn Tyr Gly Thr Ala Asp Asp Leu Lys Ala Leu Ser Ser Ala Leu His
 115 120 125
 Glu Arg Gly Met Tyr Leu Met Val Asp Val Val Ala Asn His Met Gly
 130 135 140
 Tyr Asp Gly Ala Gly Ser Ser Val Asp Tyr Ser Val Phe Lys Pro Phe
 145 150 155 160
 Ser Ser Gln Asp Tyr Phe His Pro Phe Cys Phe Ile Gln Asn Tyr Glu
 165 170 175
 Asp Gln Thr Gln Val Glu Asp Cys Trp Leu Gly Asp Asn Thr Val Ser
 180 185 190
 Leu Pro Asp Leu Asp Thr Thr Lys Asp Val Val Lys Asn Glu Trp Tyr
 195 200 205
 Asp Trp Val Gly Ser Leu Val Ser Asn Tyr Ser Ile Asp Gly Leu Arg
 210 215 220
 Ile Asp Thr Val Lys His Val Gln Lys Asp Phe Trp Pro Gly Tyr Asn
 225 230 235 240
 Lys Ala Ala Gly Val Tyr Cys Ile Gly Glu Val Leu Asp Gly Asp Pro

Ser Ser Gly Arg Thr Thr Thr Thr Thr Thr Ala Ala Ala Thr Ser Thr
 500 505 510

Ser Lys Ala Thr Thr Ser Ser Ser Ser Ser Ser Ala Ala Ala Thr Thr
 515 520 525

Ser Ser Ser Cys Thr Ala Thr Ser Thr Thr Leu Pro Ile Thr Phe Glu
 530 535 540

Glu Leu Val Thr Thr Thr Tyr Gly Glu Glu Val Tyr Leu Ser Gly Ser
 545 550 555 560

Ile Ser Gln Leu Gly Glu Trp Asp Thr Ser Asp Ala Val Lys Leu Ser
 565 570 575

Ala Asp Asp Tyr Thr Ser Ser Asn Pro Glu Trp Ser Val Thr Val Ser
 580 585 590

Leu Pro Val Gly Thr Thr Phe Glu Tyr Lys Phe Ile Lys Val Asp Glu
 595 600 605

Gly Gly Ser Val Thr Trp Glu Ser Asp Pro Asn Arg Glu Tyr Thr Val
 610 615 620

Pro Glu Cys Gly Asn Gly Ser Gly Glu Thr Val Val Asp Thr Trp Arg
 625 630 635 640

<210> 37
 <211> 1830
 <212> DNA
 <213> Artificial

<220>
 <223> Artificial

<220>
 <221> CDS
 <222> (1)..(1830)

<400> 37
 atg aga tta tcg act tcg agt ctc ttc ctt tcc gtg tct ctg ctg ggg 48
 Met Arg Leu Ser Thr Ser Ser Leu Phe Leu Ser Val Ser Leu Leu Gly
 1 5 10 15

aag ctg gcc ctc ggg ctg tcg gct gca gaa tgg cgc act cag tcg att 96
 Lys Leu Ala Leu Gly Leu Ser Ala Ala Glu Trp Arg Thr Gln Ser Ile
 20 25 30

tac ttc cta ttg acg gat cgg ttc ggt agg acg gac aat tcg acg aca 144

Tyr	Phe	Leu	Leu	Thr	Asp	Arg	Phe	Gly	Arg	Thr	Asp	Asn	Ser	Thr	Thr		
		35					40					45					
gct	aca	tgc	gat	acg	ggt	gac	caa	atc	tat	tgt	ggt	ggc	agt	tgg	caa	192	
Ala	Thr	Cys	Asp	Thr	Gly	Asp	Gln	Ile	Tyr	Cys	Gly	Gly	Ser	Trp	Gln		
		50				55					60						
gga	atc	atc	aac	cat	ctg	gat	tat	atc	cag	ggc	atg	gga	ttc	acg	gcc	240	
Gly	Ile	Ile	Asn	His	Leu	Asp	Tyr	Ile	Gln	Gly	Met	Gly	Phe	Thr	Ala		
					70					75					80		
atc	tgg	atc	tcg	cct	atc	act	gaa	cag	ctg	ccc	cag	gat	act	gct	gat	288	
Ile	Trp	Ile	Ser	Pro	Ile	Thr	Glu	Gln	Leu	Pro	Gln	Asp	Thr	Ala	Asp		
				85					90					95			
ggt	gaa	gct	tac	cat	gga	tat	tgg	cag	cag	aag	ata	tac	gac	gtg	aac	336	
Gly	Glu	Ala	Tyr	His	Gly	Tyr	Trp	Gln	Gln	Lys	Ile	Tyr	Asp	Val	Asn		
			100					105					110				
tcc	aac	ttc	ggc	act	gca	gat	gac	ctc	aag	tcc	ctc	tca	gat	gcg	ctt	384	
Ser	Asn	Phe	Gly	Thr	Ala	Asp	Asp	Leu	Lys	Ser	Leu	Ser	Asp	Ala	Leu		
		115					120					125					
cat	gcc	cgc	gga	atg	tac	ctc	atg	gtg	gac	gtc	gtc	cct	aac	cac	atg	432	
His	Ala	Arg	Gly	Met	Tyr	Leu	Met	Val	Asp	Val	Val	Pro	Asn	His	Met		
		130				135					140						
ggc	tac	gcc	ggc	aac	ggc	aac	gat	gta	gac	tac	agc	gtc	ttc	gac	ccc	480	
Gly	Tyr	Ala	Gly	Asn	Gly	Asn	Asp	Val	Asp	Tyr	Ser	Val	Phe	Asp	Pro		
					150					155					160		
ttc	gat	tcc	tcc	tcc	tac	ttc	cac	cca	tac	tgc	ctg	atc	aca	gat	tgg	528	
Phe	Asp	Ser	Ser	Ser	Tyr	Phe	His	Pro	Tyr	Cys	Leu	Ile	Thr	Asp	Trp		
				165					170					175			
gac	aac	ttg	acc	atg	gtc	caa	gat	tgt	tgg	gag	ggt	gac	acc	atc	gta	576	
Asp	Asn	Leu	Thr	Met	Val	Gln	Asp	Cys	Trp	Glu	Gly	Asp	Thr	Ile	Val		
			180					185						190			
tct	ctg	cca	gac	cta	aac	acc	acc	gaa	act	gcc	gtg	aga	aca	atc	tgg	624	
Ser	Leu	Pro	Asp	Leu	Asn	Thr	Thr	Glu	Thr	Ala	Val	Arg	Thr	Ile	Trp		
				195			200					205					
tat	gac	tgg	gta	gcc	gac	ctg	gta	tcc	aat	tat	tca	gtc	gac	gga	ctc	672	
Tyr	Asp	Trp	Val	Ala	Asp	Leu	Val	Ser	Asn	Tyr	Ser	Val	Asp	Gly	Leu		
		210				215						220					
cgc	atc	gac	agt	gtc	ctc	gaa	gtc	gaa	cca	gac	ttc	ttc	ccg	ggc	tac	720	
Arg	Ile	Asp	Ser	Val	Leu	Glu	Val	Glu	Pro	Asp	Phe	Phe	Pro	Gly	Tyr		
					230					235					240		
cag	gaa	gca	gca	ggt	gtc	tac	tgc	gtc	ggc	gaa	gtc	gac	aac	ggc	aac	768	
Gln	Glu	Ala	Ala	Gly	Val	Tyr	Cys	Val	Gly	Glu	Val	Asp	Asn	Gly	Asn		
				245					250					255			
cct	gcc	ctc	gac	tgc	cca	tac	cag	aag	gtc	ctg	gac	ggc	gtc	ctc	aac	816	
Pro	Ala	Leu	Asp	Cys	Pro	Tyr	Gln	Lys	Val	Leu	Asp	Gly	Val	Leu	Asn		
			260					265					270				
tat	ccg	atc	tac	tgg	caa	ctc	ctc	tac	gcc	ttc	gaa	tcc	tcc	agc	ggc	864	
Tyr	Pro	Ile	Tyr	Trp	Gln	Leu	Leu	Tyr	Ala	Phe	Glu	Ser	Ser	Ser	Gly		

275	280	285	
agc atc agc aat ctc tac aac atg atc aaa tcc gtc gca agc gac tgc Ser Ile Ser Asn Leu Tyr Asn Met Ile Lys Ser Val Ala Ser Asp Cys 290 295 300			912
tcc gat cgc aca cta ctc ggc aac ttc atc gaa aac cac gac aat ccc Ser Asp Pro Thr Leu Leu Gly Asn Phe Ile Glu Asn His Asp Asn Pro 305 310 315 320			960
cgt ttc gcc tcc tac acc tcc gac tac tcg caa gcc aaa aac gtc ctc Arg Phe Ala Ser Tyr Thr Ser Asp Tyr Ser Gln Ala Lys Asn Val Leu 325 330 335			1008
agc tac atc ttc ctc tcc gac ggc atc ccc atc gtc tac gcc ggc gaa Ser Tyr Ile Phe Leu Ser Asp Gly Ile Pro Ile Val Tyr Ala Gly Glu 340 345 350			1056
gaa cag cac tac tcc ggc ggc aag gtg ccc tac aac cgc gaa gcg acc Glu Gln His Tyr Ser Gly Gly Lys Val Pro Tyr Asn Arg Glu Ala Thr 355 360 365			1104
tgg ctt tca ggc tac gac acc tcc gca gag ctg tac acc tgg ata gcc Trp Leu Ser Gly Tyr Asp Thr Ser Ala Glu Leu Tyr Thr Trp Ile Ala 370 375 380			1152
acc acg aac gcg atc cgc aaa cta gcc atc tca gct gac tcg gcc tac Thr Thr Asn Ala Ile Arg Lys Leu Ala Ile Ser Ala Asp Ser Ala Tyr 385 390 395 400			1200
att acc tac gcg aat gat gca ttc tac act gac agc aac acc atc gca Ile Thr Tyr Ala Asn Asp Ala Phe Tyr Thr Asp Ser Asn Thr Ile Ala 405 410 415			1248
atg cgc aaa ggc acc tca ggg agc caa gtc atc acc gtc ctc tcc aac Met Arg Lys Gly Thr Ser Gly Ser Gln Val Ile Thr Val Leu Ser Asn 420 425 430			1296
aaa ggc tcc tca gga agc agc tac acc ctg acc ctc agc gga agc ggc Lys Gly Ser Ser Gly Ser Ser Tyr Thr Leu Thr Leu Ser Gly Ser Gly 435 440 445			1344
tac aca tcc ggc acg aag ctg atc gaa gcg tac aca tgc aca tcc gtg Tyr Thr Ser Gly Thr Lys Leu Ile Glu Ala Tyr Thr Cys Thr Ser Val 450 455 460			1392
acc gtg gac tcg agc ggc gat att ccc gtg ccg atg gcg tcg gga tta Thr Val Asp Ser Ser Gly Asp Ile Pro Val Pro Met Ala Ser Gly Leu 465 470 475 480			1440
ccg aga gtt ctt ctg ccc gcg tcc gtc gtc gat agc tct tcg ctc tgt Pro Arg Val Leu Leu Pro Ala Ser Val Val Asp Ser Ser Ser Leu Cys 485 490 495			1488
ggc ggg agc gga aga ggt gct aca agc ccg ggt ggc tcc tcg ggt agt Gly Gly Ser Gly Arg Gly Ala Thr Ser Pro Gly Gly Ser Ser Gly Ser 500 505 510			1536
gtc gag gtc act ttc gac gtt tac gct acc aca gta tat ggc cag aac Val Glu Val Thr Phe Asp Val Tyr Ala Thr Thr Val Tyr Gly Gln Asn 515 520 525			1584

atc tat atc acc ggt gat gtg agt gag ctc ggc aac tgg aca ccc gcc 1632
 Ile Tyr Ile Thr Gly Asp Val Ser Glu Leu Gly Asn Trp Thr Pro Ala
 530 535 540

aat ggt gtt gca ctc tct tct gct aac tac ccc acc tgg agt gcc acg 1680
 Asn Gly Val Ala Leu Ser Ser Ala Asn Tyr Pro Thr Trp Ser Ala Thr
 545 550 555 560

atc gct ctc ccc gct gac acg aca atc cag tac aag tat gtc aac att 1728
 Ile Ala Leu Pro Ala Asp Thr Thr Ile Gln Tyr Lys Tyr Val Asn Ile
 565 570 575

gac ggc agc acc gtc atc tgg gag gat gct atc agc aat cgc gag atc 1776
 Asp Gly Ser Thr Val Ile Trp Glu Asp Ala Ile Ser Asn Arg Glu Ile
 580 585 590

acg acg ccc gcc agc ggc aca tac acc gaa aaa gac act tgg gat gaa 1824
 Thr Thr Pro Ala Ser Gly Thr Tyr Thr Glu Lys Asp Thr Trp Asp Glu
 595 600 605

tct tag 1830
 Ser

<210> 38
 <211> 609
 <212> PRT
 <213> Artificial

<220>
 <223> Artificial

<400> 38

Met Arg Leu Ser Thr Ser Ser Leu Phe Leu Ser Val Ser Leu Leu Gly
 1 5 10 15

Lys Leu Ala Leu Gly Leu Ser Ala Ala Glu Trp Arg Thr Gln Ser Ile
 20 25 30

Tyr Phe Leu Leu Thr Asp Arg Phe Gly Arg Thr Asp Asn Ser Thr Thr
 35 40 45

Ala Thr Cys Asp Thr Gly Asp Gln Ile Tyr Cys Gly Gly Ser Trp Gln
 50 55 60

Gly Ile Ile Asn His Leu Asp Tyr Ile Gln Gly Met Gly Phe Thr Ala
 65 70 75 80

Ile Trp Ile Ser Pro Ile Thr Glu Gln Leu Pro Gln Asp Thr Ala Asp
 85 90 95

Gly Glu Ala Tyr His Gly Tyr Trp Gln Gln Lys Ile Tyr Asp Val Asn

100 105 110
 Ser Asn Phe Gly Thr Ala Asp Asp Leu Lys Ser Leu Ser Asp Ala Leu
 115 120 125
 His Ala Arg Gly Met Tyr Leu Met Val Asp Val Val Pro Asn His Met
 130 135 140
 Gly Tyr Ala Gly Asn Gly Asn Asp Val Asp Tyr Ser Val Phe Asp Pro
 145 150 155 160
 Phe Asp Ser Ser Ser Tyr Phe His Pro Tyr Cys Leu Ile Thr Asp Trp
 165 170 175
 Asp Asn Leu Thr Met Val Gln Asp Cys Trp Glu Gly Asp Thr Ile Val
 180 185 190
 Ser Leu Pro Asp Leu Asn Thr Thr Glu Thr Ala Val Arg Thr Ile Trp
 195 200 205
 Tyr Asp Trp Val Ala Asp Leu Val Ser Asn Tyr Ser Val Asp Gly Leu
 210 215 220
 Arg Ile Asp Ser Val Leu Glu Val Glu Pro Asp Phe Phe Pro Gly Tyr
 225 230 235 240
 Gln Glu Ala Ala Gly Val Tyr Cys Val Gly Glu Val Asp Asn Gly Asn
 245 250 255
 Pro Ala Leu Asp Cys Pro Tyr Gln Lys Val Leu Asp Gly Val Leu Asn
 260 265 270
 Tyr Pro Ile Tyr Trp Gln Leu Leu Tyr Ala Phe Glu Ser Ser Ser Gly
 275 280 285
 Ser Ile Ser Asn Leu Tyr Asn Met Ile Lys Ser Val Ala Ser Asp Cys
 290 295 300
 Ser Asp Pro Thr Leu Leu Gly Asn Phe Ile Glu Asn His Asp Asn Pro
 305 310 315 320
 Arg Phe Ala Ser Tyr Thr Ser Asp Tyr Ser Gln Ala Lys Asn Val Leu
 325 330 335
 Ser Tyr Ile Phe Leu Ser Asp Gly Ile Pro Ile Val Tyr Ala Gly Glu
 340 345 350

Glu Gln His Tyr Ser Gly Gly Lys Val Pro Tyr Asn Arg Glu Ala Thr
 355 360 365

Trp Leu Ser Gly Tyr Asp Thr Ser Ala Glu Leu Tyr Thr Trp Ile Ala
 370 375 380

Thr Thr Asn Ala Ile Arg Lys Leu Ala Ile Ser Ala Asp Ser Ala Tyr
 385 390 395 400

Ile Thr Tyr Ala Asn Asp Ala Phe Tyr Thr Asp Ser Asn Thr Ile Ala
 405 410 415

Met Arg Lys Gly Thr Ser Gly Ser Gln Val Ile Thr Val Leu Ser Asn
 420 425 430

Lys Gly Ser Ser Gly Ser Ser Tyr Thr Leu Thr Leu Ser Gly Ser Gly
 435 440 445

Tyr Thr Ser Gly Thr Lys Leu Ile Glu Ala Tyr Thr Cys Thr Ser Val
 450 455 460

Thr Val Asp Ser Ser Gly Asp Ile Pro Val Pro Met Ala Ser Gly Leu
 465 470 475 480

Pro Arg Val Leu Leu Pro Ala Ser Val Val Asp Ser Ser Ser Leu Cys
 485 490 495

Gly Gly Ser Gly Arg Gly Ala Thr Ser Pro Gly Gly Ser Ser Gly Ser
 500 505 510

Val Glu Val Thr Phe Asp Val Tyr Ala Thr Thr Val Tyr Gly Gln Asn
 515 520 525

Ile Tyr Ile Thr Gly Asp Val Ser Glu Leu Gly Asn Trp Thr Pro Ala
 530 535 540

Asn Gly Val Ala Leu Ser Ser Ala Asn Tyr Pro Thr Trp Ser Ala Thr
 545 550 555 560

Ile Ala Leu Pro Ala Asp Thr Thr Ile Gln Tyr Lys Tyr Val Asn Ile
 565 570 575

Asp Gly Ser Thr Val Ile Trp Glu Asp Ala Ile Ser Asn Arg Glu Ile
 580 585 590

Thr Thr Pro Ala Ser Gly Thr Tyr Thr Glu Lys Asp Thr Trp Asp Glu
 595 600 605

Ser

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 <212> DNA
 <213> Artificial

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 <222> (1)..(1827)

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 Met Val Ala Trp Trp Ser Leu Phe Leu Tyr Gly Leu Gln Val Ala Ala
 1 5 10 15

cct gct ttg gct gca acg cct gcg gac tgg cga tcg caa tcc att tat 96
 Pro Ala Leu Ala Ala Thr Pro Ala Asp Trp Arg Ser Gln Ser Ile Tyr
 20 25 30

ttc ctt ctc acg gat cga ttt gca agg acg gat ggg tcg acg act gcg 144
 Phe Leu Leu Thr Asp Arg Phe Ala Arg Thr Asp Gly Ser Thr Thr Ala
 35 40 45

act tgt aat act gcg gat cag aaa tac tgt ggt gga aca tgg cag ggc 192
 Thr Cys Asn Thr Ala Asp Gln Lys Tyr Cys Gly Gly Thr Trp Gln Gly
 50 55 60

atc atc gac aag ttg gac tat atc cag gga atg ggc ttc aca gcc atc 240
 Ile Ile Asp Lys Leu Asp Tyr Ile Gln Gly Met Gly Phe Thr Ala Ile
 65 70 75 80

tgg atc acc ccc gtt aca gcc cag ctg ccc cag acc acc gca tat gga 288
 Trp Ile Thr Pro Val Thr Ala Gln Leu Pro Gln Thr Thr Ala Tyr Gly
 85 90 95

gat gcc tac cat ggc tac tgg cag cag gat ata tac tct ctg aac gaa 336
 Asp Ala Tyr His Gly Tyr Trp Gln Gln Asp Ile Tyr Ser Leu Asn Glu
 100 105 110

aac tac ggc act gca gat gac ttg aag gcg ctc tct tcg gcc ctt cat 384
 Asn Tyr Gly Thr Ala Asp Asp Leu Lys Ala Leu Ser Ser Ala Leu His
 115 120 125

gag agg ggg atg tat ctt atg gtc gat gtg gtt gct aac cat atg ggc 432
 Glu Arg Gly Met Tyr Leu Met Val Asp Val Val Ala Asn His Met Gly
 130 135 140

tat gat gga gcg ggt agc tca gtc gat tac agt gtg ttt aaa ccg ttc 480
 Tyr Asp Gly Ala Gly Ser Ser Val Asp Tyr Ser Val Phe Lys Pro Phe

145	150	155	160	
agt tcc caa gac tac ttc cac ccg ttc tgt ttc att caa aac tat gaa				528
Ser Ser Gln Asp Tyr Phe His Pro Phe Cys Phe Ile Gln Asn Tyr Glu	165	170	175	
gat cag act cag gtt gag gat tgc tgg cta gga gat aac act gtc tcc				576
Asp Gln Thr Gln Val Glu Asp Cys Trp Leu Gly Asp Asn Thr Val Ser	180	185	190	
ttg cct gat ctc gat acc acc aag gat gtg gtc aag aat gaa tgg tac				624
Leu Pro Asp Leu Asp Thr Thr Lys Asp Val Val Lys Asn Glu Trp Tyr	195	200	205	
gac tgg gtg gga tca ttg gta tcg aac tac tcc att gac ggc ctc cgt				672
Asp Trp Val Gly Ser Leu Val Ser Asn Tyr Ser Ile Asp Gly Leu Arg	210	215	220	
atc gac aca gta aaa cac gtc cag aag gac ttc tgg ccc ggg tac aac				720
Ile Asp Thr Val Lys His Val Gln Lys Asp Phe Trp Pro Gly Tyr Asn	225	230	235	240
aaa gcc gca ggc gtg tac tgt atc ggc gag gtg ctc gac ggt gat ccg				768
Lys Ala Ala Gly Val Tyr Cys Ile Gly Glu Val Leu Asp Gly Asp Pro	245	250	255	
gcc tac act tgt ccc tac cag aac gtc atg gac ggc gta ctg aac tat				816
Ala Tyr Thr Cys Pro Tyr Gln Asn Val Met Asp Gly Val Leu Asn Tyr	260	265	270	
ccc att tac tat cca ctc ctc aac gcc ttc aag tca acc tcc ggc agc				864
Pro Ile Tyr Tyr Pro Leu Leu Asn Ala Phe Lys Ser Thr Ser Gly Ser	275	280	285	
atg gac gac ctc tac aac atg atc aac acc gtc aaa tcc gac tgt cca				912
Met Asp Asp Leu Tyr Asn Met Ile Asn Thr Val Lys Ser Asp Cys Pro	290	295	300	
gac tca aca ctc ctg ggc aca ttc gtc gag aac cac gac aac cca cgg				960
Asp Ser Thr Leu Leu Gly Thr Phe Val Glu Asn His Asp Asn Pro Arg	305	310	315	320
ttc gct tct tac acc aac gac ata gcc ctc gcc aag aac gtc gca gca				1008
Phe Ala Ser Tyr Thr Asn Asp Ile Ala Leu Ala Lys Asn Val Ala Ala	325	330	335	
ttc atc atc ctc aac gac gga atc ccc atc atc tac gcc ggc caa gaa				1056
Phe Ile Ile Leu Asn Asp Gly Ile Pro Ile Ile Tyr Ala Gly Gln Glu	340	345	350	
cag cac tac gcc ggc gga aac gac ccc gcg aac cgc gaa gca acc tgg				1104
Gln His Tyr Ala Gly Gly Asn Asp Pro Ala Asn Arg Glu Ala Thr Trp	355	360	365	
ctc tcg ggc tac ccg acc gac agc gag ctg tac aag tta att gcc tcc				1152
Leu Ser Gly Tyr Pro Thr Asp Ser Glu Leu Tyr Lys Leu Ile Ala Ser	370	375	380	
gcg aac gca atc cgg aac tat gcc att agc aaa gat aca gga ttc gtg				1200
Ala Asn Ala Ile Arg Asn Tyr Ala Ile Ser Lys Asp Thr Gly Phe Val	385	390	395	400

acc tac aag aac tgg ccc atc tac aaa gac gac aca acg atc gcc atg 1248
 Thr Tyr Lys Asn Trp Pro Ile Tyr Lys Asp Asp Thr Thr Ile Ala Met
 405 410 415

cgc aag ggc aca gat ggg tcg cag atc gtg act atc ttg tcc aac aag 1296
 Arg Lys Gly Thr Asp Gly Ser Gln Ile Val Thr Ile Leu Ser Asn Lys
 420 425 430

ggt gct tcg ggt gat tcg tat acc ctc tcc ttg agt ggt gcg ggt tac 1344
 Gly Ala Ser Gly Asp Ser Tyr Thr Leu Ser Leu Ser Gly Ala Gly Tyr
 435 440 445

aca gcc ggc cag caa ttg acg gag gtc att ggc tgc acg acc gtg acg 1392
 Thr Ala Gly Gln Gln Leu Thr Glu Val Ile Gly Cys Thr Thr Val Thr
 450 455 460

gtt ggt tcg gat gga aat gtg cct gtt cct atg gca ggt ggg cta cct 1440
 Val Gly Ser Asp Gly Asn Val Pro Val Pro Met Ala Gly Gly Leu Pro
 465 470 475 480

agg gta ttg tat ccg act gag aag ttg gca ggt agc aag atc tgt agt 1488
 Arg Val Leu Tyr Pro Thr Glu Lys Leu Ala Gly Ser Lys Ile Cys Ser
 485 490 495

agc tcg gga aga ggt gct aca agc ccg ggt ggc tcc tcg ggt agt gtc 1536
 Ser Ser Gly Arg Gly Ala Thr Ser Pro Gly Gly Ser Ser Gly Ser Val
 500 505 510

gag gtc act ttc gac gtt tac gct acc aca gta tat ggc cag aac atc 1584
 Glu Val Thr Phe Asp Val Tyr Ala Thr Thr Val Tyr Gly Gln Asn Ile
 515 520 525

tat atc acc ggt gat gtg agt gag ctc ggc aac tgg aca ccc gcc aat 1632
 Tyr Ile Thr Gly Asp Val Ser Glu Leu Gly Asn Trp Thr Pro Ala Asn
 530 535 540

ggt gtt gca ctc tct tct gct aac tac ccc acc tgg agt gcc acg atc 1680
 Gly Val Ala Leu Ser Ser Ala Asn Tyr Pro Thr Trp Ser Ala Thr Ile
 545 550 555 560

gct ctc ccc gct gac acg aca atc cag tac aag tat gtc aac att gac 1728
 Ala Leu Pro Ala Asp Thr Thr Ile Gln Tyr Lys Tyr Val Asn Ile Asp
 565 570 575

ggc agc acc gtc atc tgg gag gat gct atc agc aat cgc gag atc acg 1776
 Gly Ser Thr Val Ile Trp Glu Asp Ala Ile Ser Asn Arg Glu Ile Thr
 580 585 590

acg ccc gcc agc ggc aca tac acc gaa aaa gac act tgg gat gaa tct 1824
 Thr Pro Ala Ser Gly Thr Tyr Thr Glu Lys Asp Thr Trp Asp Glu Ser
 595 600 605

tag 1827

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 <211> 608
 <212> PRT
 <213> Artificial

<220>

<223> Artificial

<400> 40

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Pro Ala Leu Ala Ala Thr Pro Ala Asp Trp Arg Ser Gln Ser Ile Tyr
 20 25 30

Phe Leu Leu Thr Asp Arg Phe Ala Arg Thr Asp Gly Ser Thr Thr Ala
 35 40 45

Thr Cys Asn Thr Ala Asp Gln Lys Tyr Cys Gly Gly Thr Trp Gln Gly
 50 55 60

Ile Ile Asp Lys Leu Asp Tyr Ile Gln Gly Met Gly Phe Thr Ala Ile
 65 70 75 80

Trp Ile Thr Pro Val Thr Ala Gln Leu Pro Gln Thr Thr Ala Tyr Gly
 85 90 95

Asp Ala Tyr His Gly Tyr Trp Gln Gln Asp Ile Tyr Ser Leu Asn Glu
 100 105 110

Asn Tyr Gly Thr Ala Asp Asp Leu Lys Ala Leu Ser Ser Ala Leu His
 115 120 125

Glu Arg Gly Met Tyr Leu Met Val Asp Val Val Ala Asn His Met Gly
 130 135 140

Tyr Asp Gly Ala Gly Ser Ser Val Asp Tyr Ser Val Phe Lys Pro Phe
 145 150 155 160

Ser Ser Gln Asp Tyr Phe His Pro Phe Cys Phe Ile Gln Asn Tyr Glu
 165 170 175

Asp Gln Thr Gln Val Glu Asp Cys Trp Leu Gly Asp Asn Thr Val Ser
 180 185 190

Leu Pro Asp Leu Asp Thr Thr Lys Asp Val Val Lys Asn Glu Trp Tyr
 195 200 205

Asp Trp Val Gly Ser Leu Val Ser Asn Tyr Ser Ile Asp Gly Leu Arg
 210 215 220

Ile Asp Thr Val Lys His Val Gln Lys Asp Phe Trp Pro Gly Tyr Asn
 225 230 235 240

Lys Ala Ala Gly Val Tyr Cys Ile Gly Glu Val Leu Asp Gly Asp Pro
 245 250 255

Ala Tyr Thr Cys Pro Tyr Gln Asn Val Met Asp Gly Val Leu Asn Tyr
 260 265 270

Pro Ile Tyr Tyr Pro Leu Leu Asn Ala Phe Lys Ser Thr Ser Gly Ser
 275 280 285

Met Asp Asp Leu Tyr Asn Met Ile Asn Thr Val Lys Ser Asp Cys Pro
 290 295 300

Asp Ser Thr Leu Leu Gly Thr Phe Val Glu Asn His Asp Asn Pro Arg
 305 310 315 320

Phe Ala Ser Tyr Thr Asn Asp Ile Ala Leu Ala Lys Asn Val Ala Ala
 325 330 335

Phe Ile Ile Leu Asn Asp Gly Ile Pro Ile Ile Tyr Ala Gly Gln Glu
 340 345 350

Gln His Tyr Ala Gly Gly Asn Asp Pro Ala Asn Arg Glu Ala Thr Trp
 355 360 365

Leu Ser Gly Tyr Pro Thr Asp Ser Glu Leu Tyr Lys Leu Ile Ala Ser
 370 375 380

Ala Asn Ala Ile Arg Asn Tyr Ala Ile Ser Lys Asp Thr Gly Phe Val
 385 390 395 400

Thr Tyr Lys Asn Trp Pro Ile Tyr Lys Asp Asp Thr Thr Ile Ala Met
 405 410 415

Arg Lys Gly Thr Asp Gly Ser Gln Ile Val Thr Ile Leu Ser Asn Lys
 420 425 430

Gly Ala Ser Gly Asp Ser Tyr Thr Leu Ser Leu Ser Gly Ala Gly Tyr
 435 440 445

Thr Ala Gly Gln Gln Leu Thr Glu Val Ile Gly Cys Thr Thr Val Thr
 450 455 460

Val Gly Ser Asp Gly Asn Val Pro Val Pro Met Ala Gly Gly Leu Pro

Gly Ile Ile Asn His Leu Asp Tyr Ile Gln Gly Met Gly Phe Thr Ala
 45 50 55

Ile Trp Ile Ser Pro Ile Thr Glu Gln Leu Pro Gln Asp Thr Ser Asp
 60 65 70 75

Gly Glu Ala Tyr His Gly Tyr Trp Gln Gln Lys Ile Tyr Tyr Val Asn
 80 85 90

Ser Asn Phe Gly Thr Ala Asp Asp Leu Lys Ser Leu Ser Asp Ala Leu
 95 100 105

His Ala Arg Gly Met Tyr Leu Met Val Asp Val Val Pro Asn His Met
 110 115 120

Gly Tyr Ala Gly Asn Gly Asn Asp Val Asp Tyr Ser Val Phe Asp Pro
 125 130 135

Phe Asp Ser Ser Ser Tyr Phe His Pro Tyr Cys Leu Ile Thr Asp Trp
 140 145 150 155

Asp Asn Leu Thr Met Val Gln Asp Cys Trp Glu Gly Asp Thr Ile Val
 160 165 170

Ser Leu Pro Asp Leu Asn Thr Thr Glu Thr Ala Val Arg Thr Ile Trp
 175 180 185

Tyr Asp Trp Val Ala Asp Leu Val Ser Asn Tyr Ser Val Asp Gly Leu
 190 195 200

Arg Ile Asp Ser Val Glu Glu Val Glu Pro Asp Phe Phe Pro Gly Tyr
 205 210 215

Gln Glu Ala Ala Gly Val Tyr Cys Val Gly Glu Val Asp Asn Gly Asn
 220 225 230 235

Pro Ala Leu Asp Cys Pro Tyr Gln Lys Tyr Leu Asp Gly Val Leu Asn
 240 245 250

Tyr Pro Ile Tyr Trp Gln Leu Leu Tyr Ala Phe Glu Ser Ser Ser Gly
 255 260 265

Ser Ile Ser Asn Leu Tyr Asn Met Ile Lys Ser Val Ala Ser Asp Cys
 270 275 280

Ser Asp Pro Thr Leu Leu Gly Asn Phe Ile Glu Asn His Asp Asn Pro
 285 290 295

Arg Phe Ala Ser Tyr Thr Ser Asp Tyr Ser Gln Ala Lys Asn Val Leu
 300 305 310 315

Ser Tyr Ile Phe Leu Ser Asp Gly Ile Pro Ile Val Tyr Ala Gly Glu
 320 325 330

Glu Gln His Tyr Ser Gly Gly Asp Val Pro Tyr Asn Arg Glu Ala Thr
 335 340 345

Trp Leu Ser Gly Tyr Asp Thr Ser Ala Glu Leu Tyr Thr Trp Ile Ala
 350 355 360

Thr Thr Asn Ala Ile Arg Lys Leu Ala Ile Ser Ala Asp Ser Asp Tyr
 365 370 375

Ile Thr Tyr Lys Asn Asp Pro Ile Tyr Thr Asp Ser Asn Thr Ile Ala
 380 385 390 395

Met Arg Lys Gly Thr Ser Gly Ser Gln Ile Ile Thr Val Leu Ser Asn
 400 405 410

Lys Gly Ser Ser Gly Ser Ser Tyr Thr Leu Thr Leu Ser Gly Ser Gly
 415 420 425

Tyr Thr Ser Gly Thr Lys Leu Ile Glu Ala Tyr Thr Cys Thr Ser Val
 430 435 440

Thr Val Asp Ser Asn Gly Asp Ile Pro Val Pro Met Ala Ser Gly Leu
 445 450 455

Pro Arg Val Leu Leu Pro Ala Ser Val Val Asp Ser Ser Ser Leu Cys
 460 465 470 475

Gly Gly Ser Gly Asn Thr Thr Thr Thr Thr Thr Ala Ala Thr Ser Thr
 480 485 490

Ser Lys Ala Thr Thr Ser Ser Ser Ser Ser Ala Ala Ala Thr Thr
 495 500 505

Ser Ser Ser Cys Thr Ala Thr Ser Thr Thr Leu Pro Ile Thr Phe Glu
 510 515 520

Glu Leu Val Thr Thr Thr Tyr Gly Glu Glu Val Tyr Leu Ser Gly Ser
 525 530 535

Ile Ser Gln Leu Gly Glu Trp His Thr Ser Asp Ala Val Lys Leu Ser
 540 545 550 555

Ala Asp Asp Tyr Thr Ser Ser Asn Pro Glu Trp Ser Val Thr Val Ser
 560 565 570

Leu Pro Val Gly Thr Thr Phe Glu Tyr Lys Phe Ile Lys Val Asp Glu
 575 580 585

Gly Gly Ser Val Thr Trp Glu Ser Asp Pro Asn Arg Glu Tyr Thr Val
 590 595 600

Pro Glu Cys Gly Ser Gly Ser Gly Glu Thr Val Val Asp Thr Trp Arg
 605 610 615

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 <211> 505
 <212> PRT
 <213> Aspergillus niger

<220>
 <221> mat_peptide
 <222> (22)..(505)

<400> 42

Met Arg Leu Ser Thr Ser Ser Leu Phe Leu Ser Val Ser Leu Leu Gly
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Lys Leu Ala Leu Gly Leu Ser Ala Ala Glu Trp Arg Thr Gln Ser Ile
 -5 -1 1 5 10

Tyr Phe Leu Leu Thr Asp Arg Phe Gly Arg Thr Asp Asn Ser Thr Thr
 15 20 25

Ala Thr Cys Asp Thr Gly Asp Gln Ile Tyr Cys Gly Gly Ser Trp Gln
 30 35 40

Gly Ile Ile Asn His Leu Asp Tyr Ile Gln Gly Met Gly Phe Thr Ala
 45 50 55

Ile Trp Ile Ser Pro Ile Thr Glu Gln Leu Pro Gln Asp Thr Ala Asp
 60 65 70 75

Gly Glu Ala Tyr His Gly Tyr Trp Gln Gln Lys Ile Tyr Asp Val Asn

80 85 90
 Ser Asn Phe Gly Thr Ala Asp Asp Leu Lys Ser Leu Ser Asp Ala Leu
 95 100 105
 His Ala Arg Gly Met Tyr Leu Met Val Asp Val Val Pro Asn His Met
 110 115 120
 Gly Tyr Ala Gly Asn Gly Asn Asp Val Asp Tyr Ser Val Phe Asp Pro
 125 130 135
 Phe Asp Ser Ser Ser Tyr Phe His Pro Tyr Cys Leu Ile Thr Asp Trp
 140 145 150 155
 Asp Asn Leu Thr Met Val Gln Asp Cys Trp Glu Gly Asp Thr Ile Val
 160 165 170
 Ser Leu Pro Asp Leu Asn Thr Thr Glu Thr Ala Val Arg Thr Ile Trp
 175 180 185
 Tyr Asp Trp Val Ala Asp Leu Val Ser Asn Tyr Ser Val Asp Gly Leu
 190 195 200
 Arg Ile Asp Ser Val Leu Glu Val Glu Pro Asp Phe Phe Pro Gly Tyr
 205 210 215
 Gln Glu Ala Ala Gly Val Tyr Cys Val Gly Glu Val Asp Asn Gly Asn
 220 225 230 235
 Pro Ala Leu Asp Cys Pro Tyr Gln Lys Val Leu Asp Gly Val Leu Asn
 240 245 250
 Tyr Pro Ile Tyr Trp Gln Leu Leu Tyr Ala Phe Glu Ser Ser Ser Gly
 255 260 265
 Ser Ile Ser Asn Leu Tyr Asn Met Ile Lys Ser Val Ala Ser Asp Cys
 270 275 280
 Ser Asp Pro Thr Leu Leu Gly Asn Phe Ile Glu Asn His Asp Asn Pro
 285 290 295
 Arg Phe Ala Ser Tyr Thr Ser Asp Tyr Ser Gln Ala Lys Asn Val Leu
 300 305 310 315
 Ser Tyr Ile Phe Leu Ser Asp Gly Ile Pro Ile Val Tyr Ala Gly Glu
 320 325 330

Glu Gln His Tyr Ser Gly Gly Lys Val Pro Tyr Asn Arg Glu Ala Thr
 335 340 345

Trp Leu Ser Gly Tyr Asp Thr Ser Ala Glu Leu Tyr Thr Trp Ile Ala
 350 355 360

Thr Thr Asn Ala Ile Arg Lys Leu Ala Ile Ser Ala Asp Ser Ala Tyr
 365 370 375

Ile Thr Tyr Ala Asn Asp Ala Phe Tyr Thr Asp Ser Asn Thr Ile Ala
 380 385 390 395

Met Arg Lys Gly Thr Ser Gly Ser Gln Val Ile Thr Val Leu Ser Asn
 400 405 410

Lys Gly Ser Ser Gly Ser Ser Tyr Thr Leu Thr Leu Ser Gly Ser Gly
 415 420 425

Tyr Thr Ser Gly Thr Lys Leu Ile Glu Ala Tyr Thr Cys Thr Ser Val
 430 435 440

Thr Val Asp Ser Ser Gly Asp Ile Pro Val Pro Met Ala Ser Gly Leu
 445 450 455

Pro Arg Val Leu Leu Pro Ala Ser Val Val Asp Ser Ser Ser Leu Cys
 460 465 470 475

Gly Gly Ser Gly Arg Leu Tyr Val Glu
 480

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- <211> 476
- <212> PRT
- <213> *Aspergillus oryzae*

- <220>
- <221> mat_peptide
- <222> (1)..(476)

<400> 43

Ala Thr Pro Ala Asp Trp Arg Ser Gln Ser Ile Tyr Phe Leu Leu Thr
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Asp Arg Phe Ala Arg Thr Asp Gly Ser Thr Thr Ala Thr Cys Asn Thr
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Ala Asp Gln Lys Tyr Cys Gly Gly Thr Trp Gln Gly Ile Ile Asp Lys
35 40 45

Leu Asp Tyr Ile Gln Gly Met Gly Phe Thr Ala Ile Trp Ile Thr Pro
50 55 60

Val Thr Ala Gln Leu Pro Gln Thr Thr Ala Tyr Gly Asp Ala Tyr His
65 70 75 80

Gly Tyr Trp Gln Gln Asp Ile Tyr Ser Leu Asn Glu Asn Tyr Gly Thr
85 90 95

Ala Asp Asp Leu Lys Ala Leu Ser Ser Ala Leu His Glu Arg Gly Met
100 105 110

Tyr Leu Met Val Asp Val Val Ala Asn His Met Gly Tyr Asp Gly Ala
115 120 125

Gly Ser Ser Val Asp Tyr Ser Val Phe Lys Pro Phe Ser Ser Gln Asp
130 135 140

Tyr Phe His Pro Phe Cys Phe Ile Gln Asn Tyr Glu Asp Gln Thr Gln
145 150 155 160

Val Glu Asp Cys Trp Leu Gly Asp Asn Thr Val Ser Leu Pro Asp Leu
165 170 175

Asp Thr Thr Lys Asp Val Val Lys Asn Glu Trp Tyr Asp Trp Val Gly
180 185 190

Ser Leu Val Ser Asn Tyr Ser Ile Asp Gly Leu Arg Ile Asp Thr Val
195 200 205

Lys His Val Gln Lys Asp Phe Trp Pro Gly Tyr Asn Lys Ala Ala Gly
210 215 220

Val Tyr Cys Ile Gly Glu Val Leu Asp Gly Asp Pro Ala Tyr Thr Cys
225 230 235 240

Pro Tyr Gln Asn Val Met Asp Gly Val Leu Asn Tyr Pro Ile Tyr Tyr
245 250 255

Pro Leu Leu Asn Ala Phe Lys Ser Thr Ser Gly Ser Met Asp Asp Leu
260 265 270

Tyr Asn Met Ile Asn Thr Val Lys Ser Asp Cys Pro Asp Ser Thr Leu
 275 280 285

Leu Gly Thr Phe Val Glu Asn His Asp Asn Pro Arg Phe Ala Ser Tyr
 290 295 300

Thr Asn Asp Ile Ala Leu Ala Lys Asn Val Ala Ala Phe Ile Ile Leu
 305 310 315 320

Asn Asp Gly Ile Pro Ile Ile Tyr Ala Gly Gln Glu Gln His Tyr Ala
 325 330 335

Gly Gly Asn Asp Pro Ala Asn Arg Glu Ala Thr Trp Leu Ser Gly Tyr
 340 345 350

Pro Thr Asp Ser Glu Leu Tyr Lys Leu Ile Ala Ser Ala Asn Ala Ile
 355 360 365

Arg Asn Tyr Ala Ile Ser Lys Asp Thr Gly Phe Val Thr Tyr Lys Asn
 370 375 380

Trp Pro Ile Tyr Lys Asp Asp Thr Thr Ile Ala Met Arg Lys Gly Thr
 385 390 395 400

Asp Gly Ser Gln Ile Val Thr Ile Leu Ser Asn Lys Gly Ala Ser Gly
 405 410 415

Asp Ser Tyr Thr Leu Ser Leu Ser Gly Ala Gly Tyr Thr Ala Gly Gln
 420 425 430

Gln Leu Thr Glu Val Ile Gly Cys Thr Thr Val Thr Val Gly Ser Asp
 435 440 445

Gly Asn Val Pro Val Pro Met Ala Gly Gly Leu Pro Arg Val Leu Tyr
 450 455 460

Pro Thr Glu Lys Leu Ala Gly Ser Lys Ile Cys Ser
 465 470 475