The invention relates to a novel Termamyl-like alpha-amylase, and Termamyl-like alpha-amylases comprising mutations in two, three, four, five or six regions/positions. The variants have increased thermostability at acidic pH and/or at low Ca²⁺ concentrations (relative to the parent). The invention also relates to a DNA construct comprising a DNA sequence encoding an alpha-amylase variant of the invention, a recombinant expression vector which carries a DNA construct of the invention, a cell which is transformed with a DNA construct of the invention, the use of an alpha-amylase variant of the invention for washing and/or dishwashing, textile desizing, starch liquefaction, a detergent additive comprising an alpha-amylase variant of the invention, a manual or automatic dishwashing detergent composition comprising an alpha-amylase variant of the invention, a method for generating a variant of a parent Termamyl-like alpha-amylase, which variant exhibits increased thermostability at acidic pH and/or at low Ca²⁺ concentrations (relative to the parent).
Fig. 1
Fig. 1A
Fig. 2
Fig. 5

<table>
<thead>
<tr>
<th></th>
<th>Delta Remission</th>
</tr>
</thead>
<tbody>
<tr>
<td>Termamyl</td>
<td>2.0</td>
</tr>
<tr>
<td>AAS60</td>
<td>7.0</td>
</tr>
<tr>
<td>SP722</td>
<td>4.0</td>
</tr>
<tr>
<td>SP690</td>
<td>5.0</td>
</tr>
</tbody>
</table>
ALPHA-AMYLASE AND ALPHA-AMYLASE VARIANTS

CROSS-REFERENCE TO RELATED APPLICATIONS


FIELD OF THE INVENTION

The present invention relates to a novel alpha-amylase and to novel alpha-amylolytic variants (mutants) of a Termamyl-like alpha-amylase, in particular variants exhibiting increased thermostability (relative to the parent) which are advantageous in connection with the industrial processing of starch (starch liquefaction, saccharification and the like). The novel alpha-amylase is suitable for bakery applications.

BACKGROUND OF THE INVENTION

Alpha-Amylases (alpha-1,4-glucan-4-glucanohydrolases, EC 3.2.1.1) constitute a group of enzymes which catalyze hydrolysis of starch and other linear and branched 1,4-glucosidic oligo- and polysaccharides.

There is a very extensive body of patent and scientific literature relating to this industrially very important class of enzymes.

A number of alpha-amylase such as Termamyl-like alpha-amylases variants are known from, e.g., WO 90/11352, WO 95/06005, WO 95/26397, WO 96/23873 and WO 96/23874.

Among more recent disclosures relating to alpha-amylases, WO 96/23874 provides three-dimensional, X-ray crystal structural data for a Termamyl-like alpha-amylase which consists of the 300 N-terminal amino acid residues of the B. amylo liquefaciens alpha-amylase and amino acids 301-483 of the C-terminal end of the B. licheniformis alpha-amylase comprising the amino acid sequence (the latter being available commercially under the tradename TermamylPM®), and which is thus closely related to the industrially important Bacillus alpha-amylases (which in the present context are embraced within the meaning of the term “Termamyl-like alpha-amylases”; and which include, inter alia, the B. licheniformis, B. amyloliquefaciens and B. stea rothermophilus alpha-amylases). WO 96/23874 further describes methodol-
FIG. 2 shows the pH Profile of the AA560 alpha-amylase compared to the SP722 and SP690 alpha-amylases. The pH profile was measured at 37° C. The activity is shown in absolute values as Abs650/mg.

FIG. 3 shows the Temperature Profile of the AA560 alpha-amylase compared to the SP722 and SP690 alpha-amylases. The temperature profile shown as Abs650/mg.

FIG. 4 shows the wash performance of AA560 in the AP Model Detergent 97 in comparison to SP722, SP690 and Termaryn®.

FIG. 5 shows the wash performance of AA560 in the Omo Multi Aceo in comparison to SP722, SP690 and Termaryn®.

FIG. 6 shows the wash performance of AA560 in the Omo Concentrated in comparison to SP722, SP690 and Termaryn®.

FIG. 7 shows the wash performance of AA560 in the Ariel Futur liquid in comparison to SP722, SP690 and Termaryn®.

DETAILED DISCLOSURE OF THE INVENTION

Alpha-Amylase Activity Determination

Alpha-Amylases (alpha-1,4-glucan-4-glucanohydrolases, EC 3.2.1.1) constitute a group of enzymes which catalyze hydrolysis of starch and other linear and branched 1,4-glucosidic oligo- and polysaccharides. For purposes of the present invention, alpha-amylase activity may be determined using the Phadebas assay, the pNPG assay and the BS-alpha-amylase activity assay described below in the “Materials and Methods” section.

The Novel Alpha-Amylase

Microbial Source

The novel alkaline alpha-amylase of the invention may be derived from a strain of Bacillus. Preferred strains are of Bacillus sp. DSM 12649 (the AA560 alpha-amylase) or Bacillus sp. DSM 12648 (the AA349 alpha-amylase). These strains were deposited on 25 Jan. 1999 by the inventors under the terms of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure at Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ), Mascheroder Weg 1b, D-38124 Braunschweig DE.

Escherichia coli strains termed NN049467 and NN049470 containing the alpha-amylase genes in plasmids pLH1277 (AA349) and plasmid pTVB299 (AA560) have also been deposited on 7 Apr. 1999 under the terms of the Budapest Treaty with the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ), Mascheroder Weg 1b, D-38124 Braunschweig DE, and given the accession numbers DSM12761 and DSM12764, respectively.

Homology of Enzyme

In a first embodiment, the present invention relates to isolated polypeptides having an amino acid sequence which has a degree of identity to amino acids 1 to 485 of SEQ ID NO: 24 or SEQ ID NO: 26 (i.e., the mature polypeptide) of at least about 96%, preferably at least about 97%, more preferably at least about 98%, even more preferably at least about 99%, which have alpha-amylase activity (hereinafter “homologous polypeptides”). In a preferred embodiment, the homologous polypeptides have an amino acid sequence which differs by five amino acids, preferably by four amino acids, more preferably by three amino acids, even more preferably by two amino acids, and most preferably by one amino acid from amino acids 1 to 485 of SEQ ID NO: 24 or SEQ ID NO: 26. It is to be noted that SEQ ID NO: 24 and SEQ ID NO: 26 are identical. However, the DNA sequences, i.e., SEQ ID NO: 23 and SEQ ID NO: 25, respectively, encoding the alpha-amylase of the invention shown in SEQ ID NO: 24 and SEQ ID NO: 26 are not identical.

The amino acid sequence homology may be determined as the degree of identity between the two sequences indicating a derivation of the first sequence from the second. The homology may suitably be determined by means of computer programs known in the art. Thus, GAP provided in GCG version 8 (Needleman and Wunsch, 1970, Journal of Molecular Biology 48, 443-453) may be used for a pairwise alignment of the sequences and calculation of the degree of identity or degree of homology using the default settings. Alternatively, Gap from GCG version 9 may be used with a translated version 8 peptide scoring matrix, a gap creation penalty of 30, a gap extension penalty of 1 using its matrix (http://us.scripps.edu/seqweb/matrix/) without end gap penalty.

Homology (Identity) of the Novel Alpha-Amylase to Known Bacillus sp. Alpha-Amylases

A homology search of known sequences showed homologies for the sequences of the invention with a number of Bacillus amylases in the range 65-95% on amino acid basis determined as described above.

Specifically, the most homologous alpha-amylases found are SP690 (SEQ ID NO: 1 of U.S. Pat. No. 5,856,164 which is about 87% homologous), SP722 (SEQ ID NO: 2 of U.S. Pat. No. 5,856,164 which is about 87% homologous), the mature part (i.e., amino acids 31-516) of the alpha-amylase obtained from Bacillus sp. KSM-AP1378 disclosed as SEQ ID NO: 2 of WO 97/00324 which is about 86% homologous, and the alpha-amylase disclosed in Tsukamoto et al., 1988, Biochem. Biophys. Res. Commun. 151: 25-33 which is about 95% homologous to SEQ ID NO: 24 and SEQ ID NO: 26 determined as described above.

Preferably, the polypeptides of the present invention comprise the amino acid sequence of SEQ ID NO: 24 or SEQ ID NO: 26 or allelic variants thereof, or fragments thereof that have alpha-amylase activity. SEQ ID NO: 24 and SEQ ID NO: 26 show the mature part of the alkaline alpha-amylase of the invention.

A fragment of SEQ ID NO: 24 or SEQ ID NO: 26 are polypeptides having one or more amino acids deleted from the amino and/or carboxyl terminus of this amino acid sequence.

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

The amino acid sequences of the homologous polypeptides may differ from the amino acid sequence of SEQ ID NO: 24 or SEQ ID NO: 26 by an insertion or deletion
of one or more amino acid residues and/or the substitution of one or more amino acid residues by different amino acid residues. Preferably, amino acid changes are of a minor nature, that is conservative amino acid substitutions that do not significantly affect the folding and/or activity of the protein; small deletions, typically of one to about 30 amino acids; small amino- or carboxyl-terminal extensions, such as an amino-terminal methionine residue; a small linker peptide of up to about 20-25 residues; or a small extension that facilitates purification by changing net charge or another function, such as a poly-histidine tract, an antigenic epitope or a binding domain.

[0033] Examples of conservative substitutions are within the group of basic amino acids (arginine, lysine and histidine), acidic amino acids (glutamic acid and aspartic acid), polar amino acids (glutamine and asparagine), hydrophobic amino acids (leucine, isoleucine and valine), aromatic amino acids (phenylalanine, tryptophan and tyrosine), and small amino acids (glycine, alanine, serine, threonine and methionine). Amino acid substitutions which do not generally alter the specific activity are known in the art and are described, for example, by H. Neurath and R. L. Hill, 1979, In, The Proteins, Academic Press, New York. The most commonly occurring exchanges are Ala/Ser, Val/Leu, Asp/Glu, Thr/Ser, Ala/Gly, Ala/Thr, Ser/Asn, Ala/Val, Ser/Gly, Tyr/Ph, Ala/Pro, Lys/Arg, Asp/Asn, Leu/Ile, Leu/Val, Ala/Glu, and Asp/Gly as well as these in reverse.

[0034] In a second embodiment, the present invention relates to isolated polypeptides having alpha-amylase activity which are encoded by nucleic acid sequences which hybridize under medium stringency conditions, preferably medium-high stringency conditions, more preferably high stringency conditions, and most preferably very high stringency conditions with a nucleic acid probe which hybridizes under the same conditions with (i) the nucleic acid sequence of SEQ ID NO: 23 or SEQ ID NO: 25, (ii) the cDNA sequence of SEQ ID NO: 23 or SEQ ID NO: 25, (iii) a subsequence of (i) or (ii), or (iv) a complementary strand of (i), (ii), or (iii) (Sambrook et al., 1989, Molecular Cloning, A Laboratory Manual, 2d edition, Cold Spring Harbor, N.Y.). The subsequence of SEQ ID NO: 23 or SEQ ID NO: 25 may be at least 100 nucleotides or preferably at least 200 nucleotides. Moreover, the subsequence may encode a polypeptide fragment which has alpha-amylase activity. The polypeptides may also be allelic variants or fragments of the polypeptides that have alpha-amylase activity.

[0035] The nucleic acid sequence of SEQ ID NO: 23 or SEQ ID NO: 25 or a subsequence thereof, as well as the amino acid sequence of SEQ ID NO: 24 or SEQ ID NO: 26 or a fragment thereof, may be used to design a nucleic acid probe to identify and clone DNA encoding polypeptides having alpha-amylase activity from strains of different genera or species according to methods well known in the art. In particular, such probes can be used for hybridization with the genomic or cDNA of the genus or species of interest, following standard Southern blotting procedures, in order to identify and isolate the corresponding gene therein. Such probes can be considerably shorter than the entire sequence, but should be at least 15, preferably at least 25, and more preferably at least 35 nucleotides in length. Longer probes can also be used. Both DNA and RNA probes can be used. The probes are typically labelled for detecting the corresponding gene (for example, with 32P, 35S, biotin, or avidin). Such probes are encompassed by the present invention.

[0036] Thus, a genomic DNA or cDNA library prepared from such other organisms may be screened for DNA which hybridizes with the probes described above and which encodes a polypeptide having alpha-amylase activity. Genomic or other DNA from such other organisms may be separated by agarose or polyacrylamide gel electrophoresis, or other separation techniques. DNA from the libraries or the separated DNA may be transfected and immobilized on nitrocellulose or other suitable carrier material. In order to identify a clone or DNA which is homologous with SEQ ID NO: 23 or SEQ ID NO: 25 or subsequences thereof, the carrier material is used in a Southern blot.

[0037] For purposes of the present invention, hybridization indicates that the nucleic acid sequence hybridizes to a nucleic acid probe corresponding to the nucleic acid sequence shown in SEQ ID NO: 23 or SEQ ID NO: 25, its complementary strand, or subsequences thereof, under medium to very high stringency conditions. Molecules to which the nucleic acid probe hybridizes under these conditions are detected using X-ray film.

[0038] In another preferred embodiment, the nucleic acid probe is the nucleic acid sequence contained in plasmids pLIH274 (AA349) or pTVB299 (AA560) which are contained in Escherichia coli DSM12761 or Escherichia coli DSM12764, respectively, or, wherein the nucleic acid sequence encodes a polypeptide having acid alpha-amylase activity of the invention and shown in SEQ ID NO: 24 and SEQ ID NO: 26, respectively.

[0039] For long probes of at least 100 nucleotides in length, medium to very high stringency conditions are defined as prehybridization and hybridization at 42° C. in 5xSSPE, 0.3% SDS, 200 mg/ml sheared and denatured salmon sperm DNA, 35% formamide for medium and medium-high stringencies, or 50% formamide for high and very high stringencies, following standard Southern blotting procedures.

[0040] For long probes of at least 100 nucleotides in length, the carrier material is finally washed three times each for 15 minutes using 2xSSC. 0.2% SDS preferably at least at 55° C. (medium stringency), preferably at least at 60° C. (medium-high stringency), more preferably at least at 65° C. (high stringency), and most preferably at least at 70° C. (very high stringency).

[0041] For short probes which are about 15 nucleotides to about 70 nucleotides in length, stringency conditions are defined as prehybridization, hybridization, and washing post-hybridization at 5° C. to 10° C. below the calculated Tm, using the calculation according to Bolton and McCarthy (1962, Proceedings of the National Academy of Sciences USA 48:1390) in 0.9 M NaCl, 0.09 M Tris-HCl pH 7.6, 6 mM EDTA, 0.5% NP-40, 1x Denhardt’s solution, 1 mM sodium pyrophosphate, 1 mM sodium monobasic phosphate, 0.1 mM ATP, and 0.2 mg of yeast RNA per ml following standard Southern blotting procedures.

[0042] For short probes which are about 15 nucleotides to about 70 nucleotides in length, the carrier material is washed once in 6xSSC plus 0.1% SDS for 15 minutes and twice each for 15 minutes using 6xSSC at 5° C. to 10° C. below the calculated Tm.

[0043] In a third embodiment, the present invention relates to isolated polypeptides, i.e., the polypeptides shown in SEQ ID NO: 24 or SEQ ID NO: 26, having the following physicochemical properties:
A pH optimum (see FIG. 2) determined using the Phadebas method (37° C.) was found to be in the range between pH 8 and 9, more precisely at about 8.5.

A temperature optimum (see FIG. 3) determined using the Phasebax method (pH 9.0) was found to be in the range between 55 and 65° C., more precisely about 60° C.

A pl between 7-8 (See Table 1 in Example 11) was determined by isoelectric focusing (Pharmacia, Ampholine, pH 3.5-9.3).

A specific activity (see Table 1 of Example 11) of 35,000 NU/ml was determined using the Phadebas method and 6,000 NU/ml using the pNPcG method.

The alpha-amylase of the present invention have at least 20%, preferably at least 40%, more preferably at least 60%, even more preferably at least 80%, even more preferably at least 90%, and most preferably at least 100% of the alpha-amylase activity of the mature alpha-amylase shown in SEQ ID NO: 24 and SEQ ID NO: 26.

An alpha-amylase of the present invention may be obtained from microorganisms of any genus. For purposes of the present invention, the term “obtained from” as used herein in connection with a given source shall mean that the alpha-amylase encoded by the nucleic acid sequence is produced by the source or by a cell in which the nucleic acid sequence from the source has been inserted.

An alpha-amylase of the present invention is a bacterial polypeptide. For example, the polypeptide may be a gram positive bacterial polypeptide such as a Bacillus polypeptide, e.g., a Bacillus alkalophilus, Bacillus amylophilus, Bacillus brevis, Bacillus circulans, Bacillus coagulans, Bacillus lentus, Bacillus licheniformis, Bacillus megaterium, Bacillus steareothermophilus, Bacillus subtilis, or Bacillus thuringiensis polypeptide; or a Streptomyces polypeptide, e.g., a Streptomyces lividans or Streptomyces griseus polypeptide; or a gram negative bacterial polypeptide, e.g., an E. coli or a Pseudomonas sp. polypeptide.

In another preferred embodiment, the polypeptide is a Bacillus sp. polypeptide, more preferred embodiment, the polypeptide is a Bacillus sp. DSM 12648 or Bacillus sp. DSM 12649 polypeptide, e.g., the polypeptides with the amino acid sequence of SEQ ID NO: 24 and SEQ ID NO: 26, respectively.

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

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

Furthermore, such polypeptides may be identified and obtained from other sources including microorganisms isolated from nature (e.g., soil, composts, water, etc.) using the above-mentioned probes. Techniques for isolating microorganisms from natural habitats are well known in the art.

The nucleic acid sequence may then be derived by similarly screening a genomic or cDNA library of another microorganism. Once a nucleic acid sequence encoding a polypeptide has been detected with the probe(s), the sequence may be isolated or cloned by utilizing techniques which are known to those of ordinary skill in the art (see, e.g., Sambrook et al., 1989, supra).

As defined herein, an “isolated” polypeptide is a polypeptide which is essentially free of other non-alpha-amylase polypeptides, e.g., at least about 20% pure, preferably at least about 40% pure, more preferably about 60% pure, even more preferably about 80% pure, most preferably about 90% pure, and even most preferably about 95% pure, as determined by SDS-PAGE.

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

Mutants of the Novel Alpha-Amylase

Specifically contemplated mutants of the novel alpha-amylase shown in SEQ ID NO: 24 (or SEQ ID NO: 26) are described in the following. A mutant alpha-amylase of the invention is characterized by the fact that one or more of the methionine amino acid residues is exchanged with any amino acid residue except for Cys and Met. Thus, according to the invention the amino acid residues to replace the methionine amino acid residue are the following: Ala, Arg, Asn, Asp, Gln, Glu, Gly, His, Ile, Leu, Lys, Phe, Pro, Ser, Thr, Trp, Tyr, and Val.

A preferred embodiment of the mutant alpha-amylase of the invention is characterized by the fact that one or more of the methionine amino acid residues in (are) exchanged with a Leu, Thr, Ala, Gly, Ser, Ile, or Val amino acid residue, preferably a Leu, Thr, Ala, or Gly amino acid residue. In this embodiment a very satisfactory activity level and stability in the presence of oxidizing agents is obtained. Specifically this means that one or more of the methionines in the following position may be replaced or deleted using any suitable technique known in the art, including especially site-directed mutagenesis and gene shuffling. Contemplated position, using the SEQ ID NO: 24 numbering, are: 9, 10, 105, 116, 202, 208, 261, 300, 323, 382, 430, 440.

In a preferred embodiment of the mutant alpha-amylase of the invention is characterized by the fact that the methionine amino acid residue at position 202 is exchanged with any of amino acid residue except for Cys and Met, preferably with a Leu, Thr, Ala, Gly, Ser, Ile, or Asp.

Other contemplated preferred mutations include deletion of one, two or more residues of amino acids R181, G182, D183 or G184, K185, G186 or substitution of one or more of these residues. A preferred mutation is the deletion of D183-G184. Particularly relevant mutations are substitutions of G186 with Ala, Arg, Asn, Asp, Cys, Gln, Glu, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, and Val. A particularly preferred substitution is G186R.
[0061] Also contemplated is substitution of N195 with Ala, Arg, Asn, Asp, Cys, Gin, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, and Val. A particularly interesting substitution is N195F.

[0062] The following combinations of the above mentioned mutations include: deletion of D183-G184+N195F; deletion of D183-G184+G186R; deletion of D183-G184+G186R+N195F and G186R+N195F.

Nucleic Acid Sequences

[0063] The present invention also relates to isolated nucleic acid sequences which encode a polypeptide of the present invention. In a preferred embodiment, the nucleic acid sequence is set forth in SEQ ID NO: 23 or SEQ ID NO: 25. In another preferred embodiment, the nucleic acid sequence is the sequence contained in plasmid pLIH1274 (AA349) or plasmid pTVB299 (AA560) that is contained in Escherichia coli DSM 12761 and Escherichia coli DSM 12764, respectively. In a further preferred embodiment, the nucleic acid sequence is the mature polypeptide coding region of SEQ ID NO: 23 or SEQ ID NO: 25. The present invention also encompasses nucleic acid sequences which encode a polypeptide having the amino acid sequence of SEQ ID NO: 24 which differ from SEQ ID NO: 23 or SEQ ID NO: 25 by virtue of the degeneracy of the genetic code. The present invention also relates to subsequences of SEQ ID NO: 23 or SEQ ID NO: 25 which encode fragments of SEQ ID NO: 24 or SEQ ID NO: 26, respectively, that have alpha-amylase activity.

[0064] Subsequences of SEQ ID NO: 23 or SEQ ID NO: 25 are nucleic acid sequences encompassed by SEQ ID NO: 23 or SEQ ID NO: 25 except that one or more nucleotides from the 5' and/or 3' end have been deleted.

[0065] The present invention also relates to mutant nucleic acid sequences comprising at least one mutation in the mature polypeptide coding sequence of SEQ ID NO:1 or SEQ ID NO: 3, in which the mutant nucleic acid sequence encodes a polypeptide which consists of amino acids 1 to 485 of SEQ ID NO: 24 or SEQ ID NO: 26.

[0066] The techniques used to isolate or clone a nucleic acid sequence encoding a polypeptide are known in the art and include isolation from genomic DNA, preparation from cDNA, or a combination thereof. The cloning of the nucleic acid sequences of the present invention from such genomic DNA can be effected, e.g., by using the well known polymerase chain reaction (PCR) or antibody screening of expression libraries to detect cloned DNA fragments with shared structural features. See, e.g., Innis et al., 1990, PCR: A Guide to Methods and Application, Academic Press, New York. Other nucleic acid amplification procedures such as ligase chain reaction (LCR), ligated activated transcription (LAT) and nucleic acid sequence-based amplification (NASBA) may be used. The nucleic acid sequence may be cloned from a strain of Bacillus, or another or related organism and thus, for example, may be an allelic or species variant of the polypeptide encoding region of the nucleic acid sequence.

[0067] The term “isolated nucleic acid sequence” as used herein refers to a nucleic acid sequence which is essentially free of other nucleic acid 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 nucleic acid sequence can be obtained by standard cloning procedures used in genetic engineering to relocate the nucleic acid 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 nucleic acid fragment comprising the nucleic acid sequence encoding the polypeptide, 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 nucleic acid sequence will be replicated. The nucleic acid sequence may be of genomic, cDNA, RNA, semisynthetic, synthetic origin, or any combinations thereof.

Homology of DNA Sequence Encoding the Enzyme

[0068] The present invention also relates to nucleic acid sequences which have a degree of homology to the mature polypeptide coding sequence of SEQ ID NO: 23 (i.e., nucleotides 1 to 1458) or SEQ ID NO: 25 (i.e., nucleotide 1 to 1458) of at least about 96% homology on DNA level, preferably about 97%, preferably about 98%, more preferably about 99% homology, which encode an active polypeptide.

[0069] The DNA sequence homology may be determined as the degree of identity between the two sequences indicating a derivation of the first sequence from the second. The homology may suitably be determined by means of computer programs known in the art such as GAP provided in the GCG program package (described above). Thus, Gap GCGV8 may be used with the following default parameters: GAP creation penalty of 5.0 and GAP extension penalty of 0.3, default scoring matrix, GAP uses the method of Needleman/Wunsch/Sellers to make alignments.

[0070] Modification of a nucleic acid sequence encoding a polypeptide of the present invention may be necessary for the synthesis of polypeptides substantially similar to the polypeptide. The term “substantially similar” to the polypeptide refers to non-naturally occurring forms of the polypeptide. These polypeptides may differ in some engineered way from the polypeptide isolated from its native source, e.g., variants that differ in specific activity, thermostability, pH optimum, or the like. The variant sequence may be constructed on the basis of the nucleic acid sequence as presented as the polypeptide encoding part of SEQ ID NO: 23 or SEQ ID NO: 25, e.g., a subsequence thereof, and/or by introduction of nucleotide substitutions which do not give rise to another amino acid sequence of the polypeptide encoded by the nucleic acid sequence, but which correspond to the codon usage of the host organism intended for production of the enzyme, or by introduction of nucleotide substitutions which may give rise to a different amino acid sequence. For a general description of nucleotide substitution, see, e.g., Ford et al., 1991, Protein Expression and Purification 2: 95-107.

[0071] It will be apparent to those skilled in the art that such substitutions can be made outside the regions critical to the function of the molecule and still result in an active polypeptide. Amino acid residues essential to the activity of the polypeptide encoded by the isolated nucleic acid sequence of the invention, and therefore preferably not subject to substitution, may be identified according to procedures known in the art, such as site-directed mutagenesis or alanine-scanning mutagenesis (see, e.g., Cunningham and Wells, 1989, Science 244: 1081-1085). In the latter technique, mutations are introduced at every positively charged residue in the molecule, and the resultant mutant molecules are tested for [enzyme] activity to identify amino acid residues that are critical to the activity of the molecule: Sites of substrate-enzyme interac-
tion can also be determined by analysis of the three-dimen-
sional structure as determined by such techniques as nuclear
magnetic resonance analysis, crystallography or photoaffin-
ity labelling (see, e.g., de Vos et al., 1992, Science 255:
306-312; Smith et al., 1992, Journal of Molecular Biology
224: 899-904; Wlodaver et al., 1992, FEBS Letters 309:
59-64).

[0072] The present invention also relates to isolated nucleic
acid sequences encoding a polypeptide of the present inven-
tion, which hybridize under medium stringency conditions,
preferably medium-high stringency conditions, more prefer-
ably high stringency conditions, and most preferably very
high stringency conditions with a nucleic acid probe which
hybridizes under the same conditions with the nucleic acid
sequence of SEQ ID NO: 1 or SEQ ID NO: 3 or its comple-
mentary strand; or allelic variants and subsequences thereof
(Sambrook et al., 1989, supra), as defined herein.

[0073] The present invention also relates to isolated nucleic
acid sequences produced by (a) hybridizing a DNA under
medium, medium-high, high, or very high stringency condi-
tions with the sequence of SEQ ID NO: 23 or SEQ ID NO: 25;
or their complementary strands, or a subsequence thereof;
and (b) isolating the nucleic acid sequence. The subsequence
is preferably a sequence of at least 100 nucleotides such as a
sequence which encodes a polypeptide fragment which has
alpha-amylase activity.

Methods for Producing Mutant Nucleic Acid Sequences

[0074] The present invention further relates to methods for
producing a mutant nucleic acid sequence, comprising intro-
ducing at least one mutation into the mature polypeptide
coding sequence of SEQ ID NO: 23 or SEQ ID NO: 25 or a
subsequence thereof, wherein the mutant nucleic acid
sequence encodes a polypeptide which consists of 1 to 485 of
SEQ ID NO: 24 or SEQ ID NO: 26 or a fragment thereof
which has alpha-amylase activity.

[0075] The introduction of a mutation into the nucleic acid
sequence to exchange one nucleotide for another nucleotide
may be accomplished by site-directed mutagenesis using any
of the methods known in the art. Particularly useful is the
procedure which utilizes a supercoiled, double stranded DNA
vector with an insert of interest and two synthetic primers
containing the desired mutation. The oligonucleotide primers,
each complementary to opposite strands of the vector,
extend during temperature cycling by means of Pfu DNA
polymerase. On incorporation of the primers, a mutated plas-
mid containing staggered nicks is generated. Following tem-
perature cycling, the product is treated with DpnI which is
specific for methylated and hemimethylated DNA to digest
the parental DNA template and to select for mutation-con-
taining synthesized DNA. Other procedures known in the art
may also be used. These other procedures include gene shuf-
fling, e.g., as described in WO 95/22625 (from Affymax
Technologies N.V.) and WO 96/00343 (from Novo Nordisk
A/S).

Nucleic Acid Constructs

[0076] The present invention also relates to nucleic acid
constructs comprising a nucleic acid sequence of the present
invention operably linked to one or more control sequences
which direct the expression of the coding sequence in a suit-
able host cell under conditions compatible with the control
sequences. Expression will be understood to include any step
involved in the production of the polypeptide including, but
not limited to, transcription, post-transcriptional modifica-
tion, translation, post-translational modification, and secre-
tion.

[0077] “Nucleic acid construct” is defined herein as a
nucleic acid molecule, either single- or double-stranded,
which is isolated from a naturally occurring gene or which has
been modified to contain segments of nucleic acid which are
combined and juxtaposed in a manner which would not oth-
ervise exist in nature. The term nucleic acid construct is
 synonymous with the term expression cassette when the
nucleic acid construct contains all the control sequences
required for expression of a coding sequence of the present
invention. The term “coding sequence” is defined herein as a
portion of a nucleic acid sequence which directly specifies the
amino acid sequence of its protein product. The boundaries
of the coding sequence are generally determined by a ribosome
binding site (proxarytes) or by the ATG start codon (eukary-
otes) located just upstream of the open reading frame at the 5'
end of the mRNA and a transcription terminator sequence
located just downstream of the open reading frame at the 3'
end of the mRNA. A coding sequence can include, but is not
limited to, DNA, cDNA, and recombinant nucleic acid
sequences.

[0078] An isolated nucleic acid sequence encoding a
polypeptide of the present invention may be manipulated in a
variety of ways to provide for expression of the polypeptide.
Manipulation of the nucleic acid sequence prior to its inser-
tion into a vector may be desirable or necessary depending on
the expression vector. The techniques for modifying nucleic
acid sequences utilizing recombinant DNA methods are well
known in the art.

[0079] The term “control sequences” is defined herein to
include all components which are necessary or advantageous
for the expression of a polypeptide of the present invention.
Each control sequence may be native or foreign to the nucleic
acid sequence encoding the polypeptide. Such control
sequences include, but are not limited to, a leader, polyade-
ylation sequence, propeptide sequence, promoter, signal
peptide sequence, and transcription terminator. At a mini-
mum, the control sequences include a promoter, and tran-
scriptional and translational stop signals. The control
sequences may be provided with linkers for the purpose of
introducing specific restriction sites facilitating ligation of the
coding sequences with the coding region of the nucleic acid
sequence encoding a polypeptide. The term “operably linked” is
defined herein as a configuration in which a control
sequence is appropriately placed at a position relative to the
coding sequence of the DNA sequence such that the control
sequence directs the expression of a polypeptide.

Promoter Sequence

[0080] The control sequence may be an appropriate pro-
moter sequence, a nucleic acid sequence which is recognized
by a host cell for expression of the nucleic acid sequence.
The promoter sequence contains transcriptional control
sequences which mediate the expression of the polypeptide.
The promoter may be any nucleic acid sequence which shows
transcriptional activity in the host cell of choice including
mutant, truncated, and hybrid promoters, and may be
obtained from genes encoding extracellular or intracellular
polypeptides either homologous or heterologous to the host
cell.
Examples of suitable promoters for directing the transcription of the nucleic acid constructs of the present invention, especially in a bacterial host cell, are the promoters obtained from the E. coli lac operator, Streptomyces coelicolor arginase gene (dagA), Bacillus subtilis levansucrase gene (saeB), Bacillus licheniformis alpha-amylase gene (amyL), Bacillus steareothermophilus maltogenic amylase gene (amyM), Bacillus amyloliquefaciens alpha-amylase gene (amyQ), Bacillus licheniformis penicillinase gene (penP), Bacillus subtilis xylA and xylB genes, and prokaryotic beta-lactamase gene (Villa-Kamaroff et al., 1978, Proceedings of the National Academy of Sciences USA 75: 3727-3731), as well as the tac promoter (DeBoer et al., 1983, Proceedings of the National Academy of Sciences USA 80: 21-25). Further promoters are described in “Useful proteins from recombinant bacteria” in Scientific American, 1980, 242: 74-94; and in Sambrook et al., 1989, supra.

Terminator Sequence

The control sequence may also be a suitable transcription terminator sequence, a sequence recognized by a host cell to terminate transcription. The terminator sequence is operably linked to the 3′ terminus of the nucleic acid sequence encoding the polypeptide. Any terminator which is functional in the host cell of choice may be used in the present invention.

Signal Peptide

The control sequence may also be a signal peptide coding region that codes for an amino acid sequence linked to the amino terminus of a polypeptide and directs the encoded polypeptide into the cell’s secretory pathway. The 5′ end of the coding sequence of the nucleic acid sequence may inherently contain a signal peptide coding region naturally linked in translation reading frame with the segment of the coding region which encodes the secreted polypeptide. Alternatively, the 5′ end of the coding sequence may contain a signal peptide coding region which is foreign to the coding sequence. The foreign signal peptide coding region may be required where the coding sequence does not naturally contain a signal peptide coding region. Alternatively, the foreign signal peptide coding region may simply replace the natural signal peptide coding region in order to enhance secretion of the polypeptide. However, any signal peptide coding region which directs the expressed polypeptide into the secretory pathway of a host cell of choice may be used in the present invention.

Effective signal peptide coding regions for bacterial host cells are the signal peptide coding regions obtained from the genes for Bacillus NCIB 11837 maltogenic amylase, Bacillus steareothermophilus alpha-amylase, Bacillus licheniformis subtilisin, Bacillus licheniformis beta-lactamase, Bacillus steareothermophilus neutral proteases (npT, npR, npM), and Bacillus subtilis prsA. Further signal peptides are described by Simonen and Palva, 1993, Microbiological Reviews 57: 109-137.

Regulatory System

It may also be desirable to add regulatory sequences which allow the regulation of the expression of the polypeptide relative to the growth of the host cell. Examples of regulatory systems are those which cause the expression of the gene to be turned on or off in response to a chemical or physical stimulus, including the presence of a regulatory compound. Regulatory systems in prokaryotic systems include the lac, tac, and trp operator systems. In yeast, the ADH2 system or GAL1 system may be used. In filamentous fungi, the TAKA alpha-amylase promoter, Aspergillus niger glucoamylase promoter, and Aspergillus oryzae glucoamylase promoter may be used as regulatory sequences. Other examples of regulatory sequences are those which allow for gene amplification. In eukaryotic systems, these include the dihydrofolate reductase gene which is amplified in the presence of methotrexate, and the metallothionerin genes which are amplified with heavy metals. In these cases, the nucleic acid sequence encoding the polypeptide would be operably linked with the regulatory sequence.

Expression Vectors

The present invention also relates to recombinant expression vectors comprising a nucleic acid sequence of the present invention, a promoter, and transcriptional and translational stop signals. The various nucleic acid 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 nucleic acid sequence encoding the polypeptide at such sites. Alternatively, the nucleic acid sequence of the present invention may be expressed by inserting the nucleic acid sequence or a nucleic acid construct comprising the sequence into an appropriate vector for expression. In creating the expression vector, the coding sequence is located in the vector so that the coding sequence is operably linked with the appropriate control sequences for expression.

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 nucleic acid 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, 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. Furthermore, 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 may be used.

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 bacterial selectable markers are the dal genes from Bacillus subtilis or Bacillus licheniformis, or markers which confer antibiotic resistance such as ampicillin, kanamycin, chloramphenicol or tetracycline resistance. Suitable markers for yeast host cells are ADE2, HIS3, LEU2, LYS2, ME13, TRP1, and URA3. A selectable marker for use in a filamentous fungal host cell may be selected from the group including, but not limited to, amdS (acetamidase), argIII
(ornithine carbamoyltransferase), bar (phosphinothricin acetyltransferase), hygB (hygromycin phosphotransferase), niaD (nitrate reductase), pyrG (orotidine-5'-phosphate decarboxylase), sC (sulfate acetyltransferase), trpC (anthranilate synthase), as well as equivalents thereof. Preferred for use in an Aspergillus cell are the nmsD and pyrG genes of Aspergillus nidulans or Aspergillus oryzae and the bar gene of Streptomyces hygroscopicus.

[0090] 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.

[0091] For integration into the host cell genome, the vector may rely on the nucleic acid sequence encoding the polypeptide or any other element of the vector for stable integration of the vector into the genome by homologous or nonhomologous recombination. Alternatively, the vector may contain additional nucleic acid sequences for directing integration by homologous recombination into the genome of the host cell. The additional nucleic acid 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 international elements should preferably contain a sufficient number of nucleic acids, such as 100 to 1,500 base pairs, preferably 400 to 1,500 base pairs, and most preferably 500 to 1,500 base pairs, which are highly homologous with the corresponding target sequence to enhance the probability of homologous recombination. The international elements may be any sequence that is homologous with the target sequence in the genome of the host cell. Furthermore, the international elements may be non-encoding or encoding nucleic acid sequences. On the other hand, the vector may be integrated into the genome of the host cell by non-homologous recombination.

[0092] For autonomous replication, the vector may further comprise an origin of replication enabling the vector to replicate autonomously in the host cell in question. Examples of bacterial origins of replication are the origins of replication of plasmids pBR322, pUC19, pACYC177, and pACYC184 permitting replication in E. coli, and pUB110, pBl19, pLA1060, and pAMB1 permitting replication in Bacillus. Examples of origins of replication for use in a yeast host cell are the 2 micron origin of replication, ARS1, ARS4, the combination of ARS1 and CEN3, and the combination of ARS4 and CEN6. The origin of replication may be one having a mutation which makes its functioning temperature-sensitive in the host cell (see, e.g., Ehrlich, 1978, Proceedings of the National Academy of Sciences USA 75: 1433).

[0093] More than one copy of a nucleic acid sequence of the present invention may be inserted into the host cell to increase the production of the gene product. An increase in the copy number of the nucleic acid sequence can be obtained by integrating at least one additional copy of the sequence into the host cell genome or by including an amplifiable selectable marker gene with the nucleic acid sequence where cells containing amplified copies of the selectable marker gene, and thereby additional copies of the nucleic acid sequence, can be selected for by cultivating the cells in the presence of the appropriate selectable agent.

[0094] The procedures used to ligate the elements described above to construct the recombinant expression vectors of the present invention are well known to one skilled in the art (see, e.g., Sambrook et al., 1989, supra).

Host Cells

[0095] The present invention also relates to recombinant host cells, comprising a nucleic acid sequence of the invention, which are advantageously used in the recombinant production of the polypeptides. A vector comprising a nucleic acid sequence of the present invention is introduced into a host cell so that the vector is maintained as a chromosomal integrant or as a self-replicating extra-chromosomal vector as described earlier. The term “host cell” encompasses any progeny of a parent cell that is not identical to the parent cell due to mutations that occur during replication. The choice of a host cell will to a large extent depend upon the gene encoding the polypeptide and its source.

[0096] The host cell may be a unicellular microorganism, e.g., a prokaryote, or a non-unicellular microorganism, e.g., a eukaryote.

[0097] Useful unicellular bacteria are bacterial cells such as gram-positive bacteria including, but not limited to, a Bacillus cell, e.g., Bacillus halophilus, Bacillus anolytiquefaciens, Bacillus brevis, Bacillus circulans, Bacillus clausii, Bacillus coagulans, Bacillus lautus, Bacillus lentus, Bacillus licheniformis, Bacillus megaterium, Bacillus steaerothermophilus, Bacillus subtilis, and Bacillus thuringiensis, or a Streptomyces cell, e.g., Streptomyces lividus or Streptomyces marinus, or gram-negative bacteria such as E. coli and Pseudomonas sp. In a preferred embodiment, the bacterial host cell is a Bacillus lentus, Bacillus licheniformis, Bacillus steaerothermophilus or Bacillus subtilis cell. In another preferred embodiment, the Bacillus cell is an alkalophilic Bacillus.


Methods of Production

[0099] The present invention also relates to methods for producing an alpha-amylase of the present invention comprising (a) cultivating a strain, which in its wild-type form is capable of producing the polypeptide, to produce a supernatant comprising the polypeptide; and (b) recovering the polypeptide. Preferably, the strain is of the genus Bacillus sp.

[1000] The present invention also relates to methods for producing an alpha-amylase of the present invention comprising (a) cultivating a host cell under conditions conducive for production of the polypeptide; and (b) recovering the polypeptide.

[1001] The present invention also relates to methods for producing an alpha-amylase of the present invention comprising (a) cultivating a host cell under conditions conducive for production of the polypeptide, wherein the host cell comprises a mutant nucleic acid sequence having at least one mutation in the mature polypeptide coding region of SEQ ID NO: 23 or SEQ ID NO: 25, wherein the mutant nucleic acid sequence encodes a polypeptide which consists of amino acids 1 to 485 of SEQ ID NO: 24 or SEQ ID NO: 26, and (b) recovering the polypeptide.
Mutant Alpha-Amylase

[0102] The present invention also relates to alpha-amylase mutants.

The Termamyl-Like Alpha-Amylase

[0103] It is well known that a number of alpha-amylases produced by *Bacillus* spp. are highly homologous on the amino acid level. For instance, the *B. licheniformis* alpha-amylase comprising the amino acid sequence shown in SEQ ID NO: 4 (commercially available as Termamyl™) has been found to be about 89% homologous with the *B. amyloliquescens* alpha-amylase comprising the amino acid sequence shown in SEQ ID NO: 5 and about 79% homologous with the *B. steaithermophila* alpha-amylase comprising the amino acid sequence shown in SEQ ID NO: 3. Further homologous alpha-amylases include an alpha-amylase derived from a strain of the *Bacillus* sp. NCIB 12289, NCIB 12512, NCIB 12513 or DSM 9375, all of which are described in detail in WO 95/26397, and the alpha-amylase described by Tsukamoto et al., Biochemical and Biophysical Research Communications, 151 (1988), pp. 25-31. Also the novel alpha-amylase of the invention, of which a specific embodiment is shown in SEQ ID NO: 24 (and SEQ ID NO: 26), is contemplated as the parent alpha-amylase to be mutated according to the invention.

[0104] Still further homologous alpha-amylases include the alpha-amylase produced by the *B. licheniformis* strain described in EP 0252666 (ATCC 27811), and the alpha-amylases identified in WO 91/00353 and WO 94/18314. Other commercial Termamyl-like *B. licheniformis* alpha-amylases are Duramyl™ from Novo Nordisk, Optitherm™ and Takatherm™ (available from Solvay), Maxamyl™ (available from Gist-brocades/Genencor), Spezyme AA™ and Spezyme Delta AA™ (available from Genencor), and Keistase™ (available from Daiwa).

[0105] Because of the substantial homology found between these alpha-amylases, they are considered to belong to the same class of alpha-amylases, namely the class of “Termamyl-like alpha-amylases”.

[0106] Accordingly, in the present context, the term “Termamyl-like alpha-amylase” is intended to indicate an alpha-amylase which, at the amino acid level, exhibits a substantial homology to Termamyl™, i.e., the *B. licheniformis* alpha-amylase having the amino acid sequence shown in SEQ ID NO: 4 herein. In other words, a Termamyl-like alpha-amylase is an alpha-amylase which has the amino acid sequence shown in SEQ ID NOS: 1, 2, 3, 4, 5, 6, 7 or 8 herein, and the amino acid sequence shown in SEQ ID NO: 1 of WO 95/26397 (the same as the amino acid sequence shown as SEQ ID NO: 7 herein) or in SEQ ID NO: 2 of WO 95/26397 (the same as the amino acid sequence shown as SEQ ID NO: 8 herein) or in Tsukamoto et al., 1988, (which amino acid sequence is shown in SEQ ID NO: 6 herein) or i) which displays at least 60%, preferred at least 70%, more preferred at least 75%, even more preferred at least 80%, especially at least 85%, especially preferred at least 90%, even especially more preferred at least 95% homology with at least one of said amino acid sequences shown in SEQ ID NOS: 1 or 2 or 3 or 4 or 5 or 6 or 7 or 8 and/or ii) displays immunological cross-reactivity with an antibody raised against at least one of said alpha-amylases, and/or iii) is encoded by a DNA sequence which hybridizes to the DNA sequences encoding the above-specified alpha-amylases which are apparent from SEQ ID NOS: 9, 10, 11, 12 or 13 of the present application (which encoding sequences encode the amino acid sequences shown in SEQ ID NOS: 1, 2, 3, 4 and 5 herein, respectively), from SEQ ID NO: 4 of WO 95/26397 (which DNA sequence, together with the stop codon TAA, is shown in SEQ ID NO: 14 herein and encodes the amino acid sequence shown in SEQ ID NO: 8 herein) and from SEQ ID NO: 5 of WO 95/26397 (shown in SEQ ID NO: 15 herein), respectively.

[0107] In connection with property i), the “homology” may be determined by use of any conventional algorithm, preferably by use of the GAP programme from the GCG package version 7.3 (June 1993) using default values for GAP penalties, which is a GAP creation penalty of 3.0 and GAP extension penalty of 0.1. Genetic Computer Group (1991) Programme Manual for the GCG Package, version 7, 575 Science Drive, Madison, Wis., USA 53711.

[0108] A structural alignment between Termamyl and a Termamyl-like alpha-amylase may be used to identify equivalent/corresponding positions in other Termamyl-like alpha-amylases. One method of obtaining said structural alignment is to use the Pile Up programme from the GCG package using default values of gap penalties, i.e., a gap creation penalty of 3.0 and gap extension penalty of 0.1. Other structural alignment methods include the hydrophobic cluster analysis (Gaboriaud et al., 1987, FEBS Letters 224:149-155) and reverse threading (Huber and Torda, 1998, Protein Science 7(1): 142-149).

[0109] Property ii) of the alpha-amylase, i.e., the immunological cross-reactivity, may be assayed using an antibody raised against, or reactive with, at least one epitope of the relevant Termamyl-like alpha-amylase. The antibody, which may either be monoclonal or polyclonal, may be produced by methods known in the art, e.g., as described by Hudson et al., Practical Immunology, Third edition (1989), Blackwell Scientific Publications. The immunological cross-reactivity may be determined using assays known in the art, examples of which are Western Blotting or radial immunodiffusion assay, e.g., as described by Hudson et al., 1989. In this respect, immunological cross-reactivity between the alpha-amylases having the amino acid sequences SEQ ID NO: 1, 2, 3, 4, 5, 6, 7, or 8 respectively, have been found.

[0110] The oligonucleotide probe used in the characterization of the Termamyl-like alpha-amylase in accordance with property iii) above may suitably be prepared on the basis of the full or partial nucleotide or amino acid sequence of the alpha-amylase in question.

[0111] Suitable conditions for testing hybridization involve presoaking in 5xSSC and prehybridizing for 1 hour at ~40°C in a solution of 20% formamide, 5xDenhardt’s solution, 50 mM sodium phosphate, pH 6.8, and 50 mg of denatured sonicated calf thymus DNA, followed by hybridization in the same solution supplemented with 100 mM ATP for 18 hours at ~40°C, followed by three times washing of the filter in 2xSSC, 0.2% SDS at 40°C for 30 minutes (low stringency), preferred at 50°C (medium stringency), more preferably at 65°C (high stringency), even more preferably at ~75°C (very high stringency). More details about the hybridization method can be found in Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor, 1989.

[0112] In the present context, “derived from” is intended not only to indicate an alpha-amylase produced or producible by a strain of the organism in question, but also an alpha-amylase encoded by a DNA sequence isolated from such strain and produced in a host organism transformed with said
DNA sequence. Finally, the term is intended to indicate an alpha-amylase which is encoded by a DNA sequence of synthetic and/or cDNA origin and which has the identifying characteristics of the alpha-amylase in question. The term is also intended to indicate that the parent alpha-amylase may be a variant of a naturally occurring alpha-amylase, i.e., a variant which is the result of a modification (insertion, substitution, deletion) of one or more amino acid residues of the naturally occurring alpha-amylase.

Parent Hybrid Alpha-Amylases

[0113] The parent alpha-amylase may be a hybrid alpha-amylase, i.e., an alpha-amylase which comprises a combination of partial amino acid sequences derived from at least two alpha-amylases.

[0114] The parent hybrid alpha-amylase may be one which on the basis of amino acid homology and/or immunological cross-reactivity and/or DNA hybridization (as defined above) can be determined to belong to the Termamyl-like alpha-amylase family. In this case, the hybrid alpha-amylase is typically composed of at least one part of a Termamyl-like alpha-amylase and part(s) of one or more other alpha-amylases selected from Termamyl-like alpha-amylases or non-Termamyl-like alpha-amylases of microbial (bacterial or fungal) and/or mammalian origin.

[0115] Thus, the parent hybrid alpha-amylase may comprise a combination of partial amino acid sequences deriving from at least two Termamyl-like alpha-amylases, or from at least one Termamyl-like and at least one non-Termamyl-like bacterial alpha-amylase, or from at least one Termamyl-like and at least one fungal alpha-amylase. The Termamyl-like alpha-amylase from which a partial amino acid sequence derives may, e.g., be any of those specific Termamyl-like alpha-amylases referred to herein.

[0116] For instance, the parent alpha-amylase may comprise a C-terminal part of an alpha-amylase derived from a strain of B. licheniformis, and a N-terminal part of an alpha-amylase derived from a strain of B. amyoliquefaciens or from a strain of B. stearothermophilus. For instance, the parent alpha-amylase may comprise at least 430 amino acid residues of the C-terminal part of the B. licheniformis alpha-amylase, and may, e.g., comprise a) an amino acid segment corresponding to the 37 N-terminal amino acid residues of the B. amyoliquefaciens alpha-amylase having the amino acid sequence shown in SEQ ID NO: 5 and an amino acid segment corresponding to the 445 C-terminal amino acid residues of the B. licheniformis alpha-amylase having the amino acid sequence shown in SEQ ID NO: 4, or b) an amino acid segment corresponding to the 68 N-terminal amino acid residues of the B. stearothermophilus alpha-amylase having the amino acid sequence shown in SEQ ID NO: 3 and an amino acid segment corresponding to the 415 C-terminal amino acid residues of the B. licheniformis alpha-amylase having the amino acid sequence shown in SEQ ID NO: 4.

[0117] The non-Termamyl-like alpha-amylase may, e.g., be a fungal alpha-amylase, a mammalian or a plant alpha-amylase or a bacterial alpha-amylase (different from a Termamyl-like alpha-amylase). Specific examples of such alpha-amylases include the Aspergillus oryzae TAKA alpha-amylase, the A. niger acid alpha-amylase, the Bacillus subtilis alpha-amylase, the porcine pancreatic alpha-amylase and a barley alpha-amylase. All of these alpha-amylases have elucidated structures which are markedly different from the structure of a typical Termamyl-like alpha-amylase as referred to herein.

[0118] The fungal alpha-amylases mentioned above, i.e., derived from A. niger and A. oryzae, are highly homologous on the amino acid level and generally considered to belong to the same family of alpha-amylases. The fungal alpha-amylase derived from Aspergillus oryzae is commercially available under the tradename Fungamyl™.

[0119] Furthermore, when a particular variant of a Termamyl-like alpha-amylase (variant of the invention) is referred to—in a conventional manner—by reference to modification (e.g., deletion or substitution) of specific amino acid residues in the amino acid sequence of a specific Termamyl-like alpha-amylase, it is to be understood that variants of another Termamyl-like alpha-amylase modified in the equivalent position(s) (as determined from the best possible amino acid sequence alignment between the respective amino acid sequences) are encompassed thereby.

[0120] A preferred embodiment of a variant of the invention is one derived from a B. licheniformis alpha-amylase (as parent Termamyl-like alpha-amylase), e.g., one of those referred to above, such as the B. licheniformis alpha-amylase having the amino acid sequence shown in SEQ ID NO: 4.

Construction of Variants of the Invention

[0121] The construction of the variant of interest may be accomplished by cultivating a microorganism comprising a DNA sequence encoding the variant under conditions which are conducive for producing the variant. The variant may then subsequently be recovered from the resulting culture broth. This is described in detail further below.

Altered Properties of Variants of the Invention

[0122] The following describes the relationship between mutations which may be present in variants of the invention, and desirable alterations in properties (relative to those a parent, Termamyl-like alpha-amylase) which may result therefrom.

Increased Thermostability at Acidic pH and/or at Low Ca²⁺ Concentration

[0123] Mutations of particular relevance in relation to obtaining variants according to the invention having increased thermostability at acidic pH and/or at low Ca²⁺ concentration include mutations at the following positions (relative to B. licheniformis alpha-amylase, SEQ ID NO: 4): H156, N172, A181, N188, N190, H205, D207, A209, A210, E211, Q264, N265.

[0124] In the context of the invention the term “acidic pH” means a pH below 7.0, especially below the pH range, in which industrial starch liquefaction processes are normally performed, which is between pH 5.5 and 6.2.

[0125] In the context of the present invention the term “low Calcium concentration” means concentrations below the normal level used in industrial starch liquefaction. Normal concentrations vary depending on the concentration of free Ca²⁺ in the corn. Normally a dosage corresponding to 1 mM (40 ppm) is added which together with the level in corn gives between 40 and 60 ppm free Ca²⁺.

[0126] In the context of the invention the term “high temperatures” means temperatures between 95°C. and 160°C.,
especially the temperature range in which industrial starch liquefaction processes are normally performed, which is between 95° C. and 105° C.

[0127] The inventors have now found that the thermostability at acidic pH and/or at low Ca++ concentration may be increased even more by combining certain mutations including the above mentioned mutations and/or 1201 with each other.

[0128] Said "certain" mutations are the following (relative to B. licheniformis alpha-amylase, SEQ ID NO: 4):

N190, D207, E211, Q264 and 1201.

[0129] Said mutation may further be combined with deletions in one, preferably two or even three positions as described in WO 96/23873 (i.e., in positions R181, G182, T183, G184 in SEQ ID NO: 1 herein). According to the invention variants of a parent Termamyl-like alpha-amylase with alpha-amylase activity comprising mutations in two, three, four, five or six of the above positions are contemplated.

[0130] It should be emphasized that not only the Termamyl-like alpha-amylases mentioned specifically below are contemplated. Also other commercial Termamyl-like alpha-amylases are contemplated. An unexhaustive list of such alpha-amylases is the following: alpha-amylases produced by the B. licheniformis strain described in EP 0252666 (ATCC 27811) and the alpha-amylases identified in WO 91/00353 and WO 94/18314. Other commercial Termamyl-like B. licheniformis alpha-amylases are Optitherm™ and Takatherm™ (available from Solvay), Maxamyl™ (available from Gist-brocades/Genencor), Spezyme AA™ Spezyme Delta AA™ (available from Genencor), and Keistase™ (available from Daiwa).

[0131] It may be mentioned here that amino acid residues, respectively, at positions corresponding to N190, I201, D207 and E211, respectively, in SEQ ID NO: 4 constitute amino acid residues which are conserved in numerous Termamyl-like alpha-amylases. Thus, for example, the corresponding positions of these residues in the amino acid sequences of a number of Termamyl-like alpha-amylases which have already been mentioned (vide supra) are as follows:

<table>
<thead>
<tr>
<th>Termmamyl-like alpha-amylase</th>
<th>Pair-wise amino acid deletions among</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. amylobioferaciens (SEQ ID NO: 5)</td>
<td>R176, G177, E178, G179</td>
</tr>
<tr>
<td>B. stearothermophilus (SEQ ID NO: 2)</td>
<td>R179, G180, I181, G182</td>
</tr>
<tr>
<td>Bacillus WO 95/26397 (SEQ ID NO: 2)</td>
<td>R181, G182, T183, G184</td>
</tr>
<tr>
<td>Bacillus WO 95/26397 (SEQ ID NO: 1)</td>
<td>R181, G182, D183, G184</td>
</tr>
<tr>
<td>Bacillus sp. #707™ (SEQ ID NO: 6)</td>
<td>R181, G182, H183, G184</td>
</tr>
<tr>
<td>Bacillus sp. (AA560) (SEQ ID NO: 24)</td>
<td>R181, G182, H183, G184</td>
</tr>
</tbody>
</table>

[0134] When using SEQ ID NO: 1-6 or SEQ ID NO: 24 (or SEQ ID NO: 26) as the backbone (i.e., as the parent Termamyl-like alpha-amylase) two, three, four, five or six mutations may according to the invention be made in the following regions/positions to increase the thermostability at acidic pH and/or at low Ca ++ concentrations (relative to the parent):

1: R181*, G182*, T183*, G184*

[0135] (relative to SEQ ID NO: 2 herein):

1: R181*, G182*, D183*, G184*

[0132] Mutations of these conserved amino acid residues are very important in relation to improving thermostability at acidic pH and/or at low calcium concentration, and the following mutations are of particular interest in this connection (with reference to the numbering of the B. licheniformis amino acid sequence shown in SEQ ID NO: 4).
[0136] (Relative to SEQ ID NO: 3 herein):

1: R179*,G180,J181*,G182*


[0137] Relative to SEQ ID NO: 4 herein):

1: Q178*,G179*


[0138] (relative to SEQ ID NO: 5 herein):

1: R176*,G177*,E178,G179*


[0139] (relative to SEQ ID NO: 6 herein):

1: R181*,G182*,H183*,G184*


[0140] (relative to SEQ ID NO: 24):

1: R181*,G182*,H183*,G184*


[0141] Contemplated according to the present invention is combining three, four, five or six mutation.

[0142] Specific double mutations for backbone SEQ ID NO: 1-6 and SEQ ID NO: 24 and SEQ ID NO: 26 are listed in the following.

[0143] Using SEQ ID NO: 1 as the backbone the following double mutations are contemplated according to the invention:


Using SEQ ID NO: 2 as the backbone the following double mutations are contemplated according to the invention:

P,S,T;T,W,Y;  
P,S,T;T,W,Y;  
P,S,T;T,W,Y;  
P,S,T;T,W,Y;  
P,S,T;T,W,Y;  
P,S,T;T,W,Y;  
P,S,T;T,W,Y;  
P,S,T;T,W,Y;  
P,S,T;T,W,Y;  
P,S,T;T,W,Y;  
P,S,T;T,W,Y;  
P,S,T;T,W,Y;  
P,S,T;T,W,Y;  
P,S,T;T,W,Y;  
P,S,T;T,W,Y;  
P,S,T;T,W,Y;  
P,S,T;T,W,Y;  
P,S,T;T,W,Y;  
P,S,T;T,W,Y;  
P,S,T;T,W,Y;  
P,S,T;T,W,Y;  
P,S,T;T,W,Y;  
P,S,T;T,W,Y;  
P,S,T;T,W,Y;  
P,S,T;T,W,Y;  
P,S,T;T,W,Y;  
P,S,T;T,W,Y;  
P,S,T;T,W,Y;  
P,S,T;T,W,Y;  
P,S,T;T,W,Y;  
P,S,T;T,W,Y;  
P,S,T;T,W,Y;  
P,S,T;T,W,Y;  
P,S,T;T,W,Y;  
P,S,T;T,W,Y;  
P,S,T;T,W,Y;  
P,S,T;T,W,Y;  
P,S,T;T,W,Y;  
P,S,T;T,W,Y;  
P,S,T;T,W,Y;  
P,S,T;T,W,Y;  
P,S,T;T,W,Y;  
P,S,T;T,W,Y;  
P,S,T;T,W,Y;  
P,S,T;T,W,Y;  
P,S,T;T,W,Y;  
P,S,T;T,W,Y;  
P,S,T;T,W,Y;  
P,S,T;T,W,Y;  
P,S,T;T,W,Y;  
P,S,T;T,W,Y;  
P,S,T;T,W,Y;  
P,S,T;T,W,Y;  
P,S,T;T,W,Y;  
P,S,T;T,W,Y;  
P,S,T;T,W,Y;  
P,S,T;T,W,Y;  
P,S,T;T,W,Y;  
P,S,T;T,W,Y;  
P,S,T;T,W,Y;  
P,S,T;T,W,Y;
Using SEQ ID NO: 24 as the backbone the following double mutations are contemplated according to the invention:


In another embodiment the variant of the invention comprises the following mutations: I181*/G182*/N193F in SEQ ID NO: 3 (TVB146) or in corresponding positions in another parent Termamyl-like alpha-amylases. Said variant may further comprise a substitution in position E214Q.

In a preferred embodiment the parent Termamyl-like alpha-amylase is a hybrid alpha-amylase of SEQ ID NO: 4 and SEQ ID NO: 5. Specifically, the parent hybrid Termamyl-like alpha-amylase may be a hybrid alpha-amylase comprising the 445 C-terminal amino acid residues of the B. licheniformis alpha-amylase shown in SEQ ID NO: 4 and the 39 N-terminal amino acid residues of the alpha-amylase derived from B. amylophilus strain shown in SEQ ID NO: 5, which may suitably further have the following mutations: H156Y+A181T+N190F+A209V+Q264S (using the numbering in SEQ ID NO: 4). The latter mentioned hybrid is used in the examples below and is referred to as LE174.

General Mutations of the Invention

- It may be preferred that a variant of the invention comprises one or more modifications in addition to those outlined above. Thus, it may be advantageous that one or more proline residues present in the part of the alpha-amylase variant which is modified is/are replaced with a non-proline residue which may be any of the possible, naturally occurring non-proline residues, and which preferably is an alanine, glycine, serine, threonine, valine or leucine.

- Analogously, it may be preferred that one or more cysteine residues present among the amino acid residues with which the parent alpha-amylase is modified is/are replaced with a non-cysteine residue such as serine, alanine, threonine, glycine, valine or leucine.

- Furthermore, a variant of the invention may—either as the only modification or in combination with any of the above outlined modifications—be modified so that one or more Asp and/or Glu present in an amino acid fragment corresponding to the amino acid fragment 185-209 of SEQ ID NO: 4 is replaced by an Asn and/or Gin, respectively. Also of interest is the replacement, in the Termamyl-like alpha-amylase, of one or more of the Lys residues present in an amino acid fragment corresponding to the amino acid fragment 185-209 of SEQ ID NO: 4 by an Arg.

- It will be understood that the present invention encompasses variants incorporating two or more of the above outlined modifications.

- Furthermore, it may be advantageous to introduce point-mutations in any of the variants described herein.

Cloning a DNA Sequence Encoding an Alpha-Amylase

- 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, homologous, labelled oligonucleotide 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 a known alpha-amylase gene could be used as a probe to iden-
[0369] 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, thereby allowing clones expressing the alpha-amylase to be identified.

[0370] Alternatively, the DNA sequence encoding the enzyme may be prepared synthetically by established standard methods, e.g., the phosphoramidite method described by Beaucage and Caruthers (1981) or the method described by Matthes et al. (1984). In the phosphoramidite method, oligonucleotides are synthesized, e.g., in an automatic DNA synthesizer, purified, annealed, ligated and cloned in appropriate vectors.

[0371] 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 U.S. Pat. No. 4,683,202 or Saiki et al. (1988).

**Site-Directed Mutagenesis**

[0372] Once an 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; mutant nucleotides are inserted during oligonucleotide synthesis. In a specific method, a single-stranded gap of DNA, bridging 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). U.S. Pat. No. 4,760,025 discloses 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.

[0373] Another method for introducing mutations into alpha-amylase-encoding DNA sequences is described in Nelson and Long (1989). 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.

**Random Mutagenesis**

[0374] Random mutagenesis is suitably performed either as localised or region-specific random mutagenesis in at least three parts of the gene translating to the amino acid sequence shown in question, or within the whole gene.

[0375] The random mutagenesis of a DNA sequence encoding a parent alpha-amylase may be conveniently performed by use of any method known in the art.

[0376] In relation to the above, a further aspect of the present invention relates to a method for generating a variant of a parent alpha-amylase, e.g., wherein the variant exhibits altered or increased thermal stability relative to the parent, the method comprising:

[0377] (a) subjecting a DNA sequence encoding the parent alpha-amylase to random mutagenesis,

[0378] (b) expressing the mutated DNA sequence obtained in step (a) in a host cell, and

[0379] (c) screening for host cells expressing an alpha-amylase variant which has an altered property (i.e., thermal stability) relative to the parent alpha-amylase.

[0380] Step (a) of the above method of the invention is preferably performed using doped primers.

[0381] For instance, the random mutagenesis may be performed by use of a suitable physical or chemical mutagenizing agent, by use of a suitable oligonucleotide, or by subjecting the DNA sequence to PCR generated mutagenesis. Furthermore, the random mutagenesis may be performed by use of any combination of these mutagenizing agents. The mutagenizing agent may, e.g., be one which induces transitions, transversions, inversions, scrambling, deletions, and/or insertions.

[0382] Examples of a physical or chemical mutagenizing agent suitable for the present purpose include ultraviolet (UV) ir-radiation, hydroxylamine, N-methyl-N-nitro-N-nitrosoguanidine (MNNG), O-methyl hydroxylamine, nitrous acid, ethyl methane sulphonate (EMS), sodium bisulphite, formic acid, and nucleotide analogues. When such agents are used, the mutagenesis is typically performed by incubating the DNA sequence encoding the parent enzyme to be mutagenized in the presence of the mutagenizing agent of choice under suitable conditions for the mutagenesis to take place, and selecting for mutated DNA having the desired properties.

[0383] When the mutagenesis is performed by the use of an oligonucleotide, the oligonucleotide may be doped or spiked with the three non-parent nucleotides during the synthesis of the oligonucleotide at the positions which are to be changed. The doping or spiking may be done so that codons for unwanted amino acids are avoided. The doped or spiked oligonucleotide can be incorporated into the DNA encoding the alpha-amylase enzyme by any published technique, using e.g., PCR, LCR or any DNA polymerase and ligase as deemed appropriate.

[0384] Preferably, the doping is carried out using “constant random doping”, in which the percentage of wild-type and mutation in each position is predefined. Furthermore, the doping may be directed toward a preference for the introduction of certain nucleotides, and thereby a preference for the introduction of one or more specific amino acid residues. The doping may be made, e.g., so as to allow for the introduction of 90% wild type and 10% mutations in each position. An additional consideration in the choice of a doping scheme is based on genetic as well as protein-structural constraints. The doping scheme may be made by using the DOPE program which, inter alia, ensures that introduction of stop codons is avoided.
When PCR-generated mutagenesis is used, either a chemically treated or non-treated gene encoding a parent alpha-amylase is subjected to PCR under conditions that increase the mis-incorporation of nucleotides (Deshler 1992; Leung et al., Technique, Vol. 1, 1989, pp. 11-15).

A mutator strain of E. coli (Fowler et al., 1974, Molec. Gen. Genet. 133: 179-191), S. cerevisiae or any other microbial organism may be used for the random mutagenesis of the DNA encoding the alpha-amylase by, e.g., transforming a plasmid containing the parent glycosylase into the mutator strain, growing the mutator strain with the plasmid and isolating the mutated plasmid from the mutator strain. The mutated plasmid may be subsequently transformed into the expression organism.

The DNA sequence to be mutagenized may be conveniently present in a genomic or cDNA library prepared from an organism expressing the parent alpha-amylase. Alternatively, the DNA sequence may be present on a suitable vector such as a plasmid or a bacteriophage, which as such may be incubated with or other-wise exposed to the mutagenizing agent. The DNA to be mutagenized may also be present in a host cell either by being integrated in the genome of said cell or by being present on a vector harboured in the cell. Finally, the DNA to be mutagenized may be in isolated form. It will be understood that the DNA sequence to be subjected to random mutagenesis is preferably a cDNA or a genomic DNA sequence.

In some cases it may be convenient to amplify the mutated DNA sequence prior to performing the expression step b) or the screening step c). Such amplification may be performed in accordance with methods known in the art, the presently preferred method being PCR-generated amplification using oligonucleotide primers prepared on the basis of the DNA or amino acid sequence of the parent enzyme.

Subsequent to the incubation with or exposure to the mutagenizing agent, the mutated DNA is expressed by culturing a suitable host cell carrying the DNA sequence under conditions allowing expression to take place. The host cell used for this purpose may be one which has been transformed with the mutated DNA sequence, optionally present on a vector, or one which was carried the DNA sequence encoding the parent enzyme during the mutagenesis treatment.

Examples of suitable host cells are the following: gram positive bacteria such as Bacillus subtilis, Bacillus licheniformis, Bacillus lentus, Bacillus brevis, Bacillus stearothermophilus, Bacillus alkalophilus, Bacillus amyloliqufaciens, Bacillus coagulans, Bacillus circulans, Bacillus latus, Bacillus megaterium, Bacillus thermyndemans, Streptomyces lividans or Streptomyces marinus; and gram-negative bacteria such as E. coli.

The mutated DNA sequence may further comprise a DNA sequence encoding functions permitting expression of the mutated DNA sequence.

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.

Alternative Methods of Providing Alpha-Amylase Variants

Alternative methods for providing variants of the invention include gene shuffling method known in the art including the methods, e.g., described inWO95/22625 (from Affymax Technologies N.V.) andWO 96/00843 (from Novo Nordisk A/S).

Expression of Alpha-Amylase Variants

According to the invention, a DNA sequence encoding the variant produced by methods described above, or by any alternative methods known in the art, can be expressed, in enzyme form, using an expression vector which typically includes control sequences encoding a promoter, operator, ribosome binding site, translation initiation signal, and, optionally, a repressor gene or various activator genes.

The recombinant expression vector carrying the DNA sequence encoding an alpha-amylase variant of the invention may be any vector which may conveniently be subjected to recombinant DNA procedures, and the choice of vector will often depend on the host cell into which it is to be introduced. Thus, 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, a bacteriophage or an extrachromosomal element, minichromosome or an artificial chromosome. Alternatively, the vector may be one which, when introduced into a host cell, is integrated into the host cell genome and replicated together with the chromosome(s) into which it has been integrated.

In the vector, the DNA sequence should be operably connected to a suitable promoter sequence. The promoter may be any DNA sequence which shows transcriptional activity in the host cell of choice and may be derived from genes encoding proteins either homologous or heterologous to the host cell. Examples of suitable promoters for directing the transcription of the DNA sequence encoding an alpha-amylase variant of the invention, especially in a bacterial host, are the promoter of the lac operon ofE. coli, the Streptomyces coelicolor agarase gene dag A promoters, the promoters of the Bacillus licheniformis alpha-amylase gene (amyL), the promoters of the Bacillus stearothermophilus maltogenic amylase gene (amyM), the promoters of the Bacillus amyloliquefaciens alpha-amylase (amyQ), the promoters of the Bacillus subtilis xylA and xylB genes etc. For transcription in a fungal host, examples of useful promoters are those derived from the gene encoding A. oryzae TAKA amylase, Rhizomucor miehei aspartic protease, A. niger neutral alpha-amylase, A. niger acid stable alpha-amylase, A. niger glucoamylase, Rhizomucor miehei lipase, A. oryzae alkaline protease, A. oryzae triose phosphate isomerase or A. nidulans acetamidase.

The expression vector of the invention may also comprise a suitable transcription terminator and, in eukaryotes, polyadenylation sequences operably connected to the DNA sequence encoding the alpha-amylase variant of the
invention. Termination and polyadenylation sequences may suitably be derived from the same sources as the promoter. Termination and polyadenylation sequences may suitably be derived from the same sources as the promoter.

0398. The vector may further comprise a DNA sequence enabling the vector to replicate in the host cell in question. Examples of such sequences are the origins of replication of plasmids pUC19, pACYC177, pUB110, pL194, pAMBl and pL702.

0399. The vector may also comprise a selectable marker, e.g., a gene the product of which complements a defect in the host cell, such as the dal genes from B. subtilis or B. licheniformis, or one which confers antibiotic resistance such as ampicillin, kanamycin, chloramphenicol or tetracyclin resistance. Furthermore, the vector may comprise Aspergillus selection markers such as amdS, argB, niaD and suc, a marker giving rise to hygromycin resistance, or the selection may be accomplished by co-transformation, e.g., as described in WO 91/17243.

0400. While intracellular expression may be advantageous in some respects, e.g., when using certain bacteria as host cells, it is generally preferred that the expression is extracellular. In general, the Bacillus alpha-amylases mentioned herein comprise a prerogation permitting secretion of the expressed protease into the culture medium. If desirable, this prerogation may be replaced by a different prerogation or signal sequence, conveniently accomplished by substitution of the DNA sequences encoding the respective prerogations.

0401. The procedures used to ligate the DNA construct of the invention encoding an alpha-amylase variant, the promoter, terminator and other elements, respectively, and to insert them into suitable vectors containing the information necessary for replication, are well known to persons skilled in the art (cf., for instance, Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor, 1989).

0402. The cell of the invention, either comprising a DNA construct or an expression vector of the invention as defined above, is advantageously used as a host cell in the recombinant production of an alpha-amylase variant of the invention. The cell may be transformed with the DNA construct of the invention encoding the variant, conveniently by integrating the DNA construct (in one or more copies) in the host chromosome. This integration is generally considered to be an advantage as the DNA sequence is more likely to be stably maintained in the cell. Integration of the DNA constructs into the host chromosome can be performed according to conventional methods, e.g., by homologous or heterologous recombination. Alternatively, the cell may be transformed with an expression vector as described above in connection with the different types of host cells.

0403. The cell of the invention may be a cell of a higher organism such as a mammal or an insect, but is preferably a microbial cell, e.g., a bacterial or a fungal (including yeast) cell.

0404. Examples of suitable bacteria are gram-positive bacteria such as Bacillus subtilis, Bacillus licheniformis, Bacillus lentus, Bacillus brevis, Bacillus steatorrhophilus, Bacillus alcalophilus, Bacillus ammoniaceus, Bacillus coagulans, Bacillus circulans, Bacillus lautus, Bacillus megaterium, Bacillus thuringiensis, or Streptomyces lividans or Streptomyces marinus, or gram-negative bacteria such as E. coli. The transformation of the bacteria may, for instance, be effected by protoplast transformation or by using competent cells in a manner known per se.

0405. The yeast organism may favourably be selected from a species of Saccharomyces or Schizosaccharomyces, e.g., Saccharomyces cerevisiae. The filamentous fungus may advantageously belong to a species of Aspergillus, e.g., Aspergillus oryae or Aspergillus niger. Fungal cells may be transformed by a process involving protoplast formation and transformation of the protoplasts followed by regeneration of the cell wall in a manner known per se. A suitable procedure for transformation of Aspergillus host cells is described in EP 238 023.

0406. In yet a further aspect, the present invention relates to a method of producing an alpha-amylase variant of the invention, which method comprises cultivating a host cell as described above under conditions conducive to the production of the variant and recovering the variant from the cells and/or culture medium.

0407. The medium used to cultivate the cells may be any conventional medium suitable for growing the host cell in question and obtaining expression of the alpha-amylase variant of the invention. Suitable media are available from commercial suppliers or may be prepared according to published recipes (e.g., as described in catalogues of the American Type Culture Collection).

0408. The alpha-amylase variant secreted from the host cells may conveniently be recovered from the culture medium by well-known procedures, including separating the cells from the medium by centrifugation or filtration, and precipitating proteinaceous components of the medium by means of a salt such as ammonium sulphate, followed by the use of chromatographic procedures such as ion exchange chromatography, affinity chromatography, or the like.

Compositions

0409. In a still further aspect, the present invention relates to compositions comprising an alpha-amylase or alpha-amylase variant of the present invention. Preferably, the compositions are enriched in an alpha-amylase or alpha-amylase variant of the present invention. In the present context, the term “enriched” indicates that the alpha-amylase activity of the composition has been increased, e.g., with an enrichment factor of 1.1.

0410. The composition may comprise an alpha-amylase or alpha-amylase variant of the invention as the major enzymatic component, e.g., a mono-component composition. Alternatively, the composition may comprise multiple enzymatic activities, such as an amylase, amylase, carbohydrase, carboxypeptidase, catalase, cellulase, chitinase, cutinase, cyclodextrin glycosyltransferase, dexxyribonucleic, esterase, alpha-galactosidase, beta-galactosidase, glucomylase, alpha-glucosidase, beta-glucosidase, haloperoxidase, invertase, lactase, lipase, mannosidase, oxidase, pectinolytic enzyme, peptidoglucanase, peroxidase, phytase, polyphenoloxidase, proteolytic enzyme, ribonuclease, transglutaminase, or xylanase. The additional enzyme(s) may be producible by means of a microorganism belonging to the genus Aspergillus, preferably Aspergillus aculeatus, Aspergillus awamori, Aspergillus niger, or Aspergillus oryae, or Trichoderma, Humicola, preferably Humicola insolens, or Fusarium, preferably Fusarium barioides, Fusarium cerealis, Fusarium crookwellense, Fusarium culmorum, Fusarium graminearum, Fusarium graminearum, Fusarium heterosporum, Fusarium neogendii, Fusarium oxysporum, Fusarium reticulatum, Fusarium roseum, Fusarium sambucinum, Fusarium saccarophorum, Fusarium sulphureum, Fusarium torulosum, Fusarium trichothecoides, or Fusarium venenatum.
The alpha-amylase compositions may be prepared in accordance with methods known in the art and may be in the form of a liquid or a dry composition. For instance, the alpha-amylase composition may be in the form of a granulate or a microgranulate. The polypeptide to be included in the composition may be stabilized in accordance with methods known in the art.

Examples are given below of preferred uses of the alpha-amylase compositions of the invention. The dosage of the alpha-amylase composition of the invention and other conditions under which the composition is used may be determined on the basis of methods known in the art.

Further Compositions

The invention also relates to a composition comprising a mixture of one or more variants according to the invention derived from (as the parent Termamyl-like alpha-amylase) the B. steaorthermophilus alpha-amylase having the sequence shown in SEQ ID NO: 3 and a Termamyl-like alpha-amylase derived from the B. licheniformis alpha-amylase having the sequence shown in SEQ ID NO: 4.

Further, the invention also relates to a composition comprising a mixture of one or more variants according to the invention derived from (as the parent Termamyl-like alpha-amylase) the B. steaorthermophilus alpha-amylase having the sequence shown in SEQ ID NO: 3 and a hybrid alpha-amylase comprising a part of the B. amyloliquifaciens alpha-amylase shown in SEQ ID NO: 5 and a part of the B. licheniformis alpha-amylase shown in SEQ ID NO: 4. The latter mentioned hybrid Termamyl-like alpha-amylase comprises the 445 C-terminal amino acid residues of the B. licheniformis alpha-amylase shown in SEQ ID NO: 4 and the 37 N-terminal amino acid residues of the alpha-amylase derived from B. amyloliquifaciens shown in SEQ ID NO: 5.

In a specific embodiment of the invention the composition comprises a mixture of TVB146 and LE174, e.g., in a ratio of 2:1 to 1:2, such as 1:1.

An alpha-amylase or alpha-amylase variant of the invention or a composition of the invention may in an aspect of the invention be used for washing and/or dishwashing; for textile desizing or for starch liquefaction.

Detergent Compositions

The alpha-amylase or alpha-amylase variant of the invention may be added to and thus become a component of a detergent composition.

The detergent composition of the invention may for example be formulated as a hand or machine laundry detergent composition including a laundry additive composition suitable for pre-treatment of stained fabrics and a rinse added fabric softener composition, or be formulated as a detergent composition for use in general household hard surface cleaning operations, or be formulated for hand or machine dishwashing operations.

In a specific aspect, the invention provides a detergent additive comprising the enzyme of the invention. The detergent additive as well as the detergent composition may comprise one or more other enzymes such as a protease, a lipase, a cutinase, an amylase, a carboxylase, a cellulase, a pectinase, a mannanase, an arabinase, a galactanase, a xylanase, an oxidase, e.g., a lactase, and/or a peroxidase.

In general the properties of the chosen enzyme(s) should be compatible with the selected detergent, (i.e., pH optimum, compatibility with other enzymatic and non-enzymatic ingredients, etc.), and the enzyme(s) should be present in effective amounts.

Proteases: Suitable proteases include those of animal, vegetable or microbial origin. Microbial origin is preferred. Chemically modified or protein engineered mutants are included. The protease may be a serine protease or a metallo protease, preferably an alkaline microbial protease or a trypsin-like protease. Examples of alkaline proteases are subtilisins, especially those derived from Bacillus, e.g., subtilisin Novo, subtilisin Carlsberg, subtilisin 309, subtilisin 147 and subtilisin 168 (described in WO 89/06279). Examples of trypsin-like proteases are tryspin (e.g., of porcine or bovine origin) and the Fusarium protease described in WO 89/06270 and WO 94/25583.

Examples of useful proteases are the variants described in WO 92/10729, WO 98/20115, WO 98/20116, and WO 98/34946, especially the variants with substitutions in one or more of the following positions: 27, 36, 57, 76, 87, 97, 101, 104, 120, 123, 167, 170, 194, 206, 218, 222, 224, 235 and 274.

Preferred commercially available protease enzymes include Alcalase®, Savinase®, Primase®, Duralase®, Esperease®, and Kannase® (Novo Nordisk A/S), Maxatase®, Maxacal®, Maxapen®, Proprase®, Purafect®, Purafect OxP®, FN2®, and FN3® (Genencor International Inc.).

Lipases: Suitable lipases include those of bac-terial or fungal origin. Chemically modified or protein engineered mutants are included. Examples of useful lipases include lipases from Humicola (synonym Thermomyces), e.g., from H. lanuginosa (T. lanuginosus) as described in EP 258 068 and EP 305 216 or from H. insolens as described in WO 96/13580, a Pseudomonas lipase, e.g., from P. alcaligenes or P. pseudoalcaligenes (EP 218 272), P. cepacia (EP 331 376), P. stutzeri (GB 1,372,034), P. fluorescens, Pseudomonas sp. strain SD 705 (WO 95/06720 and WO 96/27002), P. wisconsinensis (WO 96/1201), a Bacillus lipase, e.g., from B. subtilis (Dartois et al. (1993), Biochimica et Biophysica Acta, 1131, 253-360), B. steaorthermophilus (JP 64/744992) or B. pumilus (WO 91/16422).


Preferred commercially available lipase enzymes include Lipolase™ and Lipolase Ultra™ (Novo Nordisk A/S).

Amylases: Suitable amylases (alpha- and/or beta-) include those of bacterial or fungal origin. Chemically modified or protein engineered mutants are included. Amylases include, for example, alpha-amylases obtained from Bacillus, e.g., a special strain of B. licheniformis, described in more detail in GB 1,296,839.
Examples of useful amylases are the variants described in WO 94/02597, WO 94/18314, WO 96/23873, and WO 97/43424, especially the variants with substitutions in one or more of the following positions: 15, 23, 105, 106, 124, 128, 133, 154, 156, 181, 188, 190, 197, 202, 208, 209, 243, 264, 304, 305, 391, 408, and 444.

Commercially available amylases are DuramyI™, TermamyI™, FungamyI™ and BAN™ (Novo Nordisk A/S), Rapidase™ and Purasat™ (from Genencor International Inc.).

Cellulases: Suitable cellulases include those of bacterial or fungal origin. Chemically modified or protein engineered mutants are included. Suitable cellulases include cellulases from the genera Bacillus, Pseudomonas, Huminola, Fusarium, Thielavia, Acremonium, e.g., the fungal cellulases produced from Humincola insolens, Myceliophthora thermophila and Fusarium oxyssporum disclosed in U.S. Pat. No. 4,435,307, U.S. Pat. No. 5,648,263, U.S. Pat. No. 5,691,178, U.S. Pat. No. 5,776,757 and WO 89/09259.


Commercially available cellulases include Celuhzyme®, and Carzyme® (Novo Nordisk A/S), Clazinase®, and Puradex HAE® (Genencor International Inc.), and KAC-500® (Kao Corporation).

Peroxidases/Oxidases: Suitable peroxidases/oxidases include those of plant, bacterial or fungal origin. Chemically modified or protein engineered mutants are included. Examples of useful peroxidases include peroxidases from Coprinus, e.g., from C. cinereus, and variants thereof as described in WO 93/24618, WO 95/10602, and WO 98/15257.

Commercially available peroxidases include Guardzyme® (Novo Nordisk A/S).

The detergent enzyme(s) may be included in a detergent composition by adding separate additives containing one or more enzymes, or by adding a combined additive comprising all of these enzymes. A detergent additive of the invention, i.e., a separate additive or a combined additive, can be formulated, e.g., as a granulate, a liquid, a slurry, etc. Preferred detergent additive formulations are granulates, in particular non-dusting granulates, liquids, in particular stabilized slurries, or slurries.

Non-dusting granulates may be produced, e.g., as disclosed in U.S. Pat. Nos. 4,106,991 and 4,661,452 and may optionally be coated by methods known in the art. Examples of waxy coating materials are poly(ethylene oxide) products (polyethylene glycol, PEG) with mean molar weights of 1000 to 20000; ethoxylated nonyl-phenols having from 16 to 50 ethylene oxide units; ethoxylated fatty alcohols in which the alcohol contains from 12 to 20 carbon atoms and in which there are 15 to 80 ethylene oxide units; fatty alcohols; fatty acids; and mono- and di- and triglycerides of fatty acids. Examples of film-forming coating materials suitable for application by fluid bed techniques are given in GB 1483591. Liquid enzyme preparations may, for instance, be stabilized by adding a polyol such as propylene glycol, a sugar or sugar alcohol, lactic acid or boric acid according to established methods. Protected enzymes may be prepared according to the method disclosed in EP 238,216.

The detergent composition of the invention may be in any convenient form, e.g., a bar, a tablet, a powder, a granule, a paste or a liquid. A liquid detergent may be aqueous, typically containing up to 70% water and 0-30% organic solvent, or non-aqueous.

The detergent composition comprises one or more surfactants, which may be non-ionic including semi-polar and/or anionic and/or cationic and/or zwitterionic. The surfactants are typically present at a level of from 0.1% to 60% by weight.

When included therein the detergent will usually contain from about 1% to about 40% of an anionic surfactant such as linear alkylbenzenesulfonate, alpha-olefin sulfonate, alkyl sulfate (fatty alcohol sulfate), alcohol ethoxysulfate, secondary alkanesulfonate, alpha-sulfo fatty acid methyl ester, alkyl- or alkenylsulfonic acid or soap.

When included therein the detergent will usually contain from about 0.2% to about 40% of a non-ionic surfactant such as alcohol ethoxylate, nonyl-phenol ethoxylate, alkylpolyglycoside, alkylphenylmethanol-amide, fatty acid monoethanolamide, polyhydroxy alkyl fatty acid amide, or N-acyl N-alkyl derivatives of glucosamine ("glucamides").

The detergent may contain 0-65% of a detergent builder or complexing agent such as zeolite, diphosphate, tripod-sphate, phosphonate, carbonate, citrate, nitritoltriacetic acid, ethylenediaminetetraacetic acid, diethylenetriaminepentaaetic acid, alkyl- or alkenylsulfonic acid, soluble silicates or layered silicates (e.g., SKS-6 from Hoechst).

The detergent may comprise one or more polymers. Examples are carboxymethylcellulose, poly(vinylpyrrolidone), poly (ethylene glycol), poly(vinyl alcohol), poly(vinylpyridine-N-oxide), poly(vinylimidazole), polycarboxylates such as polyacrylates, maleic acrylic acid copolymers and lauryl methacrylate/acrylic acid copolymers.

The detergent may contain a bleaching system which may comprise a H₂O₂ source such as perborate or percarbonate which may be combined with a persacid-forming bleach activator such as tetrachroylethylenediamine or nonanoyloxybenzenesulfonate. Alternatively, the bleaching system may comprise peroxyacids of, e.g., the amide, imide, or sulfone type.

The enzyme(s) of the detergent composition of the invention may be stabilized using conventional stabilizing agents, e.g., a polyol such as propylene glycol or glycerol, a sugar or sugar alcohol, lactic acid, boric acid, or a boric acid derivative, e.g., an aromatic borate ester, or a phenyl boronic acid derivative such as 4-formylphenyl boronic acid, and the conjugate position may be formulated as described in, e.g., WO 92/19709 and WO 92/19708.

The detergent may also contain other conventional detergent ingredients such as, e.g., fabric conditioners including clay, foam boosters, suds suppressors, anti-corrosion agents, soil-suspending agents, anti-soil redeposition agents, dyes, bactericides, optical brighteners, hydrotropes, tarnish inhibitors, or perlumes.

It is at present contemplated that in the detergent compositions any enzyme, in particular the enzyme of the invention, may be added in an amount corresponding to 0.01-100 mg of enzyme protein per liter of wash liquor, preferably
0.05-5 mg of enzyme protein per liter of wash liquor, in particular 0.1-1 mg of enzyme protein per liter of wash liquor. The enzyme of the invention may additionally be incorporated in the detergent formulations disclosed in WO 97/07202 which is hereby incorporated as reference.

Further Detergent Compositions

As mentioned above, variants of the invention may suitably be incorporated in detergent compositions. Increased thermostability at low calcium concentrations would be very beneficial for amylase performance in detergents, i.e., in the alkaline region. Reference is made, for example, to WO 96/23874 and WO 97/07202 for further details concerning relevant ingredients of detergent compositions (such as laundry or dishwashing detergents), appropriate methods of formulating the variants in such detergent compositions, and for examples of relevant types of detergent compositions.

An alpha-amylase or alpha-amylase variant of the invention may additionally comprise one or more other enzymes, such as a lipase, cutinase, protease, cellulase, peroxidase or laccase, and/or another alpha-amylase.

An alpha-amylase or alpha-amylase variant of the invention may be incorporated in detergents at conventionally employed concentrations. It is at present contemplated that a variant of the invention may be incorporated in an amount corresponding to 0.00001-1 mg (calculated as pure, active enzyme protein) of alpha-amylase per liter of wash/dishwash liquor using conventional dosing levels of detergent.

Dishwash Detergent Compositions

The alpha-amylase or alpha-amylase variant of the invention may also be used in dishwasher detergent compositions, including the following:

1) Powder Automatic Dishwashing Composition

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonionic surfactant</td>
<td>0.4-2.5%</td>
</tr>
<tr>
<td>Sodium metasilicate</td>
<td>0-20%</td>
</tr>
<tr>
<td>Sodium disilicate</td>
<td>3-20%</td>
</tr>
<tr>
<td>Sodium triphosphate</td>
<td>20-40%</td>
</tr>
<tr>
<td>Sodium carbonate</td>
<td>0-20%</td>
</tr>
<tr>
<td>Sodium perborate</td>
<td>2-9%</td>
</tr>
<tr>
<td>Tetraacetyl ethylene diamine (TAED)</td>
<td>1-4%</td>
</tr>
<tr>
<td>Sodium sulphate</td>
<td>5-33%</td>
</tr>
<tr>
<td>Enzymes</td>
<td>0.0001-0.1%</td>
</tr>
</tbody>
</table>

21 Powder Automatic Dishwashing Composition

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonionic surfactant</td>
<td>1-2%</td>
</tr>
<tr>
<td>(e.g., alcohol ethoxylate)</td>
<td></td>
</tr>
<tr>
<td>Sodium disilicate</td>
<td>2-30%</td>
</tr>
<tr>
<td>Sodium carbonate</td>
<td>10-50%</td>
</tr>
<tr>
<td>Sodium phosphate</td>
<td>0-5%</td>
</tr>
<tr>
<td>Trisodium citrate dihydrate</td>
<td>9-30%</td>
</tr>
<tr>
<td>Nitritotriiodoacetic acid (NTA)</td>
<td>0-20%</td>
</tr>
<tr>
<td>Sodium perborate monohydrate</td>
<td>5-10%</td>
</tr>
<tr>
<td>Tetraacetyl ethylene diamine (TAED)</td>
<td>1-2%</td>
</tr>
<tr>
<td>Polyacrylate polymer</td>
<td>6-25%</td>
</tr>
<tr>
<td>(e.g., maleic acid/acrylic acid copolymer)</td>
<td></td>
</tr>
</tbody>
</table>

3) Powder Automatic Dishwashing Composition

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonionic surfactant</td>
<td>0.5-2.0%</td>
</tr>
<tr>
<td>Sodium disilicate</td>
<td>25-40%</td>
</tr>
<tr>
<td>Sodium carbonate</td>
<td>30-55%</td>
</tr>
<tr>
<td>Sodium carbonate</td>
<td>0-29%</td>
</tr>
<tr>
<td>Sodium bicarbonate</td>
<td>0-20%</td>
</tr>
<tr>
<td>Sodium perborate monohydrate</td>
<td>0-15%</td>
</tr>
<tr>
<td>Tetraacetyl ethylene diamine (TAED)</td>
<td>0-6%</td>
</tr>
<tr>
<td>Maleic acid/acrylic acid copolymer</td>
<td>0-5%</td>
</tr>
<tr>
<td>Clay</td>
<td>1-3%</td>
</tr>
<tr>
<td>Polyanino acids</td>
<td>0-20%</td>
</tr>
<tr>
<td>Sodium polyacrylate</td>
<td>0-8%</td>
</tr>
<tr>
<td>Enzymes</td>
<td>0.0001-0.1%</td>
</tr>
</tbody>
</table>

4) Powder Automatic Dishwashing Composition

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonionic surfactant</td>
<td>1-2%</td>
</tr>
<tr>
<td>Zeolite MAP</td>
<td>15-42%</td>
</tr>
<tr>
<td>Sodium disilicate</td>
<td>30-34%</td>
</tr>
<tr>
<td>Sodium citrate</td>
<td>0-12%</td>
</tr>
<tr>
<td>Sodium carbonate</td>
<td>0-20%</td>
</tr>
<tr>
<td>Sodium perborate monohydrate</td>
<td>7-15%</td>
</tr>
<tr>
<td>Tetraacetyl ethylene diamine (TAED)</td>
<td>0-3%</td>
</tr>
<tr>
<td>Polymer</td>
<td>0-4%</td>
</tr>
<tr>
<td>Maleic acid/acrylic acid copolymer</td>
<td>0-5%</td>
</tr>
<tr>
<td>Organic phosphonate</td>
<td>0-4%</td>
</tr>
<tr>
<td>Clay</td>
<td>1-2%</td>
</tr>
<tr>
<td>Enzymes</td>
<td>0.0001-0.1%</td>
</tr>
<tr>
<td>Sodium sulphate</td>
<td>Balance</td>
</tr>
</tbody>
</table>

5) Powder Automatic Dishwashing Composition

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonionic surfactant</td>
<td>1-7%</td>
</tr>
<tr>
<td>Sodium disilicate</td>
<td>18-30%</td>
</tr>
<tr>
<td>Triscitium citrate</td>
<td>10-24%</td>
</tr>
<tr>
<td>Sodium carbonate</td>
<td>12-20%</td>
</tr>
<tr>
<td>Dipersulphate (2 KH2SO4•KH2SO4•K2SO4)</td>
<td>15-21%</td>
</tr>
<tr>
<td>Bleach stabilizer</td>
<td>0.1-2%</td>
</tr>
<tr>
<td>Maleic acid/acrylic acid copolymer</td>
<td>0-6%</td>
</tr>
<tr>
<td>Diethylene triamine pentaaetate, pentasodium salt</td>
<td>0-2.5%</td>
</tr>
<tr>
<td>Enzymes</td>
<td>0.0001-0.1%</td>
</tr>
<tr>
<td>Sodium sulphate, water</td>
<td>Balance</td>
</tr>
</tbody>
</table>

6) Powder and Liquid Dishwashing Composition with Cleaning Surfactant System

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonionic surfactant</td>
<td>0-1.5%</td>
</tr>
<tr>
<td>Octadecyl dimethylamine N-oxide dihydrate</td>
<td>0-5%</td>
</tr>
<tr>
<td>80:20 wt. C18/C16 blend of octadecyl dimethylamine N-oxide dihydrate and hexadecyl dimethylamine N-oxide dihydrate</td>
<td>0-4%</td>
</tr>
</tbody>
</table>
7) Non-Aqueous Liquid Automatic Dishwashing Composition

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>70:30 wt. C18:C16 blend of octadecyl bis (hydroxyethyl)amine N-oxide anhydrous</td>
<td>0-5%</td>
</tr>
<tr>
<td>(hydroxyethyl)amine N-oxide anhydrous and hexadecyl bis (hydroxyethyl)amine N-oxide anhydrous</td>
<td></td>
</tr>
<tr>
<td>C12-C18 allyl ethoxysulfate with an average degree of ethoxylation of 3</td>
<td>0-10%</td>
</tr>
<tr>
<td>C12-C18 allyl ethoxysulfate with an average degree of ethoxylation of 3</td>
<td>0-5%</td>
</tr>
<tr>
<td>C12-C18 ethoxylated alcohol with an average degree of ethoxylation of 12</td>
<td>0-5%</td>
</tr>
<tr>
<td>A blend of C12-C18 ethoxylated alcohols with an average degree of ethoxylation of 9</td>
<td>0-6.5%</td>
</tr>
<tr>
<td>A blend of C12-C18 ethoxylated alcohols with an average degree of ethoxylation of 30</td>
<td>0-4%</td>
</tr>
<tr>
<td>Sodium dodecyl sulfate</td>
<td>0-33%</td>
</tr>
<tr>
<td>Sodium tripolyphosphate</td>
<td>0-46%</td>
</tr>
<tr>
<td>Sodium citrate</td>
<td>0-28%</td>
</tr>
<tr>
<td>Citric acid</td>
<td>0-20%</td>
</tr>
<tr>
<td>Sodium carbonate</td>
<td>0-20%</td>
</tr>
<tr>
<td>Sodium perborate monohydrate</td>
<td>0-11.5%</td>
</tr>
<tr>
<td>Tetracetyl ethylene diamine (TAED)</td>
<td>0-4%</td>
</tr>
<tr>
<td>Malonic acid/acrylic acid copolymer</td>
<td>0-7.5%</td>
</tr>
<tr>
<td>Sodium sulphate</td>
<td>0-12.5%</td>
</tr>
<tr>
<td>Enzymes</td>
<td>0.0001-0.1%</td>
</tr>
</tbody>
</table>

8) Non-Aqueous Liquid Dishwashing Composition

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liquid nonionic surfactant (e.g., alcohol ethoxylates)</td>
<td>0-10.0%</td>
</tr>
<tr>
<td>Alkali metal silicate</td>
<td>3-0-15.0%</td>
</tr>
<tr>
<td>Alkali metal phosphate</td>
<td>0-20.0-40.0%</td>
</tr>
<tr>
<td>Liquid carrier selected from higher glycol, polyglycol, polyoxides, glycol ethers</td>
<td>25-0-45.0%</td>
</tr>
<tr>
<td>Stabilizer</td>
<td>0-5-7.0%</td>
</tr>
<tr>
<td>(e.g., a partial ester of phosphoric acid and a C16-C18 alkane)</td>
<td></td>
</tr>
<tr>
<td>Foam suppressor (e.g., silicone)</td>
<td>0-1.5%</td>
</tr>
<tr>
<td>Enzymes</td>
<td>0.0001-0.1%</td>
</tr>
</tbody>
</table>

9) Thixotropic Liquid Automatic Dishwashing Composition

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>C12-C14 fatty acid</td>
<td>0-0.5%</td>
</tr>
<tr>
<td>Block co-polymer surfactant</td>
<td>1.5-15.0%</td>
</tr>
<tr>
<td>Sodium citrate</td>
<td>0-12%</td>
</tr>
</tbody>
</table>

10) Liquid Automatic Dishwashing Composition

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alcohol ethoxylate</td>
<td>0-20%</td>
</tr>
<tr>
<td>Fatty acid ester sulphonate</td>
<td>0-30%</td>
</tr>
<tr>
<td>Sodium dodecyl sulphate</td>
<td>0-20%</td>
</tr>
<tr>
<td>Alkyl polyglycoside</td>
<td>0-21%</td>
</tr>
<tr>
<td>Oleic acid</td>
<td>0-10%</td>
</tr>
<tr>
<td>Sodium disilicate monohydrate</td>
<td>18-33%</td>
</tr>
<tr>
<td>Sodium citrate dehydrate</td>
<td>18-33%</td>
</tr>
<tr>
<td>Sodium stearate</td>
<td>0-2.5%</td>
</tr>
<tr>
<td>Sodium perborate monohydrate</td>
<td>0-13%</td>
</tr>
<tr>
<td>Tetracetyl ethylene diamine (TAED)</td>
<td>0-8%</td>
</tr>
<tr>
<td>Malonic acid/acrylic acid copolymer</td>
<td>4-8%</td>
</tr>
<tr>
<td>Enzymes</td>
<td>0.0001-0.1%</td>
</tr>
</tbody>
</table>

11) Liquid Automatic Dishwashing Composition Containing Protected Bleach Particles

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium silicate</td>
<td>5-10%</td>
</tr>
<tr>
<td>Tetrapotassium pyrophosphate</td>
<td>15-25%</td>
</tr>
<tr>
<td>Sodium tripolyphosphate</td>
<td>0-2%</td>
</tr>
<tr>
<td>Potassium carbonate</td>
<td>4-8%</td>
</tr>
<tr>
<td>Protected bleach particles, e.g., chlorine</td>
<td>5-10%</td>
</tr>
<tr>
<td>Polymeric thickener</td>
<td>0-7.1-5.5%</td>
</tr>
<tr>
<td>Potassium hydroxide</td>
<td>0-2%</td>
</tr>
<tr>
<td>Enzymes</td>
<td>0.0001-0.1%</td>
</tr>
<tr>
<td>Water</td>
<td>Balance</td>
</tr>
</tbody>
</table>

11) Automatic dishwashing compositions as described in 1), 2), 3), 4), 6) and 10), wherein perborate is replaced by percarbonate.

12) Automatic dishwashing compositions as described in 1)-6) which additionally contain a manganese catalyst. The manganese catalyst may, e.g., be one of the compounds described in “Efficient manganese catalysts for low-temperature bleaching”, Nature 369: 637-639 (1994).

Uses

[0461] The present invention is also directed to methods for using an alpha-amylosa or alpha-amylase variant of the invention in detergents, in particular laundry detergent compositions and dishwash detergent compositions.
INDUSTRIAL APPLICATIONS

[0462] An alpha-amylase and alpha-amylase variant of the invention are well suited for use in a variety of industrial processes, in particular the enzymes of the invention finds potential applications as a component in detergents, e.g., laundry, dishwash and hard surface cleaning detergent compositions, but it may also be useful in the production of sweeteners and ethanol from starch. Thus, it may be used in conventional starch-converting processes, such as liquefaction and saccharification processes described in U.S. Pat. No. 3,912,590 and EP patent publications Nos. 252,730 and 63,909.

[0463] An alpha-amylase or alpha-amylase variant of the invention may also be used in the production of lignocellulosic materials, such as pulp, paper and cardboard, from starch reinforced waste paper and cardboard, especially where repulping occurs at pH above 7 and where amylases can facilitate the disintegration of the waste material through degradation of the reinforcing starch. The alpha-amylase of the invention is especially useful in a process for producing a papermaking pulp from starch-coated printed paper. The process may be performed as described in WO 95/14807, comprising the following steps:

[0464] a) disintegrating the paper to produce a pulp,
[0465] b) treating with a starch-degrading enzyme before, during or after step a), and
[0466] c) separating ink particles from the pulp after steps a) and b).

[0467] An alpha-amylase or alpha-amylase variant of the invention may also be very useful in modifying starch where enzymatically modified starch is used in papermaking together with alkaline fillers such as calcium carbonate, kaolin and clays. With the alkaline alpha-amylases of the invention it becomes possible to modify the starch in the presence of the filler thus allowing for a simpler integrated process.

[0468] An alpha-amylase or alpha-amylase variant of the invention may also be very useful in textile desizing. In the textile processing industry, alpha-amylases are traditionally used as auxiliaries in the desizing process to facilitate the removal of starch-containing size which has served as a protective coating on weft yarns during weaving. Complete removal of the size coating after weaving is import-ant to ensure optimum results in the subsequent processes, in which the fabric is scoured, bleached and dyed. Enzymatic starch break-down is preferred because it does not involve any harmful effect on the fiber material. In order to reduce processing cost and increase mill throughput, the desizing processing is sometimes combined with the scouring and bleaching steps. In such cases, non-enzymatic auxiliaries such as alkali or oxidation agents are typically used to break down the starch, because traditional alpha-amylases are not very compatible with high pH levels and bleaching agents. The non-enzymatic breakdown of the starch size does lead to some fiber damage because of the rather aggressive chemicals used. Accordingly, it would be desirable to use the alpha-amylases of the invention as they have an improved performance in alkaline solutions. The alpha-amylases may be used alone or in combination with a cellulase when desizing cellulose-containing fabric or textile.

[0469] The alpha-amylases of the invention may also be very useful in a beer-making process; the alpha-amylases will typically be added during the mashing process.

Production of Sweeteners from Starch

[0470] A “traditional” process for conversion of starch to fructose syrups normally consists of three consecutive enzymatic processes, viz., a liquefaction process followed by a saccharification process and an isomerization process. During the liquefaction process, starch is degraded to dextrins by an alpha-amylase (e.g., Termamyl®) at pH values between 5.5 and 6.2 and at temperatures of 95°-160° C, for a period of approx. 2 hours. In order to ensure an optimal enzyme stability under these conditions, 1 mM of calcium is added (40 ppm free calcium ions).

[0471] After the liquefaction process the dextrins are converted into dextrose by addition of a glucoamylase (e.g., AMG®) and a debranching enzyme, such as an isoamylase or a pullulanase (e.g., Promozyme®). Before this step the pH is reduced to a value below 4.5, maintaining the high temperature (above 95° C.), and the liquefying alpha-amylase activity is denatured. The temperature is lowered to 60° C., and glucoamylase and debranching enzyme are added. The saccharification process proceeds for 24-72 hours.

[0472] After the saccharification process the pH is increased to a value in the range of 6-8, preferably pH 7.5, and the calcium is removed by ion exchange. The dextrose syrup is then converted into high fructose syrup using, e.g., an immobilized glucose isomerase (such as Sweetzyme®).

[0473] At least 1 enzymatic improvements of this process could be envisaged.

[0474] Reduction of the calcium dependency of the liquefying alpha-amylase. Addition of free calcium is required to ensure adequately high stability of the alpha-amylase, but free calcium strongly inhibits the activity of the glucose isomerase and needs to be removed, by means of an expensive unit operation, to an extent which reduces the level of free calcium to below 3.5 ppm. Cost savings could be obtained if such an operation could be avoided and the liquefaction process could be performed without addition of free calcium ions.

[0475] To achieve that, a less calcium-dependent Termamyl-like alpha-amylase which is stable and highly active at low concentrations of free calcium (<40 ppm) is required. Such a Termamyl-like alpha-amylase should have a pH optimum at a pH in the range of 4.5-6.5, preferably in the range of 4.5-5.5.

Materials and Methods

[0476] Chemicals used as buffers and substrates were commercial products of at least reagent grade.

Enzymes:

[0477] SP690: alpha-amylase shown in SEQ ID NO: 1
SP722: alpha-amylase shown in SEQ ID NO: 2
Termamyl®: alpha-amylase from Bacillus licheniformis shown in SEQ ID NO: 4.
AA560: alpha-amylase of the invention shown in SEQ ID NO: 24 encoded by the DNA sequence shown in SEQ ID NO: 23.
AA360: alpha-amylase shown in SEQ ID NO: 26 being identical to the AA560 alpha-amylase encoded by the DNA sequence shown in SEQ ID NO: 25.
BSG alpha-amylase: *B. steathermophilus* alpha-amylase depicted in SEQ ID NO: 3.

TVB146 alpha-amylase variant: *B. steathermophilus* alpha-amylase variant depicted in SEQ ID NO: 3 with the following mutations: with the deletion in positions 1181-1G182+1N193F.

LE174 hybrid alpha-amylase variant: LE174 is a hybrid Termamyyl-like alpha-amylase being identical to the *Termamyyl* sequence, i.e., the *Bacillus licheniformis* alpha-amylase shown in SEQ ID NO: 4, except that the N-terminal 35 amino acid residues (of the mature protein) has been replaced by the N-terminal 33 residues of BAN (mature protein), i.e., the *Bacillus amyloleucinefaciens* alpha-amylase shown in SEQ ID NO: 5, which further have the following mutations: H156Y+A181I+N190F+A209V+Q264S (using the numbering in SEQ ID NO: 4). LE174 was constructed by SOE-PCR (Higuchi et al., 1988, *Nucleic Acids Research* 16:7351).

Model Detergent:

[0478] A/P (Asia/Pacific) Model Detergent has the following composition: 20% STPP (sodium tripolyphosphate), 25% Na₂SO₄, 15% Na₂CO₃, 20% LAS (linear alkylbenzene sulfonate, NaNs 80S), 5% C₁₃-C₁₅ alcohol ethoxylate (Dobanol 25-7), 5% Na₂Si₂O₃, 0.3% NaCl.

Omo Multi Acao (Brazil),

[0479] Omo concentrated powder (Europe) (product of Unilever)

Ariel Futur liquid (Europe) (product of Procter and Gamble)

Deposit of Biological Material

[0480] The following biological material has been deposited under the terms of the Budapest Treaty with the Deutsche Sammlung von Microorganismen und Zellkulturen GmbH (DSMZ), Mascheroder Weg 1, D-38124 Braunschweig DE, and given the following accession number:

<table>
<thead>
<tr>
<th>Deposit</th>
<th>Accession Number</th>
<th>Date of Deposit</th>
</tr>
</thead>
<tbody>
<tr>
<td>NN017557</td>
<td>DSM 12648</td>
<td>25 Jan. 1999</td>
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<tr>
<td>NN017560</td>
<td>DSM 12649</td>
<td>25 Jan. 1999</td>
</tr>
<tr>
<td>NN040467</td>
<td>DSM12761</td>
<td>7th Apr. 1999</td>
</tr>
<tr>
<td>NN040470</td>
<td>DSM12764</td>
<td>7th Apr. 1999</td>
</tr>
</tbody>
</table>

[0481] The strains have been deposited under conditions that assure that access to the culture will be available during the pendency of this patent application to one determined by the Commissioner of Patents and Trademarks to be entitled thereto under 37 C.F.R. §1.14 and 35 U.S.C. §122. The deposit represents a substantially pure culture of the deposited strain. The deposit is available as required by foreign patent laws in countries wherein counterparts of the subject application, or its progeny are filed. However, it should be understood that the availability of a deposit does not constitute a license to practice the subject invention in derogation of patent rights granted by governmental action.

Host Organism

[0482] *Bacillus subtilis* strain SHa273 is disclosed in WO 95/10603

*E. coli* strain SJ2 (Diderichsen et al. (1990)).

Methods

General Molecular Biology Methods:

[0485] Unless otherwise mentioned the DNA manipulations and transformations were performed using standard methods of molecular biology (Sambrook et al. (1989); Ausubel et al. (1995); Harwood and Cutting (1990)).

Fermentation and Purification of Alpha-Amylase Variants

[0486] Fermentation may be performed by methods well known in the art or as follows.

[0487] A *B. subtilis* strain harbouring the relevant expression plasmid is streaked on an L.B-agar plate with 10 μg/ml kanamycin from ~80°C. stock, and grown overnight at 37°C.

[0488] The colonies are transferred to 100 ml BPX media supplemented with 10 μg/ml kanamycin in a 500 ml shaking flask.

Composition of BPX Medium:

[0489] |        |          |          |
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Potato starch</td>
<td>100 g/l</td>
<td></td>
</tr>
<tr>
<td>Barley flour</td>
<td>50 g/l</td>
<td></td>
</tr>
<tr>
<td>BAN 5000 SBF</td>
<td>0.1 g/l</td>
<td></td>
</tr>
<tr>
<td>Sodium caseinate</td>
<td>10 g/l</td>
<td></td>
</tr>
<tr>
<td>Soy Bean Meal</td>
<td>20 g/l</td>
<td></td>
</tr>
<tr>
<td>Na₂HPO₄, 12 H₂O</td>
<td>9 g/l</td>
<td></td>
</tr>
<tr>
<td>Prolenic™</td>
<td>0.1 g/l</td>
<td></td>
</tr>
</tbody>
</table>

[0490] The culture is shaken at 37°C. at 270 rpm for 5 days.

[0491] Cells and cell debris are removed from the fermentation broth by centrifugation at 4500 rpm in 20-25 minutes. Afterwards the supernatant is filtered to obtain a completely clear solution. The filtrate is concentrated and washed on a UF-filter (10000 cut off membrane) and the buffer is changed to 20 mM Acetate pH 5.5. The UF-filtrate is applied on a Sephrose F.F. and elution is carried out by step elution with 0.2M NaCl in the same buffer. The eluate is dialysed against 10 mM Tris, pH 9.0 and applied on a Q-sephrose F.F. and eluted with a linear gradient from 0-0.3 M NaCl over 6 column volumes. The fractions which contain the activity (measured by the Phadebas assay) are pooled, pH was adjusted to pH 7.5 and remaining color was removed by a treatment with 0.5% W/vol. active coal in 5 minutes.
Assays for Determining Alpha-Amylase Activity

Activity Determination—(KNU)

One Kilo alpha-amylase Unit (1 KNU) is the amount of enzyme which breaks down 5.26 g starch (Merk, Amylum Solubile, Eng. B 8, Batch 9947275) per hour in Novo Nordisk’s standard method for determination of alpha-amylase based upon the following condition:

- Substrate: soluble starch
- Calcium content in solvent: 0.0043M
- Reaction time: 7-20 minutes
- Temperature: 37° C.
- pH: 5.6

Detailed description of Novo Nordisk's analytical method (AF 9) is available on request.

BS-Amylase Activity Determination—KNU(S)

1. Application Field

This method is used to determine alpha-amylase activity in fermentation and recovery samples and formulated and granulated products.

2. Principle

BS-amylase breaks down the substrate (4,6-ethyldiene(G1)-p-nitrophenyl(G1)-alpha-D-maltotetraoside (written as ethyldiene-G1-PNP) into, among other things, G1-PNP and G3-PNP, where G denoted glucose and PNP p-nitrophenol.

G2-PNP and G3-PNP are broken down by alpha-glucosidase, which is added in excess, into glucose and the yellow-coloured p-nitrophenol.

The colour reaction is monitored in situ and the change in absorbance over time calculated as an expression of the spread of the reaction and thus of the activity of the enzyme. See the Boehringer Mannheim 1442 309 guidelines for further details.

2.1 Reaction Conditions

Reaction:
- Temperature: 37° C.
- pH: 7.1
- Pre-incubation time: 2 minutes

Detection:
- Wavelength: 405 nm
- Measurement time: 3 minutes

3. Definition of Units

Bacillus stearothermophilus alpha-amylase (BS-amylase) activity is determined relative to a standard of declared activity and stated in Kilo Novo Units (Stearothermophilus) or KNU(S)).

4. Specificity and Sensitivity

Limit of determination: approx. 0.4 KNU(s)/g

5. Apparatus

- Cobas Fara analyser
- Diluted (e.g., Hamilton Microlab 1000)
- Analytical balance (e.g., Mettler AE 100)
- Stirrer plates

6. Reagents/Substrates

A ready-made kit is used in this analysis to determine alpha-amylase activity. Note that the reagents specified for the substrate and alpha-glucosidase are not used as described in the Boehringer Mannheim guidelines. However, the designations “buffer”, “glass 1”, “glass 1a” and “Glass 2” are those referred to in those guidelines.

6.1. Substrate

4,6-ethyldiene(G1)-p-nitrophenyl(G1)-alpha-D-maltotetraoside (written as ethyldiene-G1-PNP), e.g., Boehringer Mannheim 1442 309

6.2 Alpha-Glucosidase Help Reagent

alpha-glucosidase, e.g., Boehringer Mannheim 1442 309

6.3 BRIJ 35 Solution

BRIJ 35 (30% W/V Sigma 430 AG-6) 1000 mL
Demineralized water up to 2000 mL

6.4 Stabiliser

Brij 35 solution 33 mL
CaCl2·2H2O (Merck 2382) 882 g
Demineralized water up to 2000 mL

7. Samples and Standards

7.1 Standard Curve

Example: Preparation of BS-Amylase Standard Curve

The relevant standard is diluted to 0.60 KNU(s)/mL as follows. A calculated quantity of standard is weighed out and added to 200 mL volumetric flask, which is filled to around the 5th mark with demineralized water. Stabiliser corresponding to 1% of the volume of the flask is added and the flask is filled to the mark with demineralized water.

A Hamilton Microlab 1000 is used to produce the dilutions shown below. Demineralized water with 1% stabiliser is used as the diluent.

<table>
<thead>
<tr>
<th>Dilution No.</th>
<th>Enzyme stock solution</th>
<th>1% stabilizer</th>
<th>KNU(s)/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>20 µL</td>
<td>580 µL</td>
<td>0.02</td>
</tr>
<tr>
<td>2</td>
<td>30 µL</td>
<td>570 µL</td>
<td>0.03</td>
</tr>
</tbody>
</table>
7.2 Level Control

[0514] A Novo Nordisk A/S BS amylase level control is included in all runs using the Cobas Fara. The control is diluted with 1% stabilizer so that the final dilution is within the range of the standard curve. All weights and dilutions are noted on the worklist.

7.3 Sample Solutions

Single Determination

[0515] Fermentation samples (not final samples) from production, all fermentation samples from pilot plants and storage stability samples are weighed out and analyzed once only. Double determination over 1 run:

Process samples, final fermentation samples from production, samples from GLP studies and R&D samples are weighed out and analyzed twice.

Double determinations over 2 runs:

Finished product samples are weighed out and analyzed twice over two separate runs.

Maximum concentration of samples in powder form: 5%

Test samples are diluted with demineralized water with 1% stabilizer to approx. 0.037 KNU(S)/mL on the basis of their expected activity. The final dilution is made direct into the sample cup.

8. Procedure

8.1 Cobas Menu Program

[0516] The Cobas Menu Program is used to suggest the weight/dilutions of samples and level control to be used.

[0517] The samples are entered into the program with a unique identification code and a worklist is printed out.

[0518] The samples and control are weighed out and diluted as stated on the worklist with hand-written weight data is inserted into the BS-amylase analysis logbook.

[0519] The results are computerized automatically by the Cobas Fara as described in item 9 and printed out along with the standard curve.

[0520] Worklists and results printouts are inserted into the BS-amylase analysis logbook.

8.2 Cobas Fara Set-Up

[0521] The samples are placed in the sample rack.

[0522] The five standards are placed in the calibration rack at position 1 to 5 (strongest standard at position 5), and control placed in the same rack at position 10.

[0523] The substrate is transferred to a 30 mL reagent container and placed in that reagent rack at position 2 (holder 1).

[0524] The alpha-glucosidase help reagent is transferred to a 50 mL reagent container and placed in the reagent rack at position 2 (holder C).

8.3 Cobas Fara Analysis

[0525] The main principles of the analysis are as follows:

[0526] 20 µL sample and 10µ rinse-water are pipetted into the cuvette along with 250 µL alpha-glucosidase help reagent. The cuvette rotates for 10 seconds and the reagents are thrown out into the horizontal cuvettes. 25 µL substrate and 20 µL rinse-water are pipetted off. After a 1 second wait to ensure that the temperature is 37°C, the cuvette rotates again and the substrate is mixed into the horizontal cuvettes. Absorbance is measured for the first time after 120 seconds and then every 5 seconds. Absorbance is measured a total of 37 times for each sample.

9. Calculations

[0527] The activity of the samples is calculated relative to Novo Nordisk A/S standard.

[0528] The standard curve is plotted by the analyzer. The curve is to be gently curved, rising steadily to an absorbance of around 0.25 for standard no. 5.

[0529] The activity of the samples in KNU(S)/mL is read off the standard curve by the analyzer.

[0530] The final calculations to allow for the weights/dilutions used employ the following formula:

\[
\text{Activity in KNU(S)/g} = \frac{S \times V \times F \times W}{V \times F \times W}
\]

S = analysis result read off (KNU(S)/mL)

V = volume of volumetric flask used in mL

F = dilution factor for second dilution

W = weight of enzyme sample in g

9.2 Calculation of Mean Values

[0531] Results are stated with 3 significant digits. However, for sample activity <10 KNU(S)/g, only 2 significant digits are given.

[0532] The following rules apply on calculation of mean values:

1. Data which deviates more than 2 standard deviations from the mean value is not included in the calculation.

2. Single and double determination over one run:

[0533] The mean value is calculated on basis of results lying within the standard curve’s activity area.

3. Double determinations over two runs: All values are included in the mean value. Outliers are omitted.

10. Accuracy and Precision

[0534] The coefficient of variation is 2.9% based on retrospective validation of analysis results for a number of finished products and the level control.

Phadebas Assay (for Alpha-Amylase Activity Determination)

[0535] Alpha-amylase activity is determined by a method employing Phadebas® tablets as substrate. Phadebas tablets (Phadebas® Amylase Test, supplied by Pharmacia Diagnostic) contain a cross-linked insoluble blue-colored starch polymer which has been mixed with bovine serum albumin and a buffer substance and tableted.

[0536] For every single measurement one tablet is suspended in a tube containing 5 ml 50 mM Britton-Robinson buffer (50 mM acetic acid, 50 mM phosphoric acid, 50 mM boric acid, 0.1 mM CaCl₂, pH adjusted to the value of interest with NaOH). The test is performed in a water bath at the temperature of interest. The alpha-amylase to be tested is
diluted in x ml of 50 mM Britton-Robinson buffer. 1 ml of this alpha-amylase solution is added to the 5 ml 50 mM Britton-Robinson buffer. The starch is hydrolyzed by the alpha-amylase giving soluble blue fragments. The absorbance of the resulting blue solution, measured spectrophotometrically at 620 nm, is a function of the alpha-amylase activity.

[0537] It is important that the measured 620 nm absorbance after 10 or 15 minutes of incubation (testing time) is in the range of 0.2 to 2.0 absorbance units at 620 nm. In this absorbance range there is linearity between activity and absorbance (Lambert-Beer law). The dilution of the enzyme must therefore be adjusted to fit this criterion. Under a specified set of conditions (temp., pH, reaction time, buffer conditions) 1 mg of a given alpha-amylase will hydrolyze a certain amount of substrate and a blue colour will be produced. The colour intensity is measured at 620 nm. The measured absorbance is directly proportional to the specific activity (activity/mg of pure alpha-amylase protein) of the alpha-amylase in question under the given set of conditions.

Alternative Alpha-Amylase Activity Method (PNP-G7 Assay)

[0538] Alpha-amylase activity is determined by a method employing the PNP-G7 substrate. PNP-G7 which is an abbreviation for p-nitrophenyl-alpha,D-maltotriose is a blocked oligosaccharide which can be cleaved by an endo-amylase. Following the cleavage, the alpha-glucosidase included in the kit digest the substrate to liberate a free PNP molecule which has a yellow colour and thus can be measured by visible spectophotometry at λ=405 nm (400-420 nm). Kits containing PNP-G7 substrate and alpha-glucosidase are manufactured by Boehringer-Mannheim (cat. no. 10546345).

[0539] To prepare the substrate one bottle of substrate (BM 1442309) is added to 5 ml buffer (BM1442309). To prepare the alpha-glucosidase one bottle of alpha-glucosidase (BM 1462309) is added to 45 ml buffer (BM1442309). The working solution is made by mixing 5 ml alpha-glucosidase solution with 0.5 ml substrate.

[0540] The assay is performed by transferring 20 μl enzyme solution to a 96 well microtiter plate and incubating at 25 °C. 200 μl working solution, 25 °C is added. The solution is mixed and pre-incubated 1 minute and absorption is measured every 15 sec: over 3 minutes at OD 405 nm.

[0541] The slope of the time dependent absorption-curve is directly proportional to the specific activity (activity per mg enzyme) of the alpha-amylase in question under the given set of conditions.

EXAMPLES

Example 1

Construction of Variants of BSG Alpha-Amylase

(SEQ ID NO: 3)

[0542] The gene encoding BSG, amyS, is located in plasmid pPL1117. This plasmid contains also the gene conferring resistance towards kanamycin and an origin of replication, both obtained from plasmid pUB110 (Gryczan et al., 1978. J. Bact. 134:318-329).

[0543] The DNA sequence of the mature part of amyS is shown as SEQ ID NO: 11 and the amino acid sequence of the mature protein is shown as SEQ ID NO: 3.

[0544] BSG variant TVB145, which contains a deletion of 6 nucleotides corresponding to amino acids I181-G182 in the mature protein, is constructed as follows:

[0545] Polymerase Chain Reaction (PCR) is utilized to amplify the part of the amyS gene (from plasmid pPL1117), located between DNA primers BSG1 (SEQ ID NO: 16) and BSGM2 (SEQ ID NO: 19). BSG1 is identical to a part of the amyS gene whereas BSGM2 contains the 6 bp nucleotide deletion. A standard PCR reaction is carried out at 94 °C for 5 minutes, 25 cycles of (94 °C for 45 sec, 50 °C for 45 sec, 72 °C for 90 sec, 72 °C for 7 minutes using the Pwo polymerase under conditions as recommended by the manufacturer, Boehringer Mannheim GmbH.

[0546] The resulting approximately 550 bp amplified band was used as a megaprimer (Barik and Galiński, 1991, Biotechniques 10: 489-490) together with primer BSG3 in a second PCR with pPL1117 as template resulting in a DNA fragment of approximately 1080 bp.

[0547] This DNA fragment is digested with restriction endonucleases Acc65I and Sall and the resulting approximately 550 bp fragment is ligated into plasmid pPL1117 digested with the same enzymes and transformed into the protease- and amylase-deleted Bacillus subtilis strain SHA273 (described in WO 92/11357 and WO 95/10603).

[0548] Kanamycin resistant and starch degrading transformants were analysed for the presence of the desired mutations (restriction digest to verify the introduction of a HindIII site in the gene). The DNA sequence between restriction sites Acc65I and Sall was verified by DNA sequencing to ensure the presence of only the desired mutations.

[0549] BSG variant TVB146 which contains the same 6 nucleotide deletion as TVB145 and an additional substitution of asparagine 193 for a phenylalanine, N193F, was constructed in a similar way as TVB145 utilizing primer BSGM3 (SEQ ID NO: 20) in the first PCR.

[0550] BSG variant TVB161, containing the deletion of I181-G182, N193F, and L204F, is constructed in a similar way as the two previous variants except that the template for the PCR reactions is plasmid pTVB146 (pPL1117 containing the TVB146-mutations within amyS and the mutagenic oligonucleotide for the first PCR is BSGM3.

[0551] BSG variant TVB162, containing the deletion of I181-G182, N193F, and E210H, is constructed in a similar way as TVB161 except that the mutagenic oligonucleotide is BSGM4 (SEQ ID NO: 21).

[0552] BSG variant TVB163, containing the deletion of I181-G182, N193F, and E214Q, is constructed in a similar way as TVB161 except that the mutagenic oligonucleotide is BSGM5 (SEQ ID NO: 22).

[0553] The above constructed BSG variants were then fermented and purified as described above in the “Material and Methods” section.

Example 2

Measurement of the Calcium- and pH-Dependent Stability

[0554] Normally, the industrial liquefaction process runs using pH 6.0-6.2 as liquefaction pH and an addition of 40 ppm free calcium in order to improve the stability at 95 °C-105 °C. Some of the herein proposed substitutions have been made in order to improve the stability at 1. lower pH than pH 6.2 and/or
2. at free calcium levels lower than 40 ppm free calcium.
Two different methods have been used to measure the improvements in stability obtained by the different substitutions in the alpha-amylase from \textit{B. stearothermophilus}:

Method 1. One assay which measures the stability at reduced pH, pH 5.0, in the presence of 5 ppm free calcium.

10 µg of the variant were incubated under the following conditions: A 0.1 M acetate solution, pH adjusted to pH 5.0, containing 5 ppm calcium and 5% w/w common corn starch (free of calcium). Incubation was made in a water bath at 95°C, for 30 minutes.

Method 2. One assay which measures the stability in the absence of free calcium and where the pH is maintained at pH 6.0. This assay measures the decrease in calcium sensitivity:

10 µg of the variant were incubated under the following conditions: A 0.1 M acetate solution, pH adjusted to pH 6.0, containing 5% w/w common corn starch (free of calcium). Incubation was made in a water bath at 95°C, for 30 minutes.

### Stability Determination

All the stability trials 1, 2 have been made using the same set up. The method was: The enzyme was incubated under the relevant conditions (1-4). Samples were taken at 0, 5, 10, 15 and 30 minutes and diluted 25 times (same dilution for all taken samples) in assay buffer (0.1 M 50 mM Britton buffer pH 7.3) and the activity was measured using the Phadebus assay (Pharmacia) under standard conditions pH 7.3, 37°C.

The activity measured before incubation (0 minutes) was used as reference (100%). The decline in percent was calculated as a function of the incubation time. The table shows the residual activity after 30 minutes of incubation.

#### Stability Method 1. /Low pH Stability Improvement

<table>
<thead>
<tr>
<th>MINUTES OF INCUBATION</th>
<th>WT. SEQ ID NO: 3 AMYLASE BSG</th>
<th>SEQ. ID NO: 3 VARIANT WITH DELETION IN POS. I181-G182 (TVB145)</th>
<th>SEQ. ID NO: 3 VARIANT WITH DELETION IN POS. I181-G182 + N193F (TVB146)</th>
<th>SEQ. ID NO: 3 VARIANT WITH DELETION IN POS. I181-G182 + N193F + E214Q (TVB163)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>5</td>
<td>29</td>
<td>71</td>
<td>83</td>
<td>77</td>
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<td>50</td>
<td>72</td>
<td>67</td>
</tr>
<tr>
<td>30</td>
<td>1</td>
<td>33</td>
<td>62</td>
<td>60</td>
</tr>
</tbody>
</table>

#### Stability Method 2. /Low pH Stability Improvement

The temperature described in method 1 has been reduced from 95°C. to 70°C, since the amylases mentioned for SEQ ID NO: 1 and 2 have a lower thermostability than the one for SEQ ID NO: 3.

<table>
<thead>
<tr>
<th>MINUTES OF INCUBATION</th>
<th>WT. SEQ ID NO: 2 AMYLASE BSG</th>
<th>SEQ. ID NO: 2 VARIANT WITH DELETION IN POS. D183-G184</th>
<th>SEQ. ID NO: 1 AMYLASE</th>
<th>SEQ. ID NO: 1 VARIANT WITH DELETION IN POS. I181-G184</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>5</td>
<td>73</td>
<td>92</td>
<td>41</td>
<td>76</td>
</tr>
<tr>
<td>10</td>
<td>59</td>
<td>88</td>
<td>19</td>
<td>69</td>
</tr>
<tr>
<td>15</td>
<td>48</td>
<td>91</td>
<td>11</td>
<td>62</td>
</tr>
<tr>
<td>30</td>
<td>28</td>
<td>92</td>
<td>3</td>
<td>59</td>
</tr>
</tbody>
</table>

#### Stability Method 2. /Low Calcium Sensitivity

<table>
<thead>
<tr>
<th>MINUTES OF INCUBATION</th>
<th>WT. SEQ ID NO: 3 AMYLASE BSG</th>
<th>SEQ. ID NO: 3 VARIANT WITH DELETION IN POS. I181-G182 (TVB145)</th>
<th>SEQ. ID NO: 3 VARIANT WITH DELETION IN POS. I181-G182 + N193F (TVB146)</th>
<th>SEQ. ID NO: 3 VARIANT WITH DELETION IN POS. I181-G182 + N193F + E214Q (TVB163)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>5</td>
<td>60</td>
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<td>81</td>
<td>82</td>
</tr>
<tr>
<td>10</td>
<td>42</td>
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<tr>
<td>30</td>
<td>15</td>
<td>67</td>
<td>78</td>
<td>79</td>
</tr>
</tbody>
</table>
Specific Activity Determination.

The specific activity was determined using the Phadebas assay (Pharmacia) as activity/mg enzyme. The activity was determined using the alpha-amylase assay described in the Materials and Methods section herein.

The specific activity of the parent enzyme and a single and a double mutation was determined to:

<table>
<thead>
<tr>
<th>Mutations</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSG: SEQ ID NO: 3 (Parent enzyme)</td>
<td>20000 NU/mg</td>
</tr>
<tr>
<td>TVB146: SEQ ID NO: 3 with the deletion in positions I181-G182 (Single mutation)</td>
<td>34600 NU/mg</td>
</tr>
<tr>
<td>TVB146: SEQ ID NO: 3 with the deletion in positions I181-G182 + N193F + E214Q (Triple mutation)</td>
<td>36300 NU/mg</td>
</tr>
</tbody>
</table>

Example 3
Pilot Plant Jet Cook and Liquefaction with Alpha-Amylase Variant TVB146

The liquefaction experiments were run in the mini-jet system using a dosage of 50 NU (S)/g DS at pH 5.5 with 5 ppm added Ca**+, to compare the performance of formulated BSG alpha-amylase variant TVB146 (SEQ ID NO: 3 with deletion in positions I181-G182 + N193F) with that of parent BSG alpha-amylase (SEQ ID NO: 3). The reaction was monitored by measuring the DE increase (Neocuproine method) as a function of time.

The following enzymes were used:

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>TVB146</td>
<td>108 KNU(S)/g, 146 KNU(SM9)/g</td>
</tr>
<tr>
<td>BSG amylase</td>
<td>101 KNU(S)/g, 98 KNU(SM9)/g</td>
</tr>
</tbody>
</table>

An amount of enzyme corresponding to 50 NU (SM9)/g DS was added, and the conductivity adjusted to 300 mS using NaCl. The standard conditions were as follows:

<table>
<thead>
<tr>
<th>Condition</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Substrate concentration</td>
<td>35% w/w (initial)</td>
</tr>
<tr>
<td></td>
<td>31.6-31.9% w/w (final)</td>
</tr>
<tr>
<td>Temperature</td>
<td>105° C, 5 minutes (Primary liquefaction)</td>
</tr>
<tr>
<td></td>
<td>95° C, 90 minutes (Secondary liquefaction)</td>
</tr>
<tr>
<td>pH (initial)</td>
<td>5.5</td>
</tr>
</tbody>
</table>

After jetting, the liquefied starch was collected and transported in sealed thermos-flasks from the pilot plant to the laboratory, where secondary liquefaction was continued at 95° C.

10 ml samples were taken at 15 minute intervals from 15-90 minutes. 2 drops of 1 N HCl were added to inactivate the enzyme. From these samples, 0.3-0.1 g (according to the expected DE) were weighed out and diluted to 100 ml. Reducing sugars were then determined according to the Neocuproine method (Determination of reducing sugar with improved precision, Dygert et al., 1965, Anal. Biochem. 13: 368) and DE values determined. The development of DE as a function of time is given in the following table:

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>TVB146 DE (neocuproine)</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>2.80</td>
</tr>
<tr>
<td>30</td>
<td>4.48</td>
</tr>
<tr>
<td>45</td>
<td>6.58</td>
</tr>
<tr>
<td>60</td>
<td>8.17</td>
</tr>
<tr>
<td>75</td>
<td>9.91</td>
</tr>
<tr>
<td>90</td>
<td>11.23</td>
</tr>
</tbody>
</table>

As can be seen the alpha-amylase variant TVB146 performed significantly better under industrially relevant application conditions at low levels of calcium than the parent BSG alpha-amylase.

Example 4
Jet Cook and Liquefaction with a Combination of Alpha-Amylase Variants (TVB146 and LE174)

Jet cook and liquefaction using a combination of the alpha-amylase variants, TVB146 and LE174 (ratio 1:1) were carried out at the following conditions:

<table>
<thead>
<tr>
<th>Substrate</th>
<th>A.E. Staley food powdered corn starch (100 lbs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D.S.</td>
<td>35% using DI water</td>
</tr>
<tr>
<td>Free Ca**+</td>
<td>2.7 ppm at pH 5.3 (none added, from the starch only)</td>
</tr>
<tr>
<td>Initial pH</td>
<td>5.3</td>
</tr>
</tbody>
</table>

Dose AF9 units (AF9 is available on request) for each enzyme variant was 28 NU/g starch dry for a total dose of 56 NU/g. Temperature in primary liquefaction 105° C. Hold time in primary liquefaction 5 minutes. Temperature in secondary liquefaction 95° C.

At 15 minutes into secondary liquefaction 1.5 gms of hydrolyzate was added to a tared one liter volumetric containing 500 cc of DI water and 1 ml of one normal HCl and the exact wt. added was recorded. This was repeated at 15 minute intervals out to 90 minutes with an additional point at 127 minutes. These were diluted to one liter and determined for dextrose equivalence via Neocuproine method as described by Dygert et al., 1965, Determination of reducing sugar with improved precision, Anal. Biochem. 13: 368.

The results were as follows:

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>DE</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>3.2</td>
</tr>
<tr>
<td>30</td>
<td>4.8</td>
</tr>
<tr>
<td>45</td>
<td>6.3</td>
</tr>
<tr>
<td>60</td>
<td>7.8</td>
</tr>
<tr>
<td>75</td>
<td>9.4</td>
</tr>
<tr>
<td>90</td>
<td>10.4</td>
</tr>
<tr>
<td>127</td>
<td>13.1</td>
</tr>
</tbody>
</table>

Example 5
Isolation of Genomic DNA from DSM 12648 and DSM 12649

The strains Bacillus sp. DSM 12648 (the AA560 alpha-amylase) and Bacillus sp. DSM 12648 (the AA349 alpha-amylase) were propagated in liquid TY medium (as described in Ausubel et al. (1995)). After 16 hours incubation
at 37°C and 300 rpm, the cells were harvested, and genomic DNA isolated by the method described by Pitcher et al. (1989).

Genomic Library Construction

[0578] Genomic DNA of strain DSM 12649 was partially digested with restriction enzyme Sau3A, and size-fractionated by electrophoresis on a 0.7% agarose gel. Fragments between 2 and 10 kb in size were isolated by electrophoresis onto DEAE-cellulose paper (Dretzen et al. 1981).

[0579] Isolated DNA fragments were ligated to BamHI digested pSJ1678 plasmid DNA, and the ligation mixture was used to transform E. coli SJ2.

Transformation

[0580] E. coli SJ2 host cells were prepared for and transformed by electroporation using a gene PULSERTM electroporator from BIO-RAD as described by the supplier.

Identification of Positive Transformant:

[0581] A DNA library in E. coli SJ2, constructed as described above, was screened on LB agar plates (described in Ausbel et al. (1995)) containing 0.5% AZCL-amylase (Megazyme) and 10 μg/ml Chloramphenicol and incubated overnight at 37°C. Clones expressing amylase activity appeared with blue diffusion haloes. One such clone was named LhH1274. The DNA was further characterized by DNA sequencing of part of the cloned Sau3A DNA fragment.

Example 6

**Determination of the DNA Sequence of the Gene Encoding Alpha-Amylase from Strain DSM 12648 (AA349)**

[0582] The clone constituting a large chromosomal fragment containing the gene encoding the amylolytic activity inserted into plasmid pSJ1678, plH1247, was used as template to specifically PCR amplify internal fragments of the alpha-amylase encoding gene by the use of degenerate primers directed towards the conserved regions in known Bacillus alpha-amylases.

[0583] The degenerate primers were directed towards the following regions/amino acid sequences:

- **For36:** GITA/L/V/I/W/I/L) (SEQ ID NO: 27)
- **For97:** VY/S/A) D/V/P/L/V(M/L/1/F) HH (SEQ ID NO: 28)
- **For227:** DG(P/I)R(F/L/I/Y) DA(A/V) EH (SEQ ID NO: 29)
- **Rev235:** DG(P/I)R(F/L/I/Y) DA(A/V) EH (SEQ ID NO: 30)

[0584] The various combinations of forward (For) and reverse (Rev) primers were used in PCR and internal DNA fragments could be amplified.

[0585] The DNA fragments were purified by QIAquick spin columns (QUIGEN) and sequenced utilizing the same degenerate primers.

[0586] From sequence the DNA sequence (SEQ ID NO: 23) of the complete coding region encoding the mature AA349 alpha-amylase (SEQ ID NO: 26) was determined by a standard primers-walking approach.

Example 7

**Determination of the DNA Sequence of the Gene Encoding Alpha Amylase from Strain DSM 12649 (AA560)**

[0587] A preparation of chromosomal DNA from strain DSM 12649 was utilized as template in a similar experiment to the one described above in Example 7 in order to determine the DNA sequence of the AA560 alpha-amylase (SEQ ID NO: 24).

Example 8

**Subcloning of the AA349 Alpha Amylase into pTVB110**

[0588] pTVB110 is a plasmid replicating in Bacillus subtilis by the use of origin of replication from pUB110 (Greven, 1978, J. Bact. 134:318-329). The plasmid further encodes the cat gene, conferring resistance towards chloramphenicol, obtained from plasmid pCl194 (Horinouchi and Weisblum, 1982, J. Bact. 150: 815-825). The plasmid harbors a truncated version of the Bacillus licheniformis alpha-amylase gene, amyL, such that the amyL promoter, signal sequence and transcription terminator are present, but the plasmid does not provide an amy-plus phenotype (halo formation on starch containing agar).

[0589] In order to express high amount of the AA349 alpha-amylase the mature gene was fused precisely to the amyL signal sequence so that transcription is initiated by the amyL promoter and translation is directed by the amyL signal sequence.

[0590] A PstI site is found within the mature AA349 alpha-amylase. Since the cloning of the gene into pTVB110 would utilize the PstI site in pTVB110, the PstI site located within the AA349 alpha-amylase gene was destroyed during the cloning (by introduction of a silent mutation for amino acid Alanine 88 (GCA to GCG).

[0591] Primers 188cloningN and 188Pst- were used to amplify an approximately 280 bp fragment by PCR on plasmid plH1247 using the Pwo polymerase under conditions recommended by the manufacturer (Boehringer Mannheim). This fragment was purified from agarose gel and used as a megaprimer (Sarkar and Sommer, 1990, Biotechniques 8: 404-407) together with primer 188cloningC to amplify the full length gene encoding the mature amylase in a second PCR.
The resulting approximately 1480 bp fragment was digested with restriction endonucleases PstI and SfiI and ligated with plasmid pTVB110 digested with the same enzymes.

Protease and amylase deleted Bacillus subtilis strain SHa273 (mentioned in WO 05/10603) was transformed with the ligation mixture and the DNA sequence of the amy-Plus transformant was verified. This plasmid is denoted pTVB231.

Oligonucleotides:

Example 9
Subcloning of the AA560 Alpha-Amylase into pTVB110

DNA sequencing revealed a high DNA identity between alpha-amylases from strains DSM12648 (AA349) and DSM 12649 (AA560). Consequently the same oligonucleotides and strategy was utilized for the cloning of AA560 alpha-amylase into expression vector pTVB110 resulting in plasmid pTVB232, which was then fermented using standard techniques.

Example 10
Purification of the AA560 Alpha-Amylase

The culture broth was flocculated by adding 0.01 ml 50% (w/w) CaCl2, 2H2O, 0.0125 ml 12% (w/w) Sodium hydroxide, 0.025 ml 10% C521 and 0.075 ml 0.1% A130 pr. ml culture broth. A clear solution was obtained after centrifugation. The enzyme solution was added ammonium sulphate to a final concentration of 1.2 M and applied on a Butyl-Toyo Pearl column (100 ml) previously equilibrated in 1.2 M ammonium sulphate, 10 mM Tris-HCl, pH 7.0. The amylase was eluted using 5 mM Tris-HCl, pH 7.0 and the eluted pool was dialysed against 5 mM Tris-HCl over night. The fraction was then subjected to ion exchange chromatography using a Q-Sepharose column (200 ml) previously equilibrated in 20 mM Tris-HCl, pH 9.0. Unbound material was washed out with the equilibration buffer, and the amylase was eluted using a linear gradient 0-1 M NaCl, 20 mM Tris-HCl, pH 9.0. Purity of the amylase preparation was above 95% judged by SDS-PAGE.

Example 11
Characterization of the AA560 Alpha-Amylase

The alpha-amylase activity was measured using both the Phadebas assay (37°C, pH 7.3) and the Alternative pNPP7 Assay (25°C, pH 7.1) described above. The pH- and temperature profiles were made at selected pH- and temperature values. The pH-profile was measured at 37°C and the temperature profile was measured at pH 9.0

Isoelectric Point was determined using isoelectric focusing (Pharmacia, Ampholine, pH 3.5-9.3).

TABLE 1

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Specific activity</th>
<th>pH</th>
<th>alpha-Amylase</th>
<th>pNPP7</th>
<th>pI</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA560 (SEQ ID NO: 4)</td>
<td>35000</td>
<td>6000</td>
<td>7-8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SP722 (SEQ ID NO: 2)</td>
<td>35000</td>
<td>6000</td>
<td>7-9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SP960 (SEQ ID NO: 1)</td>
<td>35000</td>
<td>7000</td>
<td>5-6</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

E = 3.2 cm²/(g·min) for AA560, SP722 and SP960

Example 12
Washing Test

Washing performance was evaluated by washing soiled test swatches for 15 and 30 minutes at 25°C and 40°C, respectively, in detergent solutions with the AA560 alpha-amylase of the invention.

The detergents used are disclosed in Table 2 below. The A/P Model Detergent is described in the Materials section above. The other detergents are commercially available detergents. Commercial detergents containing amylase were inactivated by microwaves before wash.

The purified recombinant AA560 alpha-amylase of Example 6 was added to the detergent solutions at the concentration indicated below. The test swatches were soiled with orange rice starch (CS-28 swatches available from CFT, Center for Test Material, Holland). After washing, the swatches were evaluated by measuring the remission at 460 nm using a Elrepho Remission Spectrophotometer. The results are expressed as ΔR=remission of the swatch washed with the alpha-amylase minus the remission of a swatch washed at the same conditions without the alpha-amylase.

TABLE 2

<table>
<thead>
<tr>
<th>Area</th>
<th>Detergent</th>
<th>Det. Dose g/l</th>
<th>Inact. Enzyme mg/l</th>
<th>Temp. °C</th>
<th>Time min</th>
<th>pH</th>
<th>Water hardness dF</th>
<th>Ca:Mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/P</td>
<td>Model detergent</td>
<td>3</td>
<td>1</td>
<td>25</td>
<td>15</td>
<td>10.5</td>
<td>6</td>
<td>2:1</td>
</tr>
<tr>
<td>Latin America</td>
<td>Omo Multi Acao</td>
<td>3</td>
<td>1</td>
<td>25</td>
<td>15</td>
<td>10.6</td>
<td>6</td>
<td>2:1</td>
</tr>
</tbody>
</table>
TABLE 2-continued

<table>
<thead>
<tr>
<th>Area</th>
<th>Detergent</th>
<th>Det. Dose g/l</th>
<th>Enzyme Inactivation mg/l</th>
<th>Temp. °C</th>
<th>Time min</th>
<th>pH</th>
<th>Water hardness 'dH</th>
<th>Ca:Mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Europe</td>
<td>Omo conc. Powder</td>
<td>4 +</td>
<td>0.2</td>
<td>40</td>
<td>30</td>
<td>10.2</td>
<td>15</td>
<td>4:1</td>
</tr>
<tr>
<td>Europe</td>
<td>Ariel Futur liquid</td>
<td>5 +</td>
<td>0.2</td>
<td>40</td>
<td>30</td>
<td>9.0</td>
<td>15</td>
<td>4:1</td>
</tr>
</tbody>
</table>

[0603] The results are shown in FIGS. 4-7. The results demonstrate that the alpha-amylase of the invention is effective in both detergents at highly alkaline pH.

[0604] The invention described and claimed herein is not to be limited in scope by the specific embodiments herein disclosed, since these embodiments are intended as illustrations of several aspects of the invention. Any equivalent embodiments are intended to be within the scope of this invention. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description. Such modifications are also intended to fall within the scope of the appended claims. In the case of conflict, the present disclosure including definitions will control.

[0605] Various references are cited herein, the disclosures of which are incorporated by reference in their entirities.

REFERENCES CITED


<table>
<thead>
<tr>
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</thead>
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<tr>
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<tr>
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</tr>
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</tr>
<tr>
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</tr>
<tr>
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</tr>
<tr>
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</tr>
<tr>
<td>---------------------------------------------------------------</td>
</tr>
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</tr>
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</tr>
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<td>---------------------------------------------------------------</td>
</tr>
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</tr>
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<tr>
<td>---------------------------------------------------------------</td>
</tr>
<tr>
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</tr>
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</tr>
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<td>---------------------------------------------------------------</td>
</tr>
<tr>
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<td>---------------------------------------------------------------</td>
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<td>Gly Tyr Tyr Asp Met Arg Asn Ile Leu Ann Gly Ser Val Val Glu Lys</td>
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<213> ORGANISM: Bacillus sp.
<400> SEQUENCE: 2

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35 40 45
Lys Gly Thr Ser Glu Asn Asp Val Gly Tyr Gly Ala Tyr Asp Leu Tyr
50 55 60
Asp Leu Gly Glu Phe Asn Glu Gly Thr Val Arg Thr Lys Tyr Gly
65 70 75 80
Thr Arg Ser Glu Leu Glu Ser Ala Ile His Ala Leu Lys Asn Asn Gly
95 100 105 110
Val Glu Val Tyr Gly Asp Val Val Met Asn His Lys Gly Gly Ala Asp
115 120 125
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135 140
Gln Glu Ile Ser Gly Asp Tyr Thr Ile Glu Ala Trp Thr Lys Phe Asp
150 155 160
Phe Pro Gly Arg Gly Asn Thr Tyr Ser Asp Phe Lys Trp Trp Arg Trp Tyr
165 170 175
His Phe Asp Gly Val Asp Trp Glu Ser Arg Glu Gly Phe Glu Asn Arg
180 185 190
Ile Tyr Lys Phe Arg Gly Asp Gly Lys Ala Trp Asp Trp Glu Val Asp
195 200 205
Ser Glu Asn Gly Asn Tyr Asp Tyr Leu Met Tyr Ala Asp Val Asp Met
210 215 220
Asp His Pro Glu Val Val Asn Ala Glu Arg Trp Gly Glu Trp Tyr
225 230 235 240
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245 250 255
Ile Lys Tyr Ser Phe Thr Arg Asp Trp Leu Thr His Val Arg Asn Ala
260 265 270
Thr Gly Lys Glu Met Phe Ala Val Ala Glu Phe Trp Lys Asp Leu
275 280 285
Gly Ala Leu Glu Asn Tyr Leu Asn Lys Thr Asn Trp Asn His Ser Val
290 295 300
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| Gly Asn Tyr Asp Met Ala Lys Leu Leu Asn Gly Thr Val Val Gln Lys  |
|------------------|------------------|------------------|------------------|
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| 325              | 330              | 335              |
| Gly Glu Ser Leu Glu Ser Phe Val Gln Glu Trp Phe Lys Pro Leu Ala |
| 340              | 345              | 350              |
| Tyr Ala Leu Ile Leu Thr Arg Glu Gin Gly Tyr Pro Ser Val Phe Tyr |
| 355              | 360              | 365              |
| Gly Asp Tyr Tyr Gly Ile Pro Thr His Ser Val Pro Ala Met Lys Ala |
| 370              | 375              | 380              |
| Lys Ile Asp Pro Ile Leu Glu Ala Arg Gin Asn Phe Ala Tyr Gly Thr |
| 385              | 390              | 395              | 400              |
| Gln His Asp Tyr Phe Asp His His Asn Ile Ile Gly Trp Thr Arg Glu |
| 405              | 410              | 415              |
| Gly Asn Thr Thr His Pro Asn Ser Gly Leu Ala Thr Ile Met Ser Asp |
| 420              | 425              | 430              |
| Gly Pro Gly Glu Lys Trp Met Tyr Val Gly Gin Asn Lys Ala Gly      |
| 435              | 440              | 445              |
| Gin Val Trp His Asp Ile Thr Gly Gin Lys Pro Gly Thr Val Thr Ile |
| 450              | 455              | 460              |
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| 485              |

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<213> ORGANISM: B. stearothermophilus
<400> SEQUENCE: 3

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| Leu Ser Ser Leu Gly Ile Thr Ala Leu Trp Leu Pro Pro Ala Tyr Lys |
| 35               | 40               | 45               |
| Gly Thr Ser Arg Ser Asp Val Gly Tyr Gly Val Tyr Asp Leu Tyr Asp |
| 50               | 55               | 60               |
| Leu Gly Glu Phe Asn Gin Lys Gin Ala Val Arg Thr Lys Tyr Gly Thr |
| 65               | 70               | 75               | 80               |
| Lys Ala Gin Tyr Leu Gin Ala Gin Ala Gin Gin Gin Gin Gin Gin Gin |
| 85               | 90               |
| Gin Val Tyr Ala Asp Val Val Phe Asp His Lys Gly Gin Ala Asp Gly |
| 100              | 105              | 110              |
| Thr Glu Trp Val Ala Val Gin Val Gin Gin Gin Gin Gin Gin Gin Gin |
| 115              | 120              | 125              |
| Gin Ile Ser Gly Thr Tyr Gin Gin Gin Ala Gin Trp Thr Lys Phe Asp Phe |
| 130              | 135              | 140              |
| Pro Gly Arg Gly Gin Thr Tyr Ser Ser Phe Lys Trp Arg Trp Tyr His |
| 145              | 150              | 155              | 160              |
| Phe Asp Gly Val Gin Glu Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin |
| 165              | 170              | 175              |
Lys  Phe  Arg  Gly  Ile  Gly  Lys  Ala  Trp  Asp  Trp  Glu  Val  Asp  Thr  Glu
   180    195    190
Asn  Gly  Asn  Tyr  Asp  Tyr  Leu  Met  Tyr  Ala  Asp  Leu  Asp  Met  Asp  His
   195    200    205
Pro  Glu  Val  Val  Thr  Glu  Leu  Lys  Ser  Trp  Gly  Lys  Trp  Tyr  Val  Asn
   210    215    220
Thr  Thr  Asn  Ile  Asp  Gly  Phe  Arg  Leu  Asp  Ala  Val  Lys  His  Ile  Lys
   225    230    235    240
Phe  Ser  Phe  Phe  Pro  Asp  Trp  Leu  Ser  Asp  Val  Arg  Ser  Gln  Thr  Glu
   245    250    255
Lys  Pro  Leu  Phe  Thr  Val  Gly  Glu  Tyr  Trp  Ser  Tyr  Asp  Ile  Asn  Lys
   260    265    270
Leu  His  Asn  Tyr  Ile  Met  Lys  Thr  Asn  Gly  Thr  Met  Ser  Leu  Phe  Asp
   275    280    285
Ala  Pro  Leu  His  Asn  Lys  Phe  Tyr  Thr  Ala  Ser  Lys  Ser  Gly  Gly  Thr
   290    295    300
Phe  Asp  Met  Arg  Thr  Leu  Met  Thr  Asn  Thr  Leu  Met  Lys  Asp  Gln  Pro
   305    310    315    320
Thr  Leu  Ala  Val  Thr  Phe  Val  Asn  His  Asp  Thr  Glu  Pro  Gly  Gin
   325    330    335
Ala  Leu  Gln  Ser  Trp  Val  Asp  Pro  Trp  Phe  Lys  Pro  Leu  Ala  Tyr  Ala
   340    345    350
Phe  Ile  Leu  Thr  Arg  Gln  Glu  Gly  Tyr  Pro  Cys  Val  Phe  Tyr  Gly  Asp
   355    360    365
Tyr  Tyr  Gly  Ile  Pro  Gln  Tyr  Asn  Ile  Pro  Ser  Leu  Lys  Ser  Lys  Ile
   370    375    380
Asp  Pro  Leu  Leu  Ile  Arg  Arg  Asp  Tyr  Ala  Tyr  Gly  Thr  Gin  His
   385    390    395    400
Asp  Tyr  Leu  Asp  His  Ser  Asp  Ile  Ile  Gly  Trp  Thr  Arg  Glu  Gly  Val
   405    410    415
Thr  Glu  Lys  Pro  Gly  Ser  Gly  Leu  Ala  Ala  Leu  Ile  Thr  Asp  Gly  Pro
   420    425    430
Gly  Gly  Ser  Lys  Trp  Met  Tyr  Val  Gly  Lys  Gin  His  Ala  Gly  Lys  Val
   435    440    445
Phe  Tyr  Asp  Leu  Thr  Gly  Asn  Arg  Ser  Asp  Thr  Val  Thr  Ile  Asn  Ser
   450    455    460
Asp  Gly  Trp  Gly  Glu  Phe  Lys  Val  Asn  Gly  Ser  Val  Ser  Val  Trp
   465    470    475    480
Val  Pro  Arg  Lys  Thr  Thr  Val  Ser  Thr  Ile  Ala  Trp  Ser  Ile  Thr  Thr
   485    490    495
Arg  Pro  Trp  Thr  Asp  Glu  Phe  Val  Arg  Trp  Thr  Glu  Pro  Arg  Leu  Val
   500    505    510

Ala  Trp

<210> SEQ ID NO 4
<211> LENGTH: 483
<212> TYPE: PRT
<213> ORGANISM: B. licheniformis
<400> SEQUENCE: 4
Ala  Asn  Leu  Asn  Gly  Thr  Leu  Met  Gln  Tyr  Phe  Glu  Trp  Tyr  Met  Pro
Asp Gly Gln His Trp Arg Arg Leu Gln Asp Ser Ala Tyr Leu 20 25 30
Ala Glu His Gln Ile Thr Ala Val Trp Ile Pro Pro Ala Tyr Lys Gly 35 40 45
Thr Ser Gln Ala Asp Val Gly Tyr Gly Ala Tyr Asp Leu Tyr Asp Leu 50 55 60
Gly Glu Phe His Gln Lys Gly Thr Val Arg Thr Lys Tyr Gly Thr Lys 65 70 75 80
Gly Glu Leu Gln Ser Ala Ile Lys Ser Leu His Ser Arg Asp Ile Asn 85 90 95
Val Tyr Gly Asp Val Val Ile Asn His Lys Gly Ala Asp Ala Thr 100 105 110
Glu Asp Val Thr Ala Val Glu Val Asp Pro Ala Asp Arg Asn Arg Val 115 120 125
Ile Ser Gly Glu His Leu Ile Lys Ala Trp Thr His Phe His Phe Pro 130 135 140
Gly Arg Glu Ser Thr Tyr Ser Asp Phe Lys Trp His Trp Tyr His Phe 145 150 155 160
Asp Gly Thr Asp Trp Asp Glu Ser Arg Lys Leu Asn Arg Ile Tyr Lys 165 170 175
Phe Glu Gly Lys Ala Trp Asp Trp Glu Val Ser Asn Glu Asp Gly Asn 180 185 190
Tyr Asp Tyr Leu Met Tyr Ala Asp Tyr Asp His Pro Asp Val 195 200 205
Ala Ala Glu Ile Lys Arg Trp Gly Thr Trp Tyr Ala Asn Glu Leu Gln 210 215 220
Leu Asp Gly Phe Arg Leu Asp Ala Val Lys His Ile Lys Phe Ser Phe 225 230 235 240
Leu Arg Asp Trp Val Asn His Val Arg Glu Lys Thr Gly Lys Glu Met 245 250 255
Phe Thr Val Ala Glu Tyr Trp Gln Asn Asp Leu Gly Ala Leu Glu Asn 260 265 270
Tyr Leu Asn Lys Thr Asn Phe Asn His Ser Val Phe Asp Val Pro Leu 275 280 285
His Tyr Gln Phe His Ala Ala Ser Thr Gln Gly Gly Gly Tyr Asp Met 290 295 300
Arg Lys Leu Leu Asn Gly Thr Val Val Ser Lys His Pro Leu Lys Ser 305 310 315 320
Val Thr Phe Val Asp Asn His Thr Glu Asp Gly Gln Ser Leu Glu 325 330 335
Ser Thr Val Gln Thr Trp Phe Lys Pro Leu Ala Tyr Ala Phe Ile Leu 340 345 350
Thr Arg Glu Ser Gly Tyr Pro Gln Val Phe Tyr Gly Asp Met Tyr Gly 355 360 365
Thr Lys Gly Asp Ser Gln Arg Glu Ile Pro Ala Leu Lys His Lys Ile 370 375 380
Glu Pro Ile Leu Lys Ala Arg Lys Glu Tyr Ala Tyr Gly Ala Gln His 385 390 395 400
Asp Tyr Phe Asp His His Asp Ile Val Gly Trp Thr Arg Glu Gly Asp 405 410 415
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<210> SEQ ID NO 5
<211> LENGTH: 480
<212> TYPE: PRT
<213> ORGANISM: B. amyloliquefaciens

<400> SEQUENCE: 5
Val Asn Gly Thr Leu Met Gln Tyr Phe Glu Trp Tyr Thr Pro Asn Asp
1     5     10     15
Gly Gln His Trp Lys Arg Leu Gln Asn Asp Ala Glu His Leu Ser Asp
20    25    30
Ile Gly Ile Thr Ala Val Trp Ile Pro Pro Ala Tyr Lys Glu Leu Ser
35    40    45
Gln Ser Asp Asn Gly Tyr Gly Pro Tyr Thr Asp Leu Asp Glu Gly Glu
50    55    60
Phe Gln Gln Lys Gly Thr Val Arg Thr Lys Tyr Gly Thr Lys Ser Glu
65    70    75    80
Leu Gln Asp Ala Ile Gly Ser Leu His Ser Arg Asn Val Glu Val Tyr
85    90    95
Gly Asp Val Val Leu Asn His Lys Ala Gly Ala Asp Ala Thr Glu Asp
100   105   110
Val Thr Ala Val Glu Val Asn Pro Ala Asn Arg Asn Glu Glu Thr Ser
115   120   125
Glu Glu Tyr Gln Ile Lys Ala Trp Thr Asp Phe Arg Phe Pro Gly Arg
130   135   140
Gly Asn Thr Tyr Ser Asp Phe Lys Trp His Trp His Tyr Asp Gly
145   150   155   160
Ala Asp Trp Asp Glu Ser Arg Lys Ile Ser Asp Ile Phe Lys Phe Arg
165   170   175
Gly Glu Gly Lys Ala Trp Asp Trp Glu Val Ser Ser Glu Asn Gly Asn
180   185   190
Tyr Asp Tyr Leu Met Tyr Ala Asp Val Asp Tyr Asp His Pro Asp Val
195   200   205
Val Ala Glu Thr Lys Trp Gly Ile Trp Tyr Ala Asn Glu Leu Ser
210   215   220
Leu Asp Gly Phe Arg Ile Asp Ala Ala Lys His Ile Lys Phe Ser Phe
225   230   235   240
Leu Arg Asp Trp Val Gln Ala Val Arg Gln Ala Thr Gly Lys Glu Met
245   250   255
Phe Thr Val Ala Glu Tyr Trp Glu Asn Asn Ala Gly Lys Leu Glu Asn
260   265   270
Tyr Leu Asn Lys Thr Ser Phe Asn Glu Ser Val Phe Asp Val Pro Leu
275   280   285
His His Asn Gly Thr Asn Gly Thr Met Met Gln Tyr Phe Glu Trp Tyr
Leu Pro Asn Asp Gly Asn His Trp Asn Arg Leu Asn Ser Asp Ala Ser
Asn Leu Lys Ser Lys Gly Ile Thr Ala Val Trp Ile Pro Pro Ala Trp
Lys Gly Ala Ser Gln Asp Val Gly Tyr Gly Ala Tyr Asp Leu Tyr
Asp Leu Gly Glu Phe Asn Gln Gly Thr Val Arg Thr Lys Tyr Gly
Thr Arg Ser Gln Leu Gln Ala Val Thr Ser Leu Lys Asn Asn
Ile Gln Val Tyr Gly Asp Val Val Met Asn His Lys Gly Gly Ala Asp
Ala Thr Glu Met Val Arg Ala Val Glu Val Asn Pro Asn Arg Asn
Gln Glu Val Thr Gly Glu Tyr Thr Ile Glu Ala Trp Thr Arg Phe Asp
Phe Pro Gly Arg Gly Asn Thr His Ser Ser Phe Lys Trp Trp Arg Tyr
His Phe Asp Gly Val Asp Trp Asp Gln Ser Arg Leu Asn Asn Arg

<210> SEQ ID NO: 6
<211> LENGTH: 485
<212> TYPE: PRT
<213> ORGANISM: Bacillus sp.

<400> SEQUENCE: 6

His Phe Asn Leu Gln Ala Ala Ser Ser Gln Gln Gly Gly Tyr Asp Met
Arg Arg Leu Leu Asp Glu Thr Val Val Ser Arg His Pro Glu Lys Ala
Val Thr Phe Val Glu Asn His Arg Thr Gln Pro Gly Gln Ser Leu Glu
Ser Thr Val Gln Thr Trp Phe Lys Pro Leu Ala Tyr Ala Phe Ile Leu
Thr Arg Glu Ser Gly Tyr Pro Gln Val Phe Tyr Gly Asp Met Tyr Gly
Thr Lys Gly Thr Ser Pro Lys Glu Ile Pro Ser Leu Lys Asp Asn Ile
Glu Pro Ile Leu Lys Ala Arg Lys Glu Tyr Ala Tyr Gly Pro Gln His
Asp Tyr Ile Asp His Pro Asp Val Ile Gly Trp Thr Arg Glu Gly Asp
Ser Ser Ala Ala Lys Ser Gly Leu Ala Ala Leu Ile Thr Asp Gly Pro
Gly Gly Ser Lys Arg Met Tyr Ala Gly Leu Lys Ala Gly Glu Thr
Trp Tyr Asp Ile Thr Gly Asn Arg Ser Asp Thr Val Lys Ile Gly Ser
Asp Gly Trp Gly Glu Phe His Val Asn Asp Gly Ser Val Ser Ile Tyr
ILE TYR LYS PHE ARG GLY HIS GLY LYS ALA TRP ASP TRP GLU VAL ASP
180 185 190

Thr Glu Asn Gly Asn Tyr Asp Tyr Leu Met Tyr Ala Asp Ile Asp Met
195 200 205

Asp His Pro Glu Val Val Asn Glu Leu Arg Asn Thr Gly Val Trp Tyr
210 215 220

Thr Asn Thr Leu Gly Leu Asp Gly Phe Arg Ile Asp Ala Val Lys His
225 230 235 240

ILE LYS TYR SER PHE THR ASP TRP ILE ASN HIS VAL ARG SER ALA
245 250 255

Thr Gly Lys Asn Met Phe Ala Val Ala Glu Phe Thr Asp Lys Asp Leu
260 265 270

Gly Ala Ile Glu Asn Tyr Leu Glu Tyr Thr Asn Asn His Ser Val
275 280 285

Phe Asp Val Pro Leu His Tyr Asn Leu Tyr Val Ala Ser Lys Ser Gly
290 295 300

Gly Asn Tyr Asp Met Arg Asn Ile Phe Asn Gly Thr Val Val Gln Arg
305 310 315 320

His Pro Ser His Ala Val Thr Phe Val Asp Asn His Ser Gin Pro
325 330 335

Glu Glu Ala Leu Glu Ser Phe Glu Val Glu Trp Phe Lys Pro Leu Ala
340 345 350

Tyr Ala Leu Thr Leu Thr Arg Glu Gin Gly Tyr Pro Ser Val Phe Tyr
355 360 365

Gly Asp Tyr Tyr Gly Ile Pro Thr His Gly Val Pro Ala Met Arg Ser
370 375 380

Lys Ile Asp Pro Ile Leu Glu Ala Arg Gin Lys Tyr Ala Tyr Gly Lys
385 390 395 400

Gln Asn Asp Tyr Leu Asp His His Asn Ile Ile Gly Thr Arg Glu
405 410 415

Gly Asn Thr Ala His Pro Asn Ser Gly Leu Ala Thr Ile Met Ser Asp
420 425 430

Gly Ala Gly Gly Ser Lys Trp Met Phe Val Gly Arg Asn Lys Ala Gly
435 440 445

Gln Val Trp Ser Asp Ile Thr Gly Asn Arg Thr Tyr Val Thr Ile
450 455 460

Asn Ala Asp Gly Trp Gly Asn Phe Ser Val Asn Gly Gly Ser Val Ser
465 470 475 480

Ile Trp Val Asn Lys
485

<210> SEQ ID NO 7
<211> LENGTH: 485
<212> TYPE: PRT
<213> ORGANISM: Bacillus sp.

<400> SEQUENCE: 7

His His Asn Gly Thr Asn Gly Thr Met Met Gin Tyr Phe Glu Trp Tyr
1  5 10 15

Leu Pro Asn Asp Gly Asn His Thr Asn Arg Leu Arg Asp Ala Ala
20 25 30

Asn Leu Lys Ser Lys Gly Ile Thr Ala Val Trp Ile Pro Pro Ala Trp
Lys Gly Thr Ser Gln Asn Asp Val Gly Tyr Gly Ala Tyr Asp Leu Tyr
50 55 60
Asp Leu Gly Glu Phe Asn Gln Lys Gly Thr Val Arg Thr Lys Tyr Gly
65 70 75 80
Thr Arg Asn Gln Leu Gln Ala Ala Val Thr Ser Leu Lys Asn Asn Gly
85 90 95
Ile Gln Val Tyr Gly Asp Val Val Met Asn His Lys Gly Gly Ala Asp
100 105 110
Gly Thr Glu Ile Val Asn Ala Val Glu Val Asn Arg Ser Asn Arg Asn
115 120 125
Gln Glu Thr Ser Gly Glu Tyr Ala Ile Glu Ala Trp Thr Lys Phe Asp
130 135 140
Phe Pro Gly Arg Gly Asn His Ser Ser Phe Lys Trp Arg Trp Tyr
145 150 155 160
His Phe Asp Gly Thr Asp Trp Asp Glu Arg Ser Arg Gln Leu Gln Asn Lys
165 170 175
Ile Tyr Lys Phe Arg Gly Thr Gly Lys Ala Trp Asp Trp Glu Val Asp
180 185 190
Thr Glu Asn Gly Asn Tyr Asp Tyr Leu Met Tyr Ala Asp Val Asp Met
195 200 205
Asp His Pro Glu Val Ile His Glu Arg Asn Trp Gly Val Trp Tyr
210 215 220
Thr Asn Thr Leu Asn Leu Asp Gly Phe Arg Ile Asp Ala Val Lys His
225 230 235 240
Ile Lys Tyr Ser Phe Thr Arg Asp Trp Leu Thr His Val Arg Asn Thr
245 250 255
Thr Gly Lys Pro Met Phe Ala Val Ala Glu Phe Trp Lys Asp Leu
260 265 270
Gly Ala Ile Glu Asn Tyr Leu Asn Lys Thr Ser Trp Asn His Ser Val
275 280 285
Phe Asp Val Pro Leu His Tyr Asn Leu Tyr Asn Asn Ser Gly
290 295 300
Gly Tyr Tyr Asp Met Arg Asn Ile Leu Asn Gly Ser Val Glu Lys
305 310 315 320
His Pro Thr His Ala Val Thr Phe Val Asp Asn His Asp Ser Gln Pro
325 330 335
Gly Glu Ala Leu Glu Ser Phe Val Gln Gln Trp Phe Lys Pro Leu Ala
340 345 350
Tyr Ala Leu Val Leu Thr Arg Glu Gly Tyr Pro Ser Val Phe Tyr
355 360 365
Gly Asp Tyr Tyr Gly Ile Pro Thr His Gly Val Pro Ala Met Lys Ser
370 375 380
Lys Ile Asp Pro Leu Leu Gln Ala Arg Glu Thr Phe Ala Tyr Gly Thr
385 390 395 400
Gln His Asp Tyr Phe Asp His His Ile Ile Gly Trp Thr Arg Glu
405 410 415
Gly Asn Ser Ser His Pro Asn Ser Gly Leu Ala Thr Ile Met Ser Asp
420 425 430
Gly Pro Gly Gly Asn Lys Trp Met Tyr Val Gly Lys Asn Lys Ala Gly
435 440 445
Val Trp Val Lys Gln

<210> SEQ ID NO 8
<211> LENGTH: 485
<212> TYPE: PRT
<213> ORGANISM: Bacillus sp.

<400> SEQUENCE: 8

His His Asn Gly Thr Asn Gly Thr Met Met Gln Tyr Phe Glu Trp His
1  5  10  15

Leu Pro Asn Asp Gly Asn His Trp Asn Arg Leu Arg Asp Asp Ala Ser
20 25 30

Asn Leu Arg Asn Asp Gly Ile Thr Ala Ile Trp Ile Pro Pro Ala Trp
35 40 45

Lys Gly Thr Ser Gln Asn Asp Val Gly Tyr Gln Asp Tyr Leu Tyr
50 55 60

Asp Leu Gly Glu Phe Asn Gln Gln Gly Thr Val Arg Thr Lys Tyr Gln
65 70 75 80

Thr Arg Ser Gln Leu Glu Ser Ala Ile His Ala Leu Lys Asn Asn Gly
85 90 95

Val Gln Val Tyr Gly Asp Val Val Met Asn His Lys Gln Gln Gly Ala Asp
100 105 110

Ala Thr Glu Asn Val Leu Ala Val Glu Val Asn Pro Asn Asn Arg Asn
115 120 125

Gln Glu Ile Ser Gly Asp Tyr Thr Ile Glu Ala Trp Thr Lys Phe Asp
130 135 140

Phe Pro Gly Arg Asn Tyr Thr Ser Asp Phe Lys Trp Arg Trp Tyr
145 150 155 160

His Phe Asp Gly Val Asp Trp Gln Ser Arg Gln Phe Glu Asn Arg
165 170 175

Ile Tyr Lys Phe Arg Gly Asp Gly Lys Ala Trp Asp Trp Glu Val Asp
180 185 190

Ser Glu Asn Gly Asn Tyr Asp Tyr Leu Met Tyr Ala Asp Val Asp Met
195 200 205

Asp His Pro Glu Val Val Asn Glu Leu Arg Arg Asp Gly Glu Trp Tyr
210 215 220

Thr Asn Thr Leu Asn Leu Asp Gly Phe Arg Ile Asp Ala Val Lys His
225 230 235 240

Ile Lys Tyr Ser Phe Thr Arg Asp Trp Leu Thr His Val Arg Asn Ala
245 250 255

Thr Gly Lys Glu Met Phe Ala Val Ala Glu Phe Trp Lys Asp Leu
260 265 270

Gly Ala Leu Glu Asn Tyr Leu Asn Lys Thr Asn Trp Asn His Ser Val
275 280 285

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

Gly Asn Tyr Asp Met Ala Lys Leu Leu Asn Gly Thr Val Val Gln Lys
His Pro Met His Ala Val Thr Phe Val Asp Aen His Asp Ser Gln Pro
325 330 335
Gly Glu Ser Leu Glu Ser Phe Val Gin Glu Trp Phe Lys Pro Leu Ala
340 345 350
Tyr Ala Leu Ile Leu Thr Arg Glu Gin Gly Tyr Pro Ser Val Phe Tyr
355 360 365
Gly Asp Tyr Tyr Gly Ile Pro Thr His Ser Val Pro Ala Met Lys Ala
370 375 380
Lys Ile Asp Pro Ile Leu Glu Ala Arg Gin Asn Phe Ala Tyr Gly Thr
385 390 395 400
Gln His Asp Tyr Phe Asp His Asn Ile Ile Gly Trp Thr Arg Glu
405 410 415
Gly Aen Thr Thr His Pro Asn Ser Gly Leu Ala Thr Ile Met Ser Asp
420 425 430
Gly Pro Gly Gly Glu Trp Met Tyr Val Gly Gin Aen Lys Ala Gly
435 440 445
Gln Val Trp His Asp Ile Thr Gly Asn Lys Pro Gly Thr Val Thr Ile
450 455 460
Aen Ala Asp Gly Trp Ala Aen Phe Ser Val Aen Gly Gly Ser Val Ser
465 470 475 480
Ile Trp Val Lys Arg
485

<210> SEQ ID NO 9
<211> LENGTH: 1455
<212> ORGANISM: Bacillus sp.
<400> SEQUENCE: 9
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gcacttaga tcocacgtcg atogagggg acctcgcag ataagtcag ccaaagggcc
120
tatgaatttt ataggatttc agagttttac cagaaagggaa caagttgcac aaaaaatgga
180
aacacacccc agctacagcc tcggcgtacc tcttttaasa ataacgctat caggtatat
240
ggtgagtctc tctagatcct taagtttagc gcagatgtca cggaaattgt aaagctggta
300
gaagttcgct ggacacagcc aacccagcgg aaacctggag agatgtcatt aagaggctgg
360
aacaagttcg attttccttc gcagggaaat aacatcca gottttagct gcgcctgctg
420
cattttgag gcagcacttc gcgcggttct aacaaaacc aataaattg
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660
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720
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gaatcttttg tccaacaaag gttaaaaccg ctgtcgatag cattgtgttc gacaagggaa
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caggttatac cttccgttat ttaatgggag aatcaggtta ttcacaccga ggtgttccg
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gcatatgaa atttaataga cctctctctcg cagcgcaagtc aaacctttttg ctatggtacc
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cagcatatt aacctgtgct acaatgtatt ttcggttga caagagaggg aataagctcc
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catcaaatg aacgttcttgcc cacatttagt tccagatgttc cagttggttaa caaatggagtg
1320
tgtgtgggaa aaataaacaag ggcaacagtt cagacagata ttaacgggaat tggcaagcgc
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gttggtgta agcag
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<210> SEQ ID NO 10
<211> LENGTH: 1455
<212> TYPE: DNA
<213> ORGANISM: Bacillus sp.
<400> SEQUENCE: 10

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<210> SEQ ID NO 12
<211> LENGTH: 1920
<212> TYPE: DNA
<213> ORGANISM: B. licheniformis

<400> SEQUENCE: 12

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<210> SEQ ID NO 13
<211> LENGTH: 2084
<212> TYPE: DNA
<213> ORGANISM: B. amyoliquefaciens
<400> SEQUENCE: 13

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<211> LENGTH: 1395
<212> TYPE: DNA
<213> ORGANISM: Bacillus sp.
<400> SEQUENCE: 14

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<210> SEQ ID NO: 15
<211> LENGTH: 1455
<212> TYPE: DNA
<213> ORGANISM: Bacillus sp.
<400> SEQUENCE: 15

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gctattgga ttccgctgtc ctgggaaggg aatttgccaa atgttgctgg gttaggaccc 180
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<210> SEQ ID NO 16
<211> LENGTH: 23
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Primer
<400> SEQUENCE: 16

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<210> SEQ ID NO 17
<211> LENGTH: 22
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Primer
<400> SEQUENCE: 17

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<210> SEQ ID NO 18
<211> LENGTH: 68
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Primer
<400> SEQUENCE: 18

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<210> SEQ ID NO 19
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Primer
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<210> SEQ ID NO 20
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<212> TYPE: DNA
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<213> ORGANISM: Artificial Sequence
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<223> OTHER INFORMATION: Primer

<400> SEQUENCE: 21

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<210> SEQ ID NO: 22
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<212> TYPE: DNA
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<400> SEQUENCE: 22

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<210> SEQ ID NO: 23
<211> LENGTH: 1458
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<213> ORGANISM: Bacillus sp
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<222> LOCATION: (1..1455)

<400> SEQUENCE: 23

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His His Aan Gly Thr Aan Gly Thr Met Met Gin Tyr Phe Glu Trp Tyr
1   5   10  15

tca cca aat gac gga aac cat tgg aat aag taa aag ctc gat gca aat
Leu Pro Aan Amp Gly Aan His Trp Aan Arg Leu Arg Ser Aan Ser
20  25  30

aac cta aat aat gac ggg att act tca gcc gtt tgg att cct cct gca tgg
Aan Leu Lys Amp Lys Gly Ile Ser Ala Val Trp Ile Pro Pro Aan Ala Trp
35  40  45

aag ggt gcc tct cca aat gat gtt ggg tat ggt gct tat gat ctt ttt
Lys Ala Ser Gln Aan Ser Val Gly Tyr Ala Tyr Asp Leu Tyr
50  55  60

gat tta gga gaa tcc aat cca aac gga acc att cgt aca aca aat gga
Amp Leu Gly Phe Aan Gin Lys Thr Ile Arg Thr Lys Tyr Gly
65  70  75  80

aac cgc aat cag tta cag gct gca gtt aac gcc tgt aaa aag aat gga
Thr Arg Aan Gin Leu Gln Ala Ala Val Aan Ala Leu Ser Aan Gly
85  90  95

att cca gtt tat ggc gat gtt gta atg aat cat aac gga gca cac
Ile Gln Val Tyr Gly Amp Val Val Met Aan His Lys Gly Aan Aas
100 105 110

get acc gaa atg gtt agg gca gtt gaa gta aac cgg aat aag aat
Aan Thr Glu Met Val Arg Ala Val Glu Val Aan Pro Aan Aan Arg Aan
115 120 125

ca aat gtt tcc ggt gaa tat aca att gag gct tgg aca aag ttc gac
432
Gln Glu Val Ser Gly Glu Tyr Thr Ile Glu Ala Thr Thr Lys Phe Asp
130 135 140

-continued

ttt cca gga cga ggt att act cat tca aac ttc aas tgg aga tgg tat
Phe Pro Gly Arg Gly Asn Thr His Ser Asn Phe Lys Thr Arg Thr Tyr
145 150 155 160

cac ttt gat gga gta gat tgg gat cag tca cgt aag ctc cag aat cga
His Phe Asp Gly Val Asp Trp Asp Gln Ser Arg Lys Leu Asn Arg
165 170 175

att tat aaa ttt aga ggt gat gga aag ggg tgg gat tgg gaa gtc gat
Ile Tyr Lys Phe Arg Gly Asp Gly Lys Gly Trp Asp Trp Glu Val Asp
180 185 190

aca gaa acc ggt acc tat gat tac cta atg tat gca gat att gac atg
Thr Glu Asn Gly Asn Tyr Asp Tyr Leu Met Tyr Ala Asp Ile Asp Met
195 200 205 210

gat cac cca gac gta gtt act gat gca gtt ggt gtt tgg ttt
Asp His Pro Gly Val Val Val Val Glu Arg Arg Val Asp Trp Thr Tyr
215 220 225 230 235 240

eaat att acc cca gtt gat gtt aga aat gca gta aaa cat
Thr Arg Thr Leu Gly Leu Asp Gly Phe Arg Ile Asp Ala Val Lys His
245 250 255

ata aaa tac agc ttt act cgt gat tgg att att cat gtt aga aat gca
Ile Tyr Ser Phe Thr Arg Asp Trp Ile Asn His Val Arg Ser Ala
260 265 270 275

act ggc aat atg ttt gcc gct gtt ggc gaa taa tgg aat gat tta
Thr Gly Lys Asn Met Phe Ala Val Ala Glu Phe Trp Lys Asp Met
280 285 290

ggt gct att gaa acc tat tta aac aac cag ggt gac cat tca gtc
Gly Ala Ile Glu Asn Thr Leu Gly Leu Arg Thr Leu Arg His Ser Val
295 300 305

ttt gat gtt cag ctc cac tat aac ctc tat aat gct tca aac agc gga
Phe Asp Val Pro Leu His Tyr Asn Leu Tyr Asn Ser Tyr Ser Gly
310 315 320

ggg aat tat gat atg aag cca ata ttt aat ggt acc gtc gtt cag aag
Gly Asn Tyr Asp Met Arg Glu Ile Phe Asn Thr Val Gly Arg
325 330 335 340

cac cag atg cat gct gaa aat gct gat cat gat tgg gaa ggg ggg
cat cca ggt gat cag ctc ttt gat gat aat gct gat tgg cag cca ctc
His Pro Met His Ala Val Thr Phe Val Asp Asp His Ser Glu Pro
345 350 355 360 365

gaa gaa gct tta ggc gct ctc ttt gtt gaa gaa tgg gaa aac cca tta ggc
Glu Glu Ala Leu Gly Ser Pro Leu Ala
365 370 375 380 385

tat gct ttc aca tta cag gaa cca ggg tac cct ttc tct gta ttt tat
Tyr Ala Leu Thr Leu Thr Arg Glu Gln Gly Tyr Pro Ser Val Phe Tyr
390 395 400 405

gga gat tat ttt ggc att cca acg cat ggt tca cca ggc atg aat ttc
gly asp tyr ty r l y pro thr his gly pro ala l y s e r
410 415 420 425

aaa att gac ccg att cca gga gcc ctg caa aag tat gca tat gga aga
ly s e r
430 435 440 445

cac caa gac att cac ggg cac aac tac ccc cag aac tgg gaa ggg
ca cag tac tga gac cat ctc aat ggt gaa
Gin Asp Thr Leu Asp His His Ile Ile Gly Trp Thr Arg Glu
450 455 460 465

ggg gaa gcc gga aag tgg aat ttc gat cgg cct ctt ggt gaa ggc
1344
Gly Ala Gly Asn Lys Trp Met Met Glu Tyr Phe Glu Trp Tyr 1 5 10 15
Leu Pro Asn Asp Gly Asn His Trp Asn Arg Leu Arg Ser Asp Ala Ser 20 25 30
Asn Leu Lys Asp Lys Gly Ile Ser Ala Val Trp Ile Pro Pro Ala Trp 35 40 45
Lys Gly Ala Ser Gin Asp Val Gly Tyr Gly Ala Tyr Asp Leu Tyr 50 55 60
Asp Leu Gly Glu Phe Asn Gin Gly Tyr Thr Ile Arg Thr Lys Tyr Glu 65 70 75 80
Thr Arg Asn Gin Leu Gin Ala Ala Val Asn Ala Leu Lys Ser Asn Gly 95 100 105 110
Ile Gin Val Tyr Gly Asp Val Met Asn His Lys Gly Gly Ala Asp 120 125
Ala Thr Glu Met Val Arg Ala Val Glu Val Asn Pro Asn Asn Arg Asn 130 135 140
Gln Glu Val Ser Gly Glu Tyr Thr Ile Glu Ala Thr Lys Phe Asp 145 150 155 160
Phe Pro Gly Arg Gly Asn Thr His Ser Asn Phe Lys Trp Arg Trp Tyr 170 175
His Phe Asp Gly Val Asp Trp Asp Gin Ser Arg Lys Leu Asn Asn Arg 185 190
Ile Tyr Lys Phe Arg Gly Asp Gly Lys Gly Trp Asp Trp Glu Val Asp 200 205
Thr Glu Asn Gly Asn Tyr Asp Tyr Leu Met Tyr Ala Asp Ile Asp Met 215 220
Asp His Pro Glu Val Val Asn Leu Arg Asn Trp Gly Val Trp Tyr 230 235 240
Thr Asn Thr Leu Gly Leu Asp Gly Phe Arg Ile Asp Ala Val Lys His 245 250 255
Ile Lys Tyr Ser Phe Thr Arg Asp Trp Ile Asn His Val Arg Ser Ala 260 265 270
Thr Gly Lys Asn Met Phe Ala Val Ala Glu Phe Trp Lys Asp Leu 275 280 285
Phe Asp Val Pro Leu His Tyr Asn Leu Tyr Asn Ala Ser Lys Ser Gly
290 295 300
Gly Asn Tyr Asp Met Arg Gln Ile Phe Asn Gly Thr Val Val Gln Arg
305 310 315 320
His Pro Met His Ala Val Thr Phe Val Asp Asn His Asp Ser Gln Pro
325 330 335
Glu Glu Ala Leu Glu Ser Phe Val Glu Glu Trp Phe Lys Pro Leu Ala
340 345 350
Tyr Ala Leu Thr Leu Thr Arg Glu Gly Tyr Pro Ser Val Phe Tyr
355 360 365
Gly Asp Tyr Tyr Gly Ile Pro Thr His Gly Val Pro Ala Met Lys Ser
370 375 380
Lys Ile Asp Pro Ile Leu Glu Ala Arg Gln Lys Tyr Ala Tyr Gly Arg
385 390 395 400
Gln Asn Asp Tyr Leu Asp His His Asn Ile Ile Gly Trp Thr Arg Glu
405 410 415
Gly Asn Thr Ala His Pro Asn Ser Gly Leu Ala Thr Ile Met Ser Asp
420 425 430
Gly Ala Gly Gly Asn Lys Trp Met Phe Val Gly Arg Asn Lys Ala Gly
435 440 445
Gln Val Trp Thr Asp Ile Thr Gly Asn Arg Ala Gly Thr Val Thr Ile
450 455 460
Asn Ala Asp Gly Trp Gly Asp Ser Val Asn Gly Gly Ser Val Ser
465 470 475 480
Ile Trp Val Asn Lys
485

<210> SEQ ID NO 25
<211> LENGTH: 1458
<212> TYPE: DNA
<213> ORGANISM: Bacillus sp.
<220> FEATURE:
<221> NAME/KEY: mat_peptide
<222> LOCATION: (1) ...(1455)
<220> FEATURE:
<221> NAME/KEY: CDS
<222> LOCATION: (1) ...(1455)

<400> SEQUENCE: 25

cac cat aat ggt acg aac ggc aca atg atg cag taa ttt gag tgg tat
1  5  10  15
His His Asn Gly Thr Asn Gly Thr Met Met Gin Tyr Phe Glu Trp Tyr

cac cat aat gag gaa aac cat tgg aat aga taa ggt tct gat gca agt
20  25  30
Leu Pro Asn Asp Gly Asn His Trp Asn Arg Leu Arg Ser Asp Ala Ser

aac cac aaa gat aag ggg acc cat tca gcc gtt tgt aat cct cct gcc tgg
35  40  45
Asn Leu Lys Asp Lys Gly Ile Ser Ala Val Thr Ile Pro Pro Ala Trp

aac gcc aat cag tta cac gct gta aac gcc tgt aag aat gag
70  75  80
Thr Arg Asn Gin Leu Gin Ala Ala Val Asn Ala Leu Lys Ser Asn Gly
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<211> LENGTH: 485
<212> TYPE: PRT
<213> ORGANISM: Bacillus sp.

<400> SEQUENCE: 26
His His Asn Gly Thr Asn Gly Thr Met Met Gln Tyr Phe Glu Trp Tyr
1     5     10    15
Leu Pro Asn Arg Asn His Trp Asn Arg Leu Arg Ser Asp Ala Ser
16    20    25    30
Asn Leu Leu Asp Lys Gly Ile Ser Ala Val Trp Ile Pro Pro Ala Trp
31    35    40    45
Lys Gly Ala Ser Gln Asn Asp Val Gly Tyr Gly Ala Tyr Asp Leu Tyr
46    50    55    60
Asp Leu Gly Lys Phe Asn Gly Gly Thr Ile Arg Thr Tyr Gly Thr
61    65    70    75    80
Thr Arg Gln Leu Gly Ala Val Ala Leu Lys Ser Asn Gly
81    85    90    95
Ile Gln Val Tyr Gly Asp Val Val Met Asn His Lys Gly Gly Ala Asp
96    101   106   110
Ala Thr Gly Met Val Arg Ala Val Glu Val Asn Pro Asn Asn Arg Asn
111   115   120   125
Gln Gly Val Ser Gly Gly Tyr Thr Ile Gly Ala Trp Thr Lys Phe Asp
131   135   140
Phe Gly Arg Gly Asn Thr His Ser Asn Phe Lys Trp Arg Trp Tyr
141   145   150   155   160
His Phe Asp Gly Val Asp Pro Gln Ser Arg Lys Leu Asn Arg
161   165   170   175
Ile Tyr Lys Phe Arg Gly Asp Gly Lys Gly Trp Asp Trp Glu Val Asp
176   180   185   190
Thr Glu Asn Gly Asn Tyr Arg Tyr Leu Met Tyr Ala Asp Ile Asp Met
191   195   200   205
Asp His Pro Glu Val Val Asn Glu Leu Arg Asn Trp Gly Val Trp Tyr
206   210   215   220
Thr Asn Thr Leu Gly Leu Asp Gly Phe Arg Ile Asp Ala Val Lys His
225 230 235 240
Ile Lys Tyr Ser Phe Thr Arg Asp Trp Ile Asn His Val Arg Ser Ala
245 250 255
Thr Gly Lys Asn Met Phe Ala Val Ala Glu Phe Trp Lys Asn Asp Leu
260 265 270
Gly Ala Ile Glu Asn Tyr Leu Asn Lys Thr Asn Trp Asn His Ser Val
275 280 285
Phe Asp Val Pro Leu His Tyr Asn Leu Tyr Asn Ala Ser Lys Ser Gly
290 295 300
Gly Asn Tyr Asp Met Arg Glu Ile Phe Asn Gly Thr Val Val Gly Arg
305 310 315 320
His Pro Met His Ala Val Thr Phe Val Asp Asn His Asp Ser Gly Pro
325 330 335
Glu Ala Leu Glu Ser Phe Val Glu Glu Trp Phe Leu Ala Pro Leu Ala
340 345 350
Tyr Ala Leu Thr Leu Thr Arg Glu Gly Tyr Pro Thr Ser Val Phe Tyr
355 360 365
Gly Asp Tyr Asp Gly Ile Pro Thr His Gly Val Pro Ala Met Lys Ser
370 375 380
Lys Ile Asp Pro Ile Leu Glu Ala Arg Glu Lys Tyr Ala Tyr Gly Arg
385 390 395 400
Gln Asn Asp Tyr Leu Asp Asn His Asn Ile Ile Gly Trp Thr Arg Glu
405 410 415
Gly Asn Thr Ala His Pro Asn Ser Gly Leu Ala Thr Ile Met Ser Asp
420 425 430
Gly Ala Gly Gly Asn Lys Trp Met Phe Val Gly Arg Asn Lys Ala Gly
435 440 445
Gln Val Trp Thr Asp Ile Thr Gly Asn Arg Ala Gly Thr Val Thr Ile
455 460
Asn Ala Asp Gly Trp Gly Asn Phe Ser Val Asn Gly Ser Val Ser
465 470 475 480
Ile Trp Val Asn Lys
485

<210> SEQ ID NO 27
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<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<222> OTHER INFORMATION: Primer
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (5) .. (5)
<222> OTHER INFORMATION: Xaa can be any naturally occurring amino acid
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<222> LOCATION: (7) .. (7)
<223> OTHER INFORMATION: Xaa can be any naturally occurring amino acid
<400> SEQUENCE: 27
Gly Ile Thr Ala Xaa Trp Xaa
1 5
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Primer
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (3)...(3)
<223> OTHER INFORMATION: Xaa can be any naturally occurring amino acid
<220> FEATURE:
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<222> LOCATION: (5)...(5)
<223> OTHER INFORMATION: Xaa can be any naturally occurring amino acid
<220> FEATURE:
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<222> LOCATION: (7)...(7)
<223> OTHER INFORMATION: Xaa can be any naturally occurring amino acid
<400> SEQUENCE: 28
Val Tyr Xaa Asp Xaa Val Xaa Asn His
1 5

<210> SEQ ID NO 29
<211> LENGTH: 10
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Primer
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (3)...(3)
<223> OTHER INFORMATION: Xaa can be any naturally occurring amino acid
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<222> LOCATION: (5)...(5)
<223> OTHER INFORMATION: Xaa can be any naturally occurring amino acid
<220> FEATURE:
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<223> OTHER INFORMATION: Xaa can be any naturally occurring amino acid
<400> SEQUENCE: 29
Asp Gly Xaa Arg Xaa Asp Ala Xaa Lys His
1 5 10

<210> SEQ ID NO 30
<211> LENGTH: 10
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Primer
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<222> LOCATION: (3)...(3)
<223> OTHER INFORMATION: Xaa can be any naturally occurring amino acid
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<223> OTHER INFORMATION: Xaa can be any naturally occurring amino acid
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Asp Gly Xaa Arg Xaa Asp Ala Xaa Lys His
1 5 10

<210> SEQ ID NO 31
<211> LENGTH: 8
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
Val Thr Phe Val Xaa Asn His Asp
1  5

<210> SEQ ID NO 32
<211> LENGTH: 6
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
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<223> OTHER INFORMATION: Primer

<400> SEQUENCE: 31
Val Thr Phe Val Xaa Asn His Asp
1  5

Gly Trp Thr Arg Glu Gly
1  5

<210> SEQ ID NO 33
<211> LENGTH: 22
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Primer

<400> SEQUENCE: 33
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22

cogcttgcc cogctgggc cgtcgactta tttgattacc caaatagaaac c
51

cattctgacagcgcgggca ccataatgt acgaacg
37

<210> SEQ ID NO 35
<211> LENGTH: 37
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Primer

<400> SEQUENCE: 35
cattctgacagcgcgggca ccataatgt acgaacg
37

Aen Gly Thr Aen Gly Thr Met Met Gln Tyr Phe Glu Trp Tyr Leu Pro
1  5 10 15

Aen Aep Gly Aen His Trp Aen Arg Leu Arg Ser Asp Ala Ser Aen Leu
20 25 30
Lys Asp Lys Gly Ile Ser Ala Val Trp Ile Pro Pro Ala Trp Lys Gly 35 40 45
Ala Ser Gln Asp Val Gly Tyr Gly Ala Tyr Asp Leu Tyr Asp Leu 50 55 60
Gly Glu Phe Asn Gln Lys Gly Thr Ile Arg Thr Lys Tyr Gly Thr Arg 65 70 75 80
Asn Gln Leu Gln Ala Ala Val Asn Ala Leu Lys Ser Asn Gly Ile Gln 85 90 95
Val Tyr Gly Asp Val Val Met Asn His Lys Gly Gly Ala Asp Ala Thr 100 105 110
Glu Met Val Arg Ala Val Glu Val Asn Pro Asn Gln Asp Glu Glu 115 120 125
Val Ser Gly Glu Tyr Thr Ile Glu Ala Trp Thr Lys Phe Asp Phe Pro 130 135 140
Gly Arg Gly Asn Thr His Ser Asn Phe Lys Trp Arg Trp Tyr His Phe 145 150 155 160
Asp Gly Val Asp Trp Asp Gln Ser Arg Lys Leu Asn Arg Ile Tyr 165 170 175 180
Lys Phe Arg Gly Asp Gly Lys Gly Trp Asp Trp Glu Val Asp Thr Glu 185 190
Asn Gln Asn Tyr Asp Tyr Leu Met Tyr Ala Asp Ile Asp Met Asp His 195 200 205
Pro Glu Val Val Asn Glu Leu Arg Asn Trp Gly Val Trp Tyr Thr Asn 210 215 220
Thr Leu Gly Leu Asp Gly Phe Arg Ile Asp Ala Val Lys His Ile Lys 225 230 235 240
Tyr Ser Phe Thr Arg Asp Trp Ser Ile His Val Arg Ser AlaThr Gly 245 250 255
Lys Asn Met Phe Ala Val Ala Glu Phe Trp Lys Asn Asp Leu Gly Ala 260 265 270
Ile Glu Asn Tyr Leu Asn Lys Thr Asn Trp Asn His Ser Val Phe Asp 275 280 285
Val Pro Leu His Tyr Asn Phe Tyr Thr Ala Ser Lys Ser Gly Gly Asn 290 295 300
Tyr Asp Met Arg Gln Ile Phe Asn Gly Thr Val Val Gln Arg His Pro 305 310 315 320
Met His Ala Val Thr Phe Val Asn His Asp Ser Gln Pro Glu Glu 325 330 335
Ala Leu Glu Ser Phe Val Glu Glu Trp Phe Lys Pro Leu Ala Tyr Ala 340 345 350
Leu Thr Leu Thr Arg Glu Gln Gly Tyr Pro Ser Val Phe Tyr Gly Asp 355 360 365
Tyr Tyr Gly Ile Pro Thr His Gly Val Pro Ala Met Lys Ser Lys Ile 370 375 380
Asp Pro Ile Leu Glu Ala Arg Gln Lys Tyr Ala Tyr Gly Arg Glu Asn 385 390 395 400

<210> SEQ ID NO: 37
<211> LENGTH: 485
<212> TYPE: PRT
<213> ORGANISM: Bacillus sp.
-continued

His His Asn Gly Thr Asn Gly Thr Met Met Gln Tyr Phe Glu Trp His
1 5 10 15
Leu Pro Asn Asp Gly Asn His Trp Asn Arg Leu Arg Asp Ala Ala
20 25 30
Asn Leu Lys Ser Lys Gly Ile Thr Ala Val Trp Ile Pro Pro Ala Trp
35 40 45
Lys Gly Thr Ser Gln Asp Val Gly Tyr Gly Ala Tyr Asp Leu Tyr
50 55 60
Asp Leu Gly Glu Phe Asn Gln Lys Gly Thr Val Arg Thr Lys Tyr Gly
65 70 75 80
Thr Arg Ser Gln Leu Gln Gly Ala Val Thr Ser Leu Lys Asn Asn Gly
85 90 95
Ile Gin Val Tyr Gly Asp Val Val Met Asn His Lys Gly Gly Ala Asp
100 105 110
Gly Thr Glu Met Val Asn Ala Val Glu Val Arg Asn Arg Asn Arg Asn
115 120 125
Gln Glu Ile Ser Gly Glu Tyr Thr Ile Glu Ala Trp Thr Lys Phe Asp
130 135 140
Phe Pro Gly Arg Gly Thr His Ser Asn Phe Lys Tyr Trp Arg Trp Tyr
145 150 155 160
His Phe Asp Gly Thr Asp Trp Gln Ser Arg Gin Leu Gin Asn Lys
165 170 175
Ile Tyr Lys Phe Arg Gly Thr Gly Lys Ala Trp Asp Trp Glu Val Asp
180 185 190
Ile Glu Asn Gly Asn Tyr Asp Tyr Leu Met Tyr Ala Asp Ile Asp Met
195 200 205
Asp His Pro Glu Val Ile Asn Glu Arg Asp Gly Val Trp Tyr
210 215 220
Thr Asn Thr Leu Asn Leu Asp Gly Phe Arg Ile Asp Ala Val Lys His
225 230 235 240
Ile Lys Tyr Ser Tyr Thr Arg Asp Trp Leu Thr His Val Arg Asn Thr
245 250 255
Thr Gly Lys Pro Met Phe Ala Val Ala Glu Phe Trp Lys Asn Asp Leu
260 265 270
Ala Ala Ile Glu Asn Tyr Leu Asn Lys Thr Ser Trp Asn His Ser Val
275 280 285
Phe Asp Val Pro Leu His Tyr Asn Leu Tyr Asn Ala Ser Asn Ser Gly
290 295 300 305
Gly Tyr Phe Asp Met Arg Asn Ile Leu Asn Gly Ser Val Val Gln Lys
310 315 320
His Pro Ile His Ala Val Thr Phe Val Asp Asn His Ser Gin Pro
325 330 335
Gly Glu Ala Leu Glu Ser Phe Val Gln Ser Trp Phe Lys Pro Leu Ala
340 345 350
Tyr Ala Leu Ile Leu Thr Arg Glu Gin Gly Tyr Pro Ser Val Phe Tyr
355 360 365
Gly Asp Tyr Tyr Gly Ile Pro Thr His Gly Val Pro Ser Met Lys Ser
370 375 380
Lys Ile Asp Pro Leu Leu Gin Ala Arg Gin Thr Tyr Ala Tyr Gly Thr
385 390 395 400
1. A variant of a parent Termamyl-like α-amylase with α-amylase activity comprising mutations in two, three, four, five or six of the following regions/positions or in corresponding positions in other parent Termamyl-like α-amylases:

   (relative to SEQ ID NO: 1):
   1: R181*G182*T183*G184*  
   5: E216*A*D*C*E*Q*G*H*I*L*L*K*M*F*P*S*T*W*Y*V*  

   (relative to SEQ ID NO: 2):
   1: R181*G182*T183*G184*  
   5: E216*A*D*C*E*Q*G*H*I*L*L*K*M*F*P*S*T*W*Y*V*  

   (Relative to SEQ ID NO: 3):
   1: R179*A*G180*G181*G182*  
   3: L204*A*D*C*E*Q*G*H*I*L*L*K*M*F*P*S*T*W*Y*V*  

   (Relative to SEQ ID NO: 4):
   1: N190*A*D*C*E*Q*G*H*I*L*L*K*M*F*P*S*T*W*Y*V*  
   2: L201*A*D*C*E*Q*G*H*I*L*L*K*M*F*P*S*T*W*Y*V*  

   (Relative to SEQ ID NO: 5):
   1: R176*G177*E178*G179*  
   5: E211*A*D*C*E*Q*G*H*I*L*L*K*M*F*P*S*T*W*Y*V*  

   (Relative to SEQ ID NO: 6):
   1: R181*G182*T183*G184*  
   5: E216*A*D*C*E*Q*G*H*I*L*L*K*M*F*P*S*T*W*Y*V*  

2. The variant according to claim 1, comprising the following mutations: N190F/Q264S in SEQ ID NO: 4 or in corresponding positions in another parent α-amylase.

3. The variant according to claim 1, comprising the following mutations: N195F/N193F in SEQ ID NO: 3 or in corresponding positions in another parent Termamyl like α-amylase.

4. The variant according to claim 3, further comprising a substitution in position E214Q in SEQ ID NO: 3 or in a corresponding position in another parent Termamyl like α-amylase.

5. The variant according to claim 1, wherein the parent α-amylase is a hybrid α-amylase of SEQ ID NO: 4 and SEQ ID NO: 5.

6. The variant according to claim 5, wherein the parent hybrid α-amylase is a hybrid alpha-amylase comprising the 445 C-terminal amino acid residues of the B. licheniformis α-amylase shown in SEQ ID NO: 4 and the 37 N-terminal amino acid residues of the α-amylase derived from B. amyloliquefaciens shown in SEQ ID NO: 5.

7. The variant according to claim 6, wherein the parent hybrid Termamyl-like α-amylase further has the following mutations: H156Y+A181T+N190F+A209V+Q264S (using the numbering in SEQ ID NO: 4).

8. The variant according to claim 1, exhibiting increased stability at acidic pH and/or low Ca²⁺ concentration.

9. A DNA construct comprising a DNA sequence encoding an α-amylase variant according to claim 1.

10. A recombinant expression vector which carries a DNA construct according to claim 9.

11. A cell which is transformed with a DNA construct according to claim 9 or a vector according to claim 10.

12. A cell according to claim 11, which is a microorganism.

13. A cell according to claim 12, which is a bacterium or a fungus.

14. The cell according to claim 13, which is a grampositive bacterium such as Bacillus subtilis, Bacillus licheniformis, Bacillus lentus, Bacillus brevis, Bacillus stearothermophilus, Bacillus alkaliophilus, Bacillus amyloliquefaciens, Bacillus coagulans, Bacillus circulans, Bacillus lautus or Bacillus thuringiensis.

15. A detergent additive comprising an α-amylase variant according to claim 1, optionally in the form of a non-dusting granulate, stabilised liquid or protected enzyme.
16. A detergent additive according to claim 15 which contains 0.02-200 mg of enzyme protein/g of the additive.

17. A detergent additive according to claim 15, which additionally comprises another enzyme such as a protease, a lipase, a peroxidase, another amylolytic enzyme and/or a cellulase.

18. A detergent composition comprising an α-amylase variant according to claim 1.

19. The detergent composition according to claim 18 which additionally comprises another enzyme such as a protease, a lipase, a peroxidase, another amylolytic enzyme and/or a cellulase.

20. A manual or automatic dishwashing detergent composition comprising an α-amylase variant according to claim 1.

21. A dishwashing detergent composition according to claim 20 which additionally comprises another enzyme such as a protease, a lipase, a peroxidase, another amylolytic enzyme and/or a cellulase.

22. A manual or automatic laundry washing composition comprising an α-amylase variant according to claim 1.

23. A laundry washing composition according to claim 22 which additionally comprises another enzyme such as a protease, a lipase, a peroxidase, an amylolytic enzyme and/or a cellulase.

24. A composition comprising:

(i) a mixture of the α-amylase from B. licheniformis having the sequence shown in SEQ ID NO: 4 with one or more variants according to claim 1 derived from (as the parent Termamyl-like α-amylase) the B. stearothermophilus α-amylase having the sequence shown in SEQ ID NO: 3; or

(ii) a mixture of the α-amylase from B. stearothermophilus having the sequence shown in SEQ ID NO: 3 with one or more variants according to claim 1 derived from one or more other parent Termamyl-like α-amylases; or

(iii) a mixture of one or more variants according claim 1 derived from (as the parent Termamyl-like α-amylase) the B. stearothermophilus α-amylase having the sequence shown in SEQ ID NO: 3 with one or more variants according to the invention derived from one or more other parent Termamyl-like α-amylases.

25. A composition comprising:

(a) a mixture of one or more variants according claim 1 derived from (as the parent Termamyl-like α-amylase) the B. stearothermophilus α-amylase having the sequence shown in SEQ ID NO: 3 and a Termamyl-like alpha-amylase derived from the B. licheniformis α-amylase having the sequence shown in SEQ ID NO: 4.

(b) a hybrid alpha-amylase comprising a part of the B. amyloliquefaciens α-amylase shown in SEQ ID NO: 5 and a part of the B. licheniformis α-amylase shown in SEQ ID NO: 4.

27. The composition according to claim 26, wherein the hybrid α-amylase is a hybrid alpha-amylase comprising the 445 C-terminal amino acid residues of the B. licheniformis α-amylase shown in SEQ ID NO: 4 and the 37 N-terminal amino acid residues of the α-amylase derived from B. amyloliquefaciens shown in SEQ ID NO: 5.

28. The composition according to claim 27, wherein the hybrid α-amylase further has the following mutations: H156Y+A181T+N190F+A209V+Q264S (using the numbering in SEQ ID NO: 4).

29. The composition according to claim 26, comprising a mixture of TVB146 and LE174.

30. A method for generating a variant of a parent Termamyl-like α-amylase, which variant exhibits increased stability at low pH and at low calcium concentration relative to the parent, the method comprising:

(a) subjecting a DNA sequence encoding the parent Termamyl-like α-amylase to random mutagenesis,

(b) expressing the mutated DNA sequence obtained in step (a) in a host cell, and

(c) screening for host cells expressing a mutated α-amylase which has increased stability at low pH and low calcium concentration relative to the parent α-amylase.

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