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(54) Title: ALPHA-AMYLASE VARIANTS

(57) Abrégé/Abstract:
The invention relates to a variant of a parent Termamyl-like alpha-amylase, which variant exhibits altered properties, in particular reduced capability of cleaving a substrate close to the branching point, and improved substrate specificity and/or improved specific activity relative to the parent alpha-amylase. The variant of the parent Termamyl-like alpha-amylase, comprised an alternation at one or more positions selected from the group of W13, G48, T49, S50, Q51, A52, D53, V54, G57, G107, G108, A111, S168 and M197.

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(54) Title: ALPHA-AMYLASE VARIANTS

(57) Abstract: The invention relates to a variant of a parent Termamyl-like alpha-amylase, which variant exhibits altered properties, in particular reduced capability of cleaving a substrate close to the branching point, and improved substrate specificity and/or improved specific activity relative to the parent alpha-amylase. The variant of the parent Termamyl-like alpha-amylase, comprised an alternation at one or more positions selected from the group of W13, G48, T49, S50, Q51, A52, D53, V54, G57, G107, G108, A111, S168 and M197.

Alpha-AMYLASE VARIANTS

FIELD OF THE INVENTION

The present invention relates, *inter alia*, to novel variants of parent Termamyl-like alpha-amylases, notably variants exhibiting altered properties, in particular altered cleavage pattern (relative to the parent) which are advantageous with respect to applications of the variants in, in particular, industrial starch processing (e.g., starch liquefaction or saccharification).

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 95/10603, WO 95/26397, WO 96/23873, WO 96/23874 and WO 97/41213.

Among recent disclosure relating to alpha-amylases, WO 96/23874 provides three-dimensional, X-ray crystal structural data for a Termamyl-like alpha-amylase, reffered to as BA2, which consists of the 300 N-terminal amino acid residues of the *B. amyloliquefaciens* alpha-amylase comprising the amino acid sequence shown in SEQ ID NO: 6 herein and amino acids 301-483 of the C-terminal end of the *B. licheniformis* alpha-amylase comprising the amino acid sequence shown in SEQ ID NO: 4 herein (the latter being available commercially under the tradename Termamyl™), 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. stearothermophilus* alpha-amylases). WO 96/23874 further describes methodology for designing, on the basis of an analysis of the structure of a parent Termamyl-like alpha-amylase, variants of the parent Termamyl-like alpha-amylase which exhibit altered properties relative to the parent.

WO 96/23874 and WO 97/41213 (Novo Nordisk) discloses Termamyl-like alpha-amylase variants with an altered cleavage pattern containing mutations in the amino acid residues V54, D53, Y56, Q333, G57 and A52 of the sequence shown in SEQ ID NO: 4 herein.

BRIEF DISCLOSURE OF THE INVENTION

The present invention relates to novel alpha-amylolytic variants (mutants) of a Termamyl-like alpha-amylase, in particular variants exhibiting altered cleavage pattern (relative to the parent), which are advantageous in connection with the industrial processing of starch (starch liquefaction, saccharification and the like).

The inventors have surprisingly found variants with altered properties, in particular altered cleavage pattern which have improved reduced capability of cleaving an substrate close to the branching point, and further have improved substrate specificity and/or improved specific activity, in comparison to the WO 96/23874 and WO 97/41213 (Novo Nordisk) disclosed Termamyl-like alpha-amylase variants with an altered cleavage pattern containing mutations in the amino acid residues V54, D53, Y56, Q333, G57 and A52 of the sequence shown in SEQ ID NO: 4 herein.

The invention further relates to DNA constructs encoding variants of the invention, to composition comprising variants of the invention, to methods for preparing variants of the invention, and to the use of variants and compositions of the invention, alone or in combination with other alpha-amylolytic enzymes, in various industrial processes, *e.g.*, starch liquefaction, and in detergent compositions, such as laundry, dish washing and hard surface cleaning compositions; ethanol production, such as fuel, drinking and industrial ethanol production; desizing of textiles, fabrics or garments etc.

<u>Nomenclature</u>

In the present description and claims, the conventional one-letter and three-letter codes for amino acid residues are used. For ease of reference, alpha-amylase variants of the invention are described by use of the following nomenclature:

Original amino acid(s):position(s):substituted amino acid(s)

According to this nomenclature, for instance the substitution of alanine for asparagine in position 30 is shown as:

Ala30Asn or A30N

a deletion of alanine in the same position is shown as:

Ala30* or A30*

and insertion of an additional amino acid residue, such as lysine, is shown as:

*30aLys or *30aK

A deletion of a consecutive stretch of amino acid residues, such as amino acid residues 30-33, is indicated as (30-33)* or Δ(A30-N33) or delta(A30-N33).

Where a specific alpha-amylase contains a "deletion" in comparison with other alpha-amylases and an insertion is made in such a position this is indicated as:

*36aAsp or *36aD

for insertion of an aspartic acid in position 36

Multiple mutations are separated by plus signs, i.e.:

Ala30Asp + Glu34Ser or A30N+E34S

representing mutations in positions 30 and 34 substituting alanine and glutamic acid for asparagine and serine, respectively. Multiple mutations may also be separated as follows, i.e., meaning the same as the plus sign:

Ala30Asp/Glu34Ser or A30N/E34S

When one or more alternative amino acid residues may be inserted in a given position it is indicated as

A30N,E or

A30N or A30E

Furthermore, when a position suitable for modification is identified herein without any specific modification being suggested, or A30X, it is to be understood that any amino acid residue may be substituted for the amino acid residue present in the position. Thus, for instance, when a modification of an alanine in position 30 is mentioned, but not specified, or specified as "X", it is to be understood that the alanine may be deleted or substituted for any other amino acid, i.e., any one of: R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows SEQ ID NO: 1 of WO95/26397;

Figure 2 shows SEQ ID NO: 2 of WO95/26397; and

Figure 3 shows the sequence of the Bacillus sp#707 alpha-amylase of Tsukamoto et al., Biomechanical and Biophysical Research Communications, 151 (1988), pp. 25-31.

The Termamyl-like alpha-amylase

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. amyloliquefaciens* alpha-amylase comprising the amino acid sequence shown in SEQ ID NO: 6 and about 79% homologous with the *B. stearothermophilus* alpha-amylase comprising the amino acid sequence shown in SEQ ID NO: 8. 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 #707 alpha-amylase described by Tsukamoto et al., Biochemical and Biophysical Research Communications, 151 (1988), pp. 25-31.

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 Optitherm™ and Takatherm™ (available from Solvay), Maxamyl™ (available from Gist-brocades/Genencor), Spezym AA™ and Spezyme Delta AA™ (available from Genencor), and Keistase™ (available from Daiwa).

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".

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 NO: 2, 4, 6, or 8 herein, and the amino acid sequence shown in SEQ ID NO: 1 or 2 of WO 95/26397 or in Tsukamoto et al., 1988, 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, more preferred at least 97%, more preferred at least 99% with at least one of said amino acid sequences 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 hybridises to the DNA sequences encoding the above-specified alpha-amylases which are apparent from SEQ ID NOS: 1, 3, 5 and 7 of the present application and SEQ ID NOS: 4 and 5 of WO 95/26397, respectively.

In connection with property i), the "homology" may be determined by use of any conventional algorithm, preferably by use of the GAP progamme 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, Wisconsin, USA 53711).

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, pp. 149-155) and reverse threading (Huber, T ; Torda, AE, PROTEIN SCIENCE Vol. 7, No. 1 pp. 142-149 (1998). 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 NOS: 2, 4, 6, or 8, respectively, have been found.

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.

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, 50mM sodium phosphate, pH 6.8, and 50mg of denatured sonicated calf thymus DNA, followed by hybridization in the same solution supplemented with 100mM 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.

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

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.

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.

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.

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. amyloliquefaciens* 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. amyloliquefaciens* alpha-amylase having the amino acid sequence shown in SEQ ID NO: 6 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: 8 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.

In a preferred embodiment the parent Termamyl-like alpha-amylase is a hybrid Termamyl-like alpha-amylase identical to the *Bacillus licheniformis* alpha-amylase shown in SEQ ID NO: 4, except that the N-terminal 35 amino acid residues (of the mature protein) is replaced with the N-terminal 33 amino acid residues of the mature protein of the *Bacillus amyloliquefaciens* alpha-amylase (BAN) shown in SEQ ID NO: 6. Said hybrid may further have the following mutations: H156Y+A181T+N190F+A209V+Q264S (using the numbering in SEQ ID NO: 4) referred to as LE174.

Another preferred parent hybrid alpha-amylase is LE429 shown in SEQ ID NO: 2.

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.

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™.

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.

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

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

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

In the first aspect the invention relates to a variant of a parent Termamyl-like alpha-amylase, comprising an alteration at one or more positions selected from the group of:

W13, G48, T49, S50, Q51, A52, D53, V54, G57, G107, G108, A111, S168, M197, wherein (a) the alteration(s) are independently

(i) an insertion of an amino acid downstream of the amino acid which occupies the position,

(ii) a deletion of the amino acid which occupies the position, or

(iii) a substitution of the amino acid which occupies the position with a different amino acid,

(b) the variant has alpha-amylase activity and (c) each position corresponds to a position of the amino acid sequence of the parent Termamyl-like alpha-amylase having the amino acid sequence of SEQ ID NO: 4.

In a preferred embodiment the above variants of the invention comprise a mutation in a position corresponding to at least one of the following mutations in the amino acid sequence shown in SEQ ID NO: 4:

V54N, A52S, A52S+V54N, T49L, T49+G107A, A52S+V54N+T49L+G107A, A52S+V54N+T49L, G107A, Q51R, Q51R+A52S, A52N; or

T49F+G107A, T49V+G107A, T49D+G107A, T49Y+G107A, T49S+G107A, T49N+G107A, T49I+G107A, T49L+A52S+G107A, T49L+A52T+G107A, T49L+A52F+G107A, T49L+A52L+G107A, T49L+A52I+G107A, T49L+A52V+G107A; or

T49V, T49I, T49D, T49N, T49S, T49Y, T49F, T49W, T49M, T49E, T49Q, T49K, T49R, A52T, A52L, A52I, A52V, A52M, A52F, A52Y, A52W, V54M, G107V, G07I, G107L, G107C.

In a preferred embodiment a variant of the invention comprises at least one mutation in a position corresponding to the following mutations in the amino acid sequence shown in SEQ ID NO: 4:

W13F,L,I,V,Y,A;

G48A,V,S,T,I,L;

*48aD or *48aY (i.e., insertion of D or Y);

T49X;

*49aX (i.e., insertion of any possible amino acid residue)

S50X, in particular D,Y,L,T,V,I;

Q51R,K;

A52X, in particular A52S,N,T,F,L,I,V;

D53E,Q,Y,I,N,S,T,V,L;

V54X, in particular V54I,N,W,Y,F,L;
G57S,A,V,L,I,F,Y,T;
G107X, in particular G107A,V,S,T,I,L,C;
G108X, in particular G108A,V,S,T,I,L;
A111V,I,L;
S168Y;
M197X, in particular Y,F,L,I,T,A,G.

In a preferred embodiment a variant of the invention comprises the following mutations corresponding to the following mutations in the amino acid sequence shown in SEQ ID NO: 4: T49X+A52X+V54N/I/L/Y/F/W+G107A, and may further comprise G108A.

In a preferred embodiment a variant of the invention comprises at least one mutation corresponding to the following mutations in the amino acid sequence shown in SEQ ID NO: 4:
T49L+G107A;
T49I+G107A;
T49L+G107A+V54I;
T49I+G107A+V54I;
A52S+V54N+T49L+G107A;
A52S+V54I+T49L+G107A;
A52S+T49L+G107A;
A52T+T49L+G107A;
A52S+V54N+T49I+G107A;
A52S+V54I+T49I+G107A;
A52S+T49I+G107A;
T49L+G108A;
T49I+G108A;
T49L+G108A+V54I;
T49I+G108A+V54I.

All of the above-mentioned variants of the invention have altered properties (meaning increased or decreased properties), in particular at least one of the following properties relative to the parent alpha-amylase: reduced ability to cleave a substrate close to the branching point, improved substrate specificity and/or improved specific activity, altered substrate binding, altered thermal stability, altered pH/activity profile, altered pH/stability profile, altered stability towards oxidation, altered Ca^{2+} dependency.

Stability

In the context of the present invention, mutations (including amino acid substitutions and/or deletions) of importance with respect to achieving altered stability, in particular improved stability (i.e., higher or lower), at especially low pH (*i.e.*, pH 4-6) include any of the mutations listed in the in ''Altered properties'' section, above and the variants mentioned right below.

The following variants: Q360A,K; N102A, N326A,L, N190G, N190K; Y262A,K,E (using the BAN, i.e., SEQ ID N: 6, numbering) were also tested for pH stability. A preferred parent alpha-amylase may be BA2 described above. The pH stability was determined as described in the "Materials & Methods" section.

Ca^{2+} stability

Altered Ca^{2+} stability means the stability of the enzyme under Ca^{2+} depletion has been improved, i.e., higher or lower stability. In the context of the present invention, mutations (including amino acid substitutions) of importance with respect to achieving altered Ca^{2+} stability, in particular improved Ca^{2+} stability, i.e., higher or lower stability, at especially low pH (*i.e.*, pH 4-6) include any of the mutations listed in the in ''Altered properties'' section above.

Specific activity

In a further aspect of the present invention, important mutations with respect to obtaining variants exhibiting altered specific activity, in particular increased or decreased specific activity, especially at temperatures from 60-100°C, preferably 70-95°C, especially 80-90°C, include any of the mutations listed in the in ''Altered properties'' section above.

The specific activity of LE174 and LE429 was determined to

16,000 NU/mg using the Phadebas® assay described in the ''Materials and Methods'' section.

Altered cleavage pattern

In the starch liquefaction process it is desirable to use an alpha-amylase, which is capable of degrading the starch molecules into long, branched oligosaccharides, rather than an alpha-amylase, which gives rise to formation of shorter, branched oligosaccharides (like conventional Termamyl-like alpha-amylases). Short, branched oligosaccharides (panose precursors) are not hydrolyzed satisfactorily by pullulanases, which are used after alpha-amylase treatment in the liquefaction process, or simultaneously with a saccharifying amyloglucosidase (glucoamylase), or before adding a saccharifying amyloglucosidase (glucoamylase). Thus, in the presence of panose precursors, the product mixture present after the glucoamylase treatment contains a significant proportion of short, branched, so-called limit-dextrin, viz. the trisaccharide panose. The presence of panose lowers the saccharification yield significantly and is thus undesirable.

It has been reported previously (US patent 5,234,823) that, when saccharifying with glucoamylase and pullulanase, the presence of residual alpha-amylase activity arising from the liquefaction process, can lead to lower yields of glucose, if the alpha-amylase is not inactivated before the saccharification stage. This inactivation can be typically carried out by adjusting the pH to below 4.7 at 95°C, before lowering the temperature to 60°C for saccharification.

The reason for this negative effect on glucose yield is not fully understood, but it is assumed that the liquefying alpha-amylase (for example Termamyl 120 L from *B.licheniformis*) generates "limit dextrins" (which are poor substrates for pullulanase), by hydrolysing 1,4-alpha-glucosidic linkages close to and on both sides of the branching points in amylopectin. Hydrolysis of these limit dextrins by glucoamylase leads to a build up of the trisaccharide panose, which is only slowly hydrolysed by glucoamylase.

The development of a thermostable alpha-amylase, which does not suffer from this disadvantage, would be a significant improvement, as no separate inactivation step would be required.

Thus, the aim of the present invention is to arrive at a mutant alpha-amylase having appropriately modified starch-degradation characteristics but retaining the thermostability of the parent Termamyl-like alpha-amylase.

Accordingly, the invention relates to a variant of a Termamyl-like alpha-amylase, which has an improved reduced ability to cleave a substrate close to the branching point, and further has improved substrate specificity and/or improved specific activity.

Of particular interest is a variant, which cleaves an amylopectin substrate, from the reducing end, more than one glucose unit from the branching point, preferably more than two or three glucose units from the branching point, i.e., at a further distance from the branching point than that obtained by use of a wild type *B. licheniformis* alpha-amylase.

It may be mentioned here that according to WO 96/23874, variants comprising at least one of the following mutations are expected to prevent cleavage close to the branching point:

V54L,I,F,Y,W,R,K,H,E,Q;

D53L,I,F,Y,W;

Y56W;

Q333W;

G57,all possible amino acid residues;

A52, amino acid residues larger than A, e.g., A52W,Y,L,F,I.

Mutations of particular interest in relation to obtaining variants according to the invention having an improved reduced ability to cleave a substrate close to the branching point, and further has improved substrate specificity and/or improved specific activity include mutations at the following positions in *B. licheniformis* alpha-amylase, SEQ ID NO: 4:

H156, A181, N190, A209, Q264 and I201.

It should be emphazised that not only the Termamyl-like alpha-amylases mentioned specifically below may be used. Also other commercial Termamyl-like alpha-amylases can be used. 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), Spezym AA™ Spezyme Delta AA™ (available from Genencor), and Keistase™ (available from Daiwa).

All Termamyl-like alpha-amylase may suitably be used as backbone for preparing variants of the invention.

In a preferred embodiment of the invention the parent Termamyl-like alpha-amylase is a hybrid alpha-amylase of SEQ ID NO: 4 and SEQ ID NO: 6. 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 37 N-terminal amino acid residues of the mature alpha-amylase derived from *B. amyloliquefaciens* shown in SEQ ID NO: 6, which may suitably further have the following mutations: H156Y+A181T+N190F+A209V+Q264S (using the numbering in SEQ ID NO: 4). This hybrid is referred to as LE174. The LE174 hybrid may be combined with a further mutation I201F to form a parent hybrid Termamyl-like alpha-amylase having the following mutations H156Y+A181T+N190F+A209V+Q264S+I201F (using SEQ ID NO: 4 for the numbering). This hybrid variant is shown in SEQ ID NO: 2 and is used in the examples below, and is referred to as LE429.

Also, LE174 or LE429 (SEQ ID NO: 2) or *B. licheniformis* alpha-amylase shown in SEQ ID NO: 4 comprising one or more of the following mutations may be used as backbone (using SEQ ID NO: 4 for the numbering of the mutations):

E119C;

S130C;

D124C;

R127C;

A52all possible amino acid residues;
S85all possible amino acid residues;
N96all possible amino acid residues;
V129all possible amino acid residues;
A269all possible amino acid residues;
A378all possible amino acid residues;
S148all possible amino acid residues, in particular S148N;
E211all possible amino acid residues, in particular E211Q;
N188all possible amino acid residues, in particular N188S, N188P
M197all possible amino acid residues, in particular M197T, M197A, M197G, M197I, M197L, M197Y, M197F, M197I;
W138all possible amino acid residues, in particular W138Y;
D207all possible amino acid residues, in particular D207Y;
H133all possible amino acid residues, in particular H133Y;
H205all possible amino acid residues, in particular H205H, H205C, H205R;
S187all possible amino acid residues, in particular S187D;
A210all possible amino acid residues, in particular A210S, A210T;
H405all possible amino acid residues, in particular H405D;
K176all possible amino acid residues, in particular K176R;
F279all possible amino acid residues, in particular F279Y;
Q298all possible amino acid residues, in particular Q298H;
G299all possible amino acid residues, in particular G299R;
L308all possible amino acid residues, in particular L308F;
T412all possible amino acid residues, in particular T412A;

Further, *B. licheniformis* alpha-amylase shown in SEQ ID NO: 4 comprising at least one of the following mutations may be used as backbone:

M15all possible amino acid residues;
A33all possible amino acid residues;

When using LE429 (shown in SEQ ID NO: 2) as the backbone (i.e., as the parent Termamyl-like alpha-amylase) by combining LE174 with the mutation I201F (SEQ ID NO: 4 numbering), the mutations/alterations, in particular substitutions, deletions and insertions, may according to the invention be made in one or more of the following positions to improve the reduced ability to cleave a substrate close to the branching point, and to improve substrate specificity and/or improved specific activity: W13, G48, T49, S50, Q51, A52, D53, V54, G57, G107, G108, A111, S168, M197 (using the SEQ ID NO: 4 numbering)

wherein (a) the alteration(s) are independently

(i) an insertion of an amino acid downstream of the amino acid which occupies the position,

(ii) a deletion of the amino acid which occupies the position, or

(iii) a substitution of the amino acid which occupies the position with a different amino acid,

(b) the variant has alpha-amylase activity and (c) each position corresponds to a position of the amino acid sequence of the parent Termamyl-like alpha-amylase having the amino acid sequence of SEQ ID NO: 4.

In a preferred embodiment a variant of the invention comprises at least one mutation in a position corresponding to the following mutations in the amino acid sequence shown in SEQ ID NO: 4:

V54N, A52S, A52S+V54N, T49L, T49+G107A, A52S+V54N+T49L+G107A, A52S+V54N+T49L, G107A, Q51R, Q51R+A52S, A52N; or

T49F+G107A, T49V+G107A, T49D+G107A, T49Y+G107A, T49S+G107A, T49N+G107A, T49I+G107A, T49L+A52S+G107A, T49L+A52T+G107A, T49L+A52F+G107A, T49L+A52L+G107A, T49L+A52I+G107A, T49L+A52V+G107A; or

T49V, T49I, T49D, T49N, T49S, T49Y, T49F, T49W, T49M, T49E, T49Q, T49K, T49R, A52T, A52L, A52I, A52V, A52M, A52F, A52Y, A52W, V54M, G107V, G07I, G107L, G107C.

In a preferred embodiment a variant of the invention comprises at least one mutation in a position corresponding to the following mutations in the amino acid sequence shown in SEQ ID NO: 4:

W13F,L,I,V,Y,A;

G48A,V,S,T,I,L;

*48aD or *48aY (i.e., insertion of D or Y);

T49X;

*49aX (i.e., insertion of any amino acid residue)

S50X, in particular D,Y,L,T,V,I;
Q51R,K;
A52X, in particular A52S,N,T,F,L,I,V;
D53E,Q,Y,I,N,S,T,V,L;
V54X, in particular V54I,N,W,Y,F,L;
G57S,A,V,L,I,F,Y,T;
G107X, in particular G107A,V,S,T,I,L,C;
G108X, in particular G108A,V,S,T,I,L;
A111V,I,L;
S168Y;
M197X, in particular Y,F,L,I,T,A,G.

In a preferred embodiment a variant of the invention comprises at least one mutation in a position corresponding to the following mutations in the amino acid sequence shown in SEQ ID NO: 4:

T49X+A52X+V54N/I/L/Y/F/W+G107A, and may further comprise G108A.

In a preferred embodiment a variant of the invention comprises at least one mutation in a position corresponding to the following mutations in the amino acid sequence shown in SEQ ID NO: 4:

T49L+G107A;
T49I+G107A;
T49L+G107A+V54I;
T49I+G107A+V54I;
A52S+V54N+T49L+G107A;
A52S+V54I+T49L+G107A;
A52S+T49L+G107A;
A52T+T49L+G107A;
A52S+V54N+T49I+G107A;
A52S+V54I+T49I+G107A;
A52S+T49I+G107A;
T49L+G108A;
T49I+G108A;
T49L+G108A+V54I;
T49I+G108A+V54I.

General mutations in variants 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 Gln, 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.

Methods for preparing alpha-amylase variants

Several methods for introducing mutations into genes are known in the art. After a brief discussion of the cloning of alpha-amylase-encoding DNA sequences, methods for generating mutations at specific sites within the alpha-amylase-encoding sequence will be discussed.

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 identify alpha-amylase-encoding clones, using hybridization and washing conditions of lower stringency.

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.

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

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

Site-directed mutagenesis

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). US 4,760,025 disclose the introduction of oligonucleotides encoding multiple mutations by performing minor alterations of the cassette. However, an even greater variety of mutations can be introduced at any one time by the Morinaga method, because a multitude of oligonucleotides, of various lengths, can be introduced.

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

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.

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.

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 a reduced capability of cleaving an oligo-saccharide substrate close to the branching point, and further exhibits improved substrate specificity and/or improved specific activity relative to the parent, the method:

(a) subjecting a DNA sequence encoding the parent alpha-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 an alpha-amylase variant which has an altered property (i.e., thermal stability) relative to the parent alpha-amylase.

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

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. 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. 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., Molec. Gen. Genet., 133, 1974, pp. 179-191), *S. cereviseae* 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 otherwise exposed to the mutagenising 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 mutagenising 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 amyloliquefaciens*, *Bacillus coagulans*, *Bacillus circulans*, *Bacillus lautus*, *Bacillus megaterium*, *Bacillus thuringiensis*, *Streptomyces lividans* or *Streptomyces murinus*; 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.

Localised random mutagenesis

The random mutagenesis may be advantageously localised 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 localised, 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 in WO 95/22625 (from Affymax Technologies N.V.) and WO 96/00343 (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 of *E.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 proteinase, *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.

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, pE194, pAMB1 and pIJ702.

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 sC, a marker giving rise to hygromycin resistance, or the selection may be accomplished by co-transformation, e.g., as described in WO 91/17243.

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 pre-region permitting secretion of the expressed protease into the culture medium. If desirable, this pre-region may be replaced by a different preregion or signal sequence, conveniently accomplished by substitution of the DNA sequences encoding the respective preregions.

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).

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 may 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.

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.

Examples of suitable bacteria are gram-positive bacteria such as *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus lentus*, *Bacillus brevis*, *Bacillus stearothermophilus*, *Bacillus alkalophilus*, *Bacillus amyloliquefaciens*, *Bacillus coagulans*, *Bacillus circulans*, *Bacillus lautus*, *Bacillus megaterium*, *Bacillus thuringiensis*, or *Streptomyces lividans* or *Streptomyces murinus*, or gramnegative 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*.

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 oryzae* 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.

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.

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).

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.

Industrial applications

The alpha-amylase variants of this invention possess valuable properties allowing for a variety of industrial applications. In particular, enzyme variants of the invention are applicable as a component in washing, dishwashing and hard surface cleaning detergent compositions. Numerous variants are particularly useful in the production of sweeteners and ethanol, e.g., fuel, drinking or industrial ethanol, from starch, and/or for textile desizing. Conditions for conventional starch-conversion processes, including starch liquefaction and/or saccharification processes, are described in, *e.g.*, US 3,912,590 and in EP patent publications Nos. 252 730 and 63 909.

Production of sweeteners from starch:

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 optimal enzyme stability under these conditions, 1 mM of calcium is added (40 ppm free calcium ions).

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.

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 immmobilized glucoseisomerase (such as Sweetzyme™).

At least one enzymatic improvement of this process could be envisaged: 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 glucoseisomerase 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.

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.

The invention also relates to a composition comprising a mixture of one or more variants of the invention derived from (as the parent Termamyl-like alpha-amylase) the *B. stearothermophilus* alpha-amylase having the sequence shown in SEQ ID NO: 8 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 the invention derived from (as the parent Termamyl-like alpha-amylase) the *B. stearothermophilus* alpha-amylase having the sequence shown in SEQ ID NO: 8 and a hybrid alpha-amylase comprising a part of the *B. amyloliquefaciens* alpha-amylase shown in SEQ ID NO: 6 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. amyloliquefaciens* shown in SEQ ID NO: 6. Said latter mentioned hybrid alpha-amylase may suitably comprise the following mutations: H156Y+A181T+N190F+A209V+Q264S (using the numbering in SEQ ID NO: 4) Preferably, said latter mentioned hybrid alpha-amylase may suitably comprise the following mutations: H156Y+A181T+N190F+A209V+Q264S+I201F (using the SEQ ID NO: 4 numbering). In the examples below said last-mentioned parent hybrid Termamyl-like alpha-amylase referred to as LE429 (shown in SEQ ID NO: 2) is used for preparing variants of the invention, which variants may be used in compositions of the invention.

An alpha-amylase variant of the invention or a composition of the invention may in an aspect of the invention be used for starch liquefaction, in detergent composition, such as laundry, dish wash compositions and hard surface cleaning, ethanol production, such as fuel, drinking and industrial ethanol production, desizing of textile, fabric and garments.

MATERIALS AND METHODS

Enzymes:

LE174: hybrid alpha-amylase variant:

LE174 is a hybrid Termamyl-like alpha-amylase being identical to the Termamyl 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 amyloliquefaciens* alpha-amylase shown in SEQ ID NO: 6, which further have following mutations:

H156Y+A181T+N190F+A209V+Q264S (SEQ ID NO: 4).

LE429 hybrid alpha-amylase variant:

LE429 is a hybrid Termamyl-like alpha-amylase being identical to the Termamyl 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 amyloliquefaciens* alpha-amylase shown in SEQ ID NO: 6, which further have following mutations: H156Y+A181T+N190F+A209V+Q264S+I201F (SEQ ID NO: 4). LE429 is shown as SEQ ID NO: 2 and was constructed by SOE-PCR (Higuchi et al. 1988, Nucleic Acids Research 16:7351).

Dextrozyme™ E: a balanced mixture of glucoamylase (AMG) and pullulanase obtainable from selected strains of *Aspergillus niger* and *Bacillus deramificans* (available from Novo Nordisk A/S)

<u>Fermentation and purification of alpha-amylase variants</u>

A *B. subtilis* strain harbouring the relevant expression plasmid is streaked on an LB-agar plate with 10 micro g/ml kanamycin from -80°C stock, and grown overnight at 37°C.

The colonies are transferred to 100 ml BPX media supplemented with 10 micro g/ml kanamycin in a 500 ml shaking flask.

Composition of BPX medium:

Potato starch	100 g/l
Barley flour	50 g/l
BAN 5000 SKB	0.1 g/l
Sodium caseinate	10 g/l
Soy Bean Meal	20 g/l
Na_2HPO_4, 12 H_2O	9 g/l
Pluronic™	0.1 g/l

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

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 an UF-filter (10000 cut off membrane) and the buffer is changed to 20mM Acetate pH 5.5. The UF-filtrate is applied on a S-sepharose™ F.F. and elution is carried out by step elution with 0.2M NaCl in the same buffer. The eluate is dialysed against 10mM Tris, pH 9.0 and applied on a Q-sepharose F.F. and eluted with a linear gradient from 0-0.3M NaCl over 6 column volumes. The fractions that 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.

Activity determination - (KNU)

One Kilo alpha-amylase Unit (1 KNU) is the amount of enzyme which breaks down 5.26 g starch (Merck, Amylum Solubile, Erg. B 6, 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	0.0043 M
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.

Assay for Alpha-Amylase Activity

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-coloured starch polymer, which has been mixed with bovine serum albumin and a buffer substance and tabletted.

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 $CaCl_2$, 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 hydrolysed 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.

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 hydrolyse 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.

Determining Specific Activity

The specific activity is determined using the Phadebas assay (Pharmacia) as activity/mg enzyme.

Measuring the pH activity profile (pH stability)

The variant is stored in 20 mM TRIS ph 7.5, 0.1 mM, $CaCl_2$ and tested at 30°C, 50 mM Britton-Robinson, 0.1 mM $CaCl_2$. The pH activity is measured at pH 4.0, 4.5, 5.0, 5.5, 6.0, 7.0, 8.0, 9.5, 9.5, 10, and 10.5, using the Phadebas assay described above.

Determination Of AGU Activity and As AGU/mg

One Novo Amyloglucosidase Unit (AGU) is defined as the amount of enzyme, which hydrolyzes 1 micromole maltose per minute at 37°C and pH 4.3. A detailed description of the analytical method (AEL-SM-0131) is available on request from Novo Nordisk.

The activity is determined as AGU/ml by a method modified after (AEL-SM-0131) using the Glucose GOD-Perid kit from Boehringer Mannheim, 124036. Standard: AMG-standard, batch 7-1195, 195 AGU/ml.

375 microL substrate (1% maltose in 50 mM Sodium acetate, pH 4.3) is incubated 5 minutes at 37°C. 25 microL enzyme diluted in sodium acetate is added. The reaction is stopped after 10 minutes by adding 100 microL 0.25 M NaOH. 20 microL is transferred to a 96 well microtitre plate and 200 microL GOD-Perid solution is added. After 30 minutes at room temperature, the absorbance is measured at 650 nm and the activity calculated in AGU/ml from the AMG-standard.

The specific activity in AGU/mg is then calculated from the activity (AGU/ml) divided with the protein concentration (mg/ml).

EXAMPLES

EXAMPLE 1

Construction of Termamyl variants in accordance with the invention

Termamyl (*B. licheniformis* alpha-amylase SEQ ID NO: 4) is expressed in *B. subtilis* from a plasmid denoted pDN1528. This plasmid contains the complete gene encoding Termamyl, *amyL*, the expression of which is directed by its own promoter. Further, the plasmid contains the origin of replication, *ori*, from plasmid pUB110 and the *cat* gene from plasmid pC194 conferring resistance towards chloramphenicol. pDN1528 is shown in Fig. 9 of WO 96/23874. A specific mutagenesis vector containing a major part of the coding region of SEQ ID NO: 3 was prepared. The important features of this vector, denoted pJeEN1, include an origin of replication derived from the pUC plasmids, the *cat* gene conferring resistance towards chloramphenicol, and a frameshift-containing version of the *bla* gene, the wild type of which normally confers resistance towards ampicillin (amp^R

phenotype). This mutated version results in an amp[S] phenotype. The plasmid pJeEN1 is shown in Fig. 10 of WO 96/23874, and the *E. coli* origin of replication, *ori*, *bla*, *cat*, the 5'-truncated version of the Termamyl amylase gene, and selected restriction sites are indicated on the plasmid.

Mutations are introduced in *amyL* by the method described by Deng and Nickoloff (1992, Anal. Biochem. 200, pp. 81-88) except that plasmids with the "selection primer" (primer #6616; see below) incorporated are selected based on the amp[R] phenotype of transformed *E. coli* cells harboring a plasmid with a repaired *bla* gene, instead of employing the selection by restriction enzyme digestion outlined by Deng and Nickoloff. Chemicals and enzymes used for the mutagenesis were obtained from the ChameleonÔ mutagenesis kit from Stratagene (catalogue number 200509).

After verification of the DNA sequence in variant plasmids, the truncated gene, containing the desired alteration, is subcloned into pDN1528 as a *Pst*I-*Eco*RI fragment and transformed into the protease- and amylase-depleted *Bacillus subtilis* strain SHA273 (described in WO92/11357 and WO95/10603) in order to express the variant enzyme.

The Termamyl variant V54W was constructed by the use of the following mutagenesis primer (written 5' to 3', left to right):

PG GTC GTA GGC ACC GTA GCC CCA ATC CGC TTG (SEQ ID NO: 9)

The Termamyl variant A52W + V54W was constructed by the use of the following mutagenesis primer (written 5' to 3', left to right):

PG GTC GTA GGC ACC GTA GCC CCA ATC CCA TTG GCT CG (SEQ ID NO: 10)

Primer #6616 (written 5' to 3', left to right; P denotes a 5' phosphate):

P CTG TGA CTG GTG AGT ACT CAA CCA AGT C (SEQ ID NO: 11)

The Termamyl variant V54E was constructed by the use of the following mutagenesis primer (written 5'-3', left to right):

PGG TCG TAG GCA CCG TAG CCC TCA TCC GCT TG (SEQ ID NO: 12)

The Termamyl variant V54M was constructed by the use of the following mutagenesis primer (written 5'-3', left to right):

PGG TCG TAG GCA CCG TAG CCC ATA TCC GCT TG (SEQ ID NO: 13)

The Termamyl variant V54I was constructed by the use of the following mutagenesis primer (written 5'-3', left to right):

PGG TCG TAG GCA CCG TAG CCA ATA TCC GCT TG (SEQ ID NO: 14)

The Termamyl variants Y290E and Y290K were constructed by the use of the following mutagenesis primer (written 5'-3', left to right):

PGC AGC ATG GAA CTG CTY ATG AAG AGG CAC GTC AAA C (SEQ ID NO:15)

Y represents an equal mixture of C and T. The presence of a codon encoding either Glutamate or Lysine in position 290 was verified by DNA sequencing.

The Termamyl variant N190F was constructed by the use of the following mutagenesis primer (written 5'-3', left to right):

PCA TAG TTG CCG AAT TCA TTG GAA ACT TCC C (SEQ ID NO: 16)

The Termamyl variant N188P+N190F was constructed by the use of the following mutagenesis primer (written 5'-3', left to right):

PCA TAG TTG CCG AAT TCA GGG GAA ACT TCC CAA TC (SEQ ID NO: 17)

The Termamyl variant H140K+H142D was constructed by the use of the following mutagenesis primer (written 5'-3', left to right):

PCC GCG CCC CGG GAA ATC AAA TTT TGT CCA GGC TTT AAT TAG (SEQ ID NO: 18)

The Termamyl variant H156Y was constructed by the use of the following mutagenesis primer (written 5'-3', left to right):

PCA AAA TGG TAC CAA TAC CAC TTA AAA TCG CTG (SEQ ID NO: 19)

The Termamyl variant A181T was constructed by the use of the following mutagenesis primer (written 5'-3', left to right):

PCT TCC CAA TCC CAA GTC TTC CCT TGA AAC (SEQ ID NO: 20)

The Termamyl variant A209V was constructed by the use of the following mutagenesis primer (written 5'-3', left to right):

PCTT AAT TTC TGC TAC GAC GTC AGG ATG GTC ATA ATC (SEQ ID NO: 21)

The Termamyl variant Q264S was constructed by the use of the following mutagenesis primer (written 5'-3', left to right):

PCG CCC AAG TCA TTC GAC CAG TAC TCA GCT ACC GTA AAC (SEQ ID NO: 22)

The Termamyl variant S187D was constructed by the use of the following mutagenesis primer (written 5'-3', left to right):

PGC CGT TTT CAT TGT CGA CTT CCC AAT CCC (SEQ ID NO: 23)

The Termamyl variant DELTA(K370-G371-D372) (i.e., deleted of amino acid residues nos. 370, 371 and 372) was constructed by the use of the following mutagenesis primer (written 5'-3', left to right):

PGG AAT TTC GCG CTG ACT AGT CCC GTA CAT ATC CCC (SEQ ID NO: 24)

The Termamyl variant DELTA(D372-S373-Q374) was constructed by the use of the following mutagenesis primer (written 5'-3', left to right):

PGG CAG GAA TTT CGC GAC CTT TCG TCC CGT ACA TAT C (SEQ ID NO: 25)

The Termamyl variants A181T and A209V were combined to A181T+A209V by digesting the A181T containing pDN1528-like plasmid (i.e., pDN1528 containing within *amyL* the mutation resulting in the A181T alteration) and the A209V-containing pDN1528-like plasmid (i.e., pDN1528 containing within *amyL* the mutation resulting in the A209V alteration) with restriction enzyme *Cla*I which cuts the pDN1528-like plasmids twice resulting in a fragment of 1116 bp and the vector-part (i.e. contains the plasmid origin of replication) of 3850 bp. The fragment containing the A209V mutation and the vector part containing the A181T mutation were purified by QIAquick gel extraction kit (purchased from QIAGEN) after separation on an agarose gel. The fragment and the vector were ligated and transformed into the protease and amylase depleted *Bacillus subtilis* strain referred to above. Plasmid from *amy*+ (clearing zones on starch containing agar-plates) and chloramphenicol resistant transformants were analysed for the presence of both mutations on the plasmid.

In a similar way as described above, H156Y and A209V were combined utilizing restriction endonucleases Acc65I and EcoRI, giving H156Y+A209V.

H156Y +A209V and A181T+A209V were combined into H156Y+ A181T+A209V by the use of restriction endonucleases Acc65I and HindIII.

The 35 N-terminal residues of the mature part of Termamyl variant H156Y+ A181T+A209V were substituted by the 33 N-terminal residues of the *B. amyloliquefaciens* alpha-amylase (SEQ ID NO: 4) (which in the present context is termed BAN) by a SOE-PCR approach (Higuchi et al. 1988, Nucleic Acids Research 16:7351) as follows:

Primer 19364 (sequence 5'-3'): CCT CAT TCT GCA GCA GCA GCC GTA AAT GGC ACG CTG (SEQ ID NO: 26)

Primer 19362: CCA GAC GGC AGT AAT ACC GAT ATC CGA TAA ATG TTC CG (SEQ ID NO: 27)

Primer 19363: CGG ATA TCG GTA TTA CTG CCG TCT GGA TTC (SEQ ID NO: 28)

Primer 1C: CTC GTC CCA ATC GGT TCC GTC (SEQ ID NO: 29)

A standard PCR, polymerase chain reaction, was carried out using the Pwo thermostable polymerase from Boehringer Mannheim according to the manufacturer's instructions and the temperature cyclus: 5 minutes at 94°C, 25 cycles of (94°C for 30 seconds, 50°C for 45 seconds, 72°C for 1 minute), 72°C for 10 minutes.

An approximately 130 bp fragment was amplified in a first PCR denoted PCR1 with primers 19364 and 19362 on a DNA fragment containing the gene encoding the *B. amyloliquefaciens* alpha-amylase.

An approximately 400 bp fragment was amplified in another PCR denoted PCR2 with primers 19363 and 1C on template pDN1528.

PCR1 and PCR2 were purified from an agarose gel and used as templates in PCR3 with primers 19364 and 1C, which resulted in a fragment of approximately 520 bp. This fragment thus contains one part of DNA encoding the N-terminus from BAN fused to a part of DNA encoding Termamyl from the 35th amino acid.

The 520 bp fragment was subcloned into a pDN1528-like plasmid (containing the gene encoding Termamyl variant H156Y+ A181T+A209V) by digestion with restriction endonucleases *Pst*I and *Sac*II, ligation and transformation of the *B. subtilis* strain as previously described. The DNA sequence between restriction sites *Pst*I and *Sac*II was verified by DNA sequencing in extracted plasmids from *amy*+ and chloramphenicol resistant transformants.

The final construct containing the correct N-terminus from BAN and H156Y+ A181T+A209V was denoted BAN(1-35)+ H156Y+ A181T+A209V.

N190F was combined with BAN(1-35)+ H156Y+ A181T+A209V giving BAN(1-35)+ H156Y+ A181T+N190F+A209V by carrying out mutagenesis as described above except that the sequence of *amyL* in pJeEN1 was substituted by the DNA sequence encoding Termamyl variant BAN(1-35)+ H156Y+ A181T+A209V

Q264S was combined with BAN(1-35)+ H156Y+ A181T+A209V giving BAN(1-35)+ H156Y+ A181T+A209V+Q264S by carrying out mutagenesis as described above except that the sequence of amyL in pJeEN was substituted by the the DNA sequence encoding Termamyl variant BAN(1-35)+ H156Y+ A181T+A209V

BAN(1-35)+ H156Y+ A181T+A209V+Q264S and BAN(1-35)+ H156Y+ A181T+N190F+A209V were combined into BAN(1-35)+ H156Y+ A181T+N190F+A209V+Q264S utilizing restriction endonucleases *Bsa*HI (*Bsa*HI site was introduced close to the A209V mutation) and *Pst*I.

I201F was combined with BAN(1-35)+ H156Y+ A181T+N190F+A209V+Q264S giving BAN(1-35)+ H156Y+ A181T+N190F+A209V+Q264S+I201F (SEQ ID NO: 2) by carrying out mutagenesis as described above. The mutagenesis primer AM100 was used, introduced the I201F substitution and removed simultaneously a Cla I restriction site, which facilitates easy pin-pointing of mutants.

primer AM100:
5'GATGTATGCCGACTTCGATTATGACC 3' (SEQ ID NO: 30

EXAMPLE 2

Construction of Termamyl-like alpha-amylase variants with an altered cleavage pattern according to the invention

The variant of the thermostable *B. licheniformis* alpha-amylase consisting comprising 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. amyloliquefaciens* shown in SEQ ID NO: 6, and further comprising the following mutations: H156Y+A181T+N190F+A209V+Q264S+I201F (the construction of this variant is described in Example 1, and the amino acid sequence shown in SEQ ID NO: 2) has a reduced capability of cleaving an substrate close to the branching point.

In an attempt to further improve the reduced capability of cleaving an substrate close to the branching point of said alpha-amylase variant site directed mutagenesis was carried out using the Mega-primer method as described by Sarkar and Sommer, 1990 (BioTechniques 8: 404-407):

Construction of LE313: BAN/Termamyl hybrid + H156Y+A181T+N190F+ A209V+Q264S+V54N:

Gene specific primer 27274 and mutagenic primer AM115 are used to amplify by PCR an approximately 440 bp DNA fragment from a pDN1528-like plasmid (harbouring the BAN(1-35)+H156Y+A181T+N190F+I201F+A209V+Q264S mutations in the gene encoding the amylase from SEQ ID NO: 4).

The 440 bp fragment is purified from an agarose gel and used as a Mega-primer together with primer 113711 in a second PCR carried out on the same template.

The resulting approximately 630 bp fragment is digested with restriction enzymes EcoR V and Acc65 I and the resulting approximately 370 bp DNA fragment is purified and ligated with the pDN1528-like plasmid digested with the same enzymes. Competent *Bacillus subtilis* SHA273 (amylase and protease low) cells are transformed with the ligation and Chlorampenicol resistant transformants are checked by DNA sequencing to verify the presence of the correct mutations on the plasmid.

Primer 27274:
5' CATAGTTGCCGAATTCATTGGAAACTTCCC 3' (SEQ ID NO: 31)

Primer 1B:
5' CCGATTGCTGACGCTGTTATTTGC 3' (SEQ ID NO: 32)

primer AM115:
5' GCCAAGCGGATAACGGCTACGGTGC 3' (SEQ ID NO:33)

Construction of LE314: BAN/Termamyl hybrid + H156Y+A181T+N190F+ A209V+Q264S + A52S is carried our in a similar way, except that mutagenic primer AM116 is used.

AM116:
5' GAACGAGCCAATCGGACGTGGGCTACGG 3' (SEQ ID NO: 34)

Construction of LE315: BAN/Termamyl hybrid + H156Y+A181T+N190F+ A209V+Q264S + A52S+V54N is carried our in a similar way, except that mutagenic primer AM117 is used.

AM117:
5' GGAACGAGCCAATCGGATAACGGCTACGGTGC 3' (SEQ ID NO: 35)

Construction of LE316: BAN/Termamyl hybrid + H156Y+A181T+N190F+ A209V+Q264S + T49L is carried our in a similar way, except that mutagenic primer AM118 is used.

AM118:
5' GCATATAAGGGACTGAGCCAAGCGG 3' (SEQ ID NO: 36)

Construction LE317: BAN/Termamyl hybrid + H156Y+A181T+N190F+ A209V+Q264S + T49L+G107A is carried our in a similar way, except that mutagenic primer AM118 and mutagenic primer AM119 are used simultaneously.

AM119:
5' CAACCACAAAGCCGGCGCTGATGCG 3' (SEQ ID NO: 37)

Construction of LE318: BAN/Termamyl hybrid + H156Y+A181T+N190F+ A209V+Q264S + A52S+V54N+T49L+G107A is carried our in a similar way, except that mutagenic primer AM120 and mutagenic primer AM119 are used simultaneously.

AM120:
5' GCATATAAGGGACTGAGCCAATCGGATAACGGCTACGGTGC 3' (SEQ ID NO: 38)

Construction of LE 319: BAN/Termamyl hybrid + H156Y+A181T+N190F+ A209V+Q264S + A52S+V54N+T49L is carried our in a similar way, except that mutagenic primer AM120 is used.

Construction of LE320: BAN/Termamyl hybrid + H156Y+A181T+N190F+ A209V+Q264S + G107A is carried our in a similar way, except that mutagenic primer AM119 is used.

Construction of LE322: BAN/Termamyl hybrid + H156Y+A181T+N190F+A209V+Q264S + Q51R+A52S is carried our in a similar way, except that mutagenic primer AM121 is used.

AM121:
5' GAACGAGCCGATCGGACGTGGGCTACGG 3' (SEQ ID NO:39)

Construction of LE323: BAN/Termamyl hybrid + H156Y+A181T+N190F+ A209V+Q264S + A52N is carried our in a similar way, except that mutagenic primer AM122 is used.

AM122:
5' GAACGAGCCAAAACGACGTGGGCTACGG 3' (SEQ ID NO: 40)

EXAMPLE 3

Testing of LE429 variants (saccharification)

The standard reaction conditions were:

Substrate concentration	30 % w/w
Temperature	60°C
Initial pH (at 60°C)	5.5
Enzyme dosage	
Glucoamylase	0.18 AGU/g DS
Pullulanase	0.06 PUN/g DS
Alpha-amylase	10 micro g enzyme/g DS

Dextrozyme™ E was used to provide glucoamylase and pullulanase activities

Substrates for saccharification were prepared by dissolving common corn starch in deionized water and adjusting the dry substance to approximately 30% w/w. The pH was adjusted to 5.5 (measured at 60°C), and aliquots of substrate corresponding to 10 g dry weight were transferred to blue cap glass flasks.

The flasks were then placed in a shaking water bath equilibrated at 60°C, and the enzymes added. The pH was readjusted to 5.5 where necessary. The samples were taken after 48 hours of saccharification; the pH was adjusted to about 3.0, and then heated in a boiling water bath for 15 minutes to inactivate the enzymes. After cooling, the samples were treated with approximately 0.1 g mixed bed ion exchange resin (BIO-RAD 501 X8 (D)) for 30 minutes on a rotary mixer to remove salts and soluble N. After filtration, the carbohydrate composition was determined by HPLC. The following results were obtained:

The parent alpha-amylase for the variants is LE429.

Added Alpha-amylase Variants	DP_1	DP_2	DP_3	SPEC. ACT. (NU/mg)
V54N	96.1	1.75	1.18	8200
A52S	95.9	1.80	1.11	18800
A52S+V54N	96.3	1.84	1.08	10000

T49L	96.3	1.77	1.11	12300
T49L+G107A	96.4	1.87	0.72	13600
A52S+V54N+T49L+G107A	80.5	2.55	0.43	10000
A52S+V54N+T49L	95.8	1.76	0.84	8400
G107A	94.4	1.89	1.04	19600
Q51R+A52S	95.9	1.77	1.27	16500
A52N	95.5	1.89	1.56	17600
LE174 (CONTROL)	95.9/ 95.8	1.87/ 1.83	1.17/ 1.35	16000

Compared with the control, the presence of an active alpha-amylase variant of the invention during liquefaction results in decreased panose levels (DP3).

Especially the T49L+G107A variant of LE429 and the A52S+V54N+T49L variant of LE429, respectively, result in a drastically decreased panose level (DP_3). If these alpha-amylase variants are used for starch liquefaction, it will not be necessary to inactivate the enzyme before the commencement of saccharification.

Example 4

Liquefaction and saccharification of LE429 variants

The experiment in Example 3 was repeated for a number of other LE429 variants under the same conditions.

The result is shown below:

Variant/sugar profile	DP1	DP2	DP3	DP4+
T49V+G107A	95.9%	1.72%	1.27%	1.11%
T49Y+G107A	95.3%	1.73%	1.29%	1.65%
T49N+G107A	95.7%	1.64%	1.51%	1.18%
T49L+A52S+G107A	95.7%	1.73%	0.95%	1.67%
T49L+A52T+G107A	95.8%	1.66%	1.03%	1.48%
T49L+A52F+G107A	95.7%	1.69%	1.16%	1.42%
T49L+A52L+G107A	95.5%	1.70%	1.40%	1.38%
T49L+A52I+G107A	95.9%	1.72%	1.31%	1.07%
T49L+A52V+G107A	94.7%	1.69%	1.16%	2.44%

T49L+A52V+G107A+A111V	94.5%	1.75%	0.72%	2.99%
LE429	94.9%	1.71%	1.85%	1.51%

Example 5

The experiment in Example 3 was repeated for a number of LE429 variants, except that the liquefaction was carried out at 95°C, pH 6.0 and the saccharification at 60°C, pH 4.5, 40 ppm $CaCl_2$, followed by inactivation. The variant referred to below are LE429 variant. The results found are as follows:

Variant/sugar profile	DP4+	DP3	DP2	DP1
T49F	1.15	0.92	1.83	96.12
T49D+G107A	0.84	1.03	1.82	96.3
T49I+G107A	0.97	0.64	1.84	96.55
T49L+G107A	0.96	0.81	1.82	96.42
T49L+A52S+G107A	1.37	0.75	1.88	96.01
T49L+A52T+G107A	0.87	0.81	1.8	96.52
T49L+A52F+G107A	0.98	0.83	1.87	96.31
T49V+G107A	0.65	0.8	2.13	96.43
T49Y+G107A	0.83	0.94	1.89	96.35
LE429	1.16	1.21	1.77	95.87

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Phe His Gln Lys Gly Thr Val Arg Thr Lys Tyr Gly Thr Lys Gly Glu
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Leu Gln Ser Ala Ile Lys Ser Leu His Ser Arg Asp Ile Asn Val Tyr
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Gly Asp Val Val Ile Asn His Lys Gly Gly Ala Asp Ala Thr Glu Asp
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Tyr Leu Met Tyr Ala Asp Phe Asp Tyr Asp His Pro Asp Val Val Ala
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Ile Leu Lys Ala Arg Lys Gln Tyr Ala Tyr Gly Ala Gln His Asp Tyr
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Thr Ser Gln Ala Asp Val Gly Tyr Gly Ala Tyr Asp Leu Tyr Asp Leu
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 65                  70                  75                  80

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His Tyr Gln Phe His Ala Ala Ser Thr Gln Gly Gly Gly Tyr Asp Met
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Val Thr Phe Val Asp Asn His Asp Thr Gln Pro Gly Gln Ser Leu Glu
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tgctgtccag actgtccgct gtgtaaaaaa taggaataaa ggggggttgt tattatttta 720

ctgatatgta aaatataatt tgtataagaa aatgagaggg agaggaaaca tgattcaaaa 780

acgaaagcgg acagtttcgt tcagacttgt gcttatgtgc acgctgttat ttgtcagttt 840

gccgattaca aaaacatcag cc gta aat ggc acg ctg atg cag tat ttt gaa 892
Val Asn Gly Thr Leu Met Gln Tyr Phe Glu
1 5 10

tgg tat acg ccg aac gac ggc cag cat tgg aaa cga ttg cag aat gat 940
Trp Tyr Thr Pro Asn Asp Gly Gln His Trp Lys Arg Leu Gln Asn Asp
15 20 25

gcg gaa cat tta tcg gat atc gga atc act gcc gtc tgg att cct ccc 988
Ala Glu His Leu Ser Asp Ile Gly Ile Thr Ala Val Trp Ile Pro Pro
30 35 40

gca tac aaa gga ttg agc caa tcc gat aac gga tac gga cct tat gat 1036
Ala Tyr Lys Gly Leu Ser Gln Ser Asp Asn Gly Tyr Gly Pro Tyr Asp
45 50 55

ttg tat gat tta gga gaa ttc cag caa aaa ggg acg gtc aga acg aaa 1084
Leu Tyr Asp Leu Gly Glu Phe Gln Gln Lys Gly Thr Val Arg Thr Lys
60 65 70

tac ggc aca aaa tca gag ctt caa gat gcg atc ggc tca ctg cat tcc 1132
Tyr Gly Thr Lys Ser Glu Leu Gln Asp Ala Ile Gly Ser Leu His Ser
75 80 85 90

cgg aac gtc caa gta tac gga gat gtg gtt ttg aat cat aag gct ggt 1180
Arg Asn Val Gln Val Tyr Gly Asp Val Val Leu Asn His Lys Ala Gly
95 100 105

gct gat gca aca gaa gat gta act gcc gtc gaa gtc aat ccg gcc aat 1228
Ala Asp Ala Thr Glu Asp Val Thr Ala Val Glu Val Asn Pro Ala Asn
110 115 120

aga aat cag gaa act tcg gag gaa tat caa atc aaa gcg tgg acg gat 1276
Arg Asn Gln Glu Thr Ser Glu Glu Tyr Gln Ile Lys Ala Trp Thr Asp
125 130 135

ttt cgt ttt ccg ggc cgt gga aac acg tac agt gat ttt aaa tgg cat 1324
Phe Arg Phe Pro Gly Arg Gly Asn Thr Tyr Ser Asp Phe Lys Trp His
140 145 150

tgg tat cat ttc gac gga gcg gac tgg gat gaa tcc cgg aag atc agc 1372
Trp Tyr His Phe Asp Gly Ala Asp Trp Asp Glu Ser Arg Lys Ile Ser
155 160 165 170

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cgc atc ttt aag ttt cgt ggg gaa gga aaa gcg tgg gat tgg gaa gta    1420
Arg Ile Phe Lys Phe Arg Gly Glu Gly Lys Ala Trp Asp Trp Glu Val
                175                 180                 185

tca agt gaa aac ggc aac tat gac tat tta atg tat gct gat gtt gac    1468
Ser Ser Glu Asn Gly Asn Tyr Asp Tyr Leu Met Tyr Ala Asp Val Asp
            190                 195                 200

tac gac cac cct gat gtc gtg gca gag aca aaa aaa tgg ggt atc tgg    1516
Tyr Asp His Pro Asp Val Val Ala Glu Thr Lys Lys Trp Gly Ile Trp
        205                 210                 215

tat gcg aat gaa ctg tca tta gac ggc ttc cgt att gat gcc gcc aaa    1564
Tyr Ala Asn Glu Leu Ser Leu Asp Gly Phe Arg Ile Asp Ala Ala Lys
    220                 225                 230

cat att aaa ttt tca ttt ctg cgt gat tgg gtt cag gcg gtc aga cag    1612
His Ile Lys Phe Ser Phe Leu Arg Asp Trp Val Gln Ala Val Arg Gln
235                 240                 245                 250

gcg acg gga aaa gaa atg ttt acg gtt gcg gag tat tgg cag aat aat    1660
Ala Thr Gly Lys Glu Met Phe Thr Val Ala Glu Tyr Trp Gln Asn Asn
                255                 260                 265

gcc ggg aaa ctc gaa aac tac ttg aat aaa aca agc ttt aat caa tcc    1708
Ala Gly Lys Leu Glu Asn Tyr Leu Asn Lys Thr Ser Phe Asn Gln Ser
            270                 275                 280

gtg ttt gat gtt ccg ctt cat ttc aat tta cag gcg gct tcc tca caa    1756
Val Phe Asp Val Pro Leu His Phe Asn Leu Gln Ala Ala Ser Ser Gln
        285                 290                 295

gga ggc gga tat gat atg agg cgt ttg ctg gac ggt acc gtt gtg tcc    1804
Gly Gly Gly Tyr Asp Met Arg Arg Leu Leu Asp Gly Thr Val Val Ser
    300                 305                 310

agg cat ccg gaa aag gcg gtt aca ttt gtt gaa aat cat gac aca cag    1852
Arg His Pro Glu Lys Ala Val Thr Phe Val Glu Asn His Asp Thr Gln
315                 320                 325                 330

ccg gga cag tca ttg gaa tcg aca gtc caa act tgg ttt aaa ccg ctt    1900
Pro Gly Gln Ser Leu Glu Ser Thr Val Gln Thr Trp Phe Lys Pro Leu
                335                 340                 345

gca tac gcc ttt att ttg aca aga gaa tcc ggt tat cct cag gtg ttc    1948
Ala Tyr Ala Phe Ile Leu Thr Arg Glu Ser Gly Tyr Pro Gln Val Phe
            350                 355                 360

tat ggg gat atg tac ggg aca aaa ggg aca tcg cca aag gaa att ccc    1996
Tyr Gly Asp Met Tyr Gly Thr Lys Gly Thr Ser Pro Lys Glu Ile Pro
        365                 370                 375

tca ctg aaa gat aat ata gag ccg att tta aaa gcg cgt aag gag tac    2044
Ser Leu Lys Asp Asn Ile Glu Pro Ile Leu Lys Ala Arg Lys Glu Tyr
    380                 385                 390

gca tac ggg ccc cag cac gat tat att gac cac ccg gat gtg atc gga    2092
Ala Tyr Gly Pro Gln His Asp Tyr Ile Asp His Pro Asp Val Ile Gly
395                 400                 405                 410

tgg acg agg gaa ggt gac agc tcc gcc gcc aaa tca ggt ttg gcc gct    2140
Trp Thr Arg Glu Gly Asp Ser Ser Ala Ala Lys Ser Gly Leu Ala Ala
                415                 420                 425

tta atc acg gac gga ccc ggc gga tca aag cgg atg tat gcc ggc ctg    2188
Leu Ile Thr Asp Gly Pro Gly Gly Ser Lys Arg Met Tyr Ala Gly Leu
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                430                       435                       440

aaa aat gcc ggc gag aca tgg tat gac ata acg ggc aac cgt tca gat    2236
Lys Asn Ala Gly Glu Thr Trp Tyr Asp Ile Thr Gly Asn Arg Ser Asp
            445                       450                       455

act gta aaa atc gga tct gac ggc tgg gga gag ttt cat gta aac gat    2284
Thr Val Lys Ile Gly Ser Asp Gly Trp Gly Glu Phe His Val Asn Asp
        460                       465                       470

ggg tcc gtc tcc att tat gtt cag aaa taa ggtaataaaa aaacacctcc     2334
Gly Ser Val Ser Ile Tyr Val Gln Lys
475                     480

aagctgagtg cgggtatcag cttggaggtg cgtttatttt ttcagccgta tgacaaggtc 2394

ggcatcaggt gtgacaaata cggtatgctg gctgtcatag gtgacaaatc cgggttttgc 2454

gccgtttggc tttttcacat gtctgatttt tgtataatca acaggcacgg agccggaatc 2514

tttcgccttg gaaaaataag cggcgatcgt agctgcttcc aatatggatt gttcatcggg 2574

atcgctgctt ttaatcacaa cgtgggatcc                                  2604


<210> 6
<211> 483
<212> PRT
<213> Bacillus amyloliquefaciens

<400> 6
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 Gly Leu Ser
        35                  40                  45

Gln Ser Asp Asn Gly Tyr Gly Pro Tyr Asp Leu Tyr Asp Leu 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 Gln 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 Gln 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 Tyr His Phe Asp Gly
145                 150                 155                 160

Ala Asp Trp Asp Glu Ser Arg Lys Ile Ser Arg 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 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 Gln Asn Asn Ala Gly Lys Leu Glu Asn
260 265 270

Tyr Leu Asn Lys Thr Ser Phe Asn Gln Ser Val Phe Asp Val Pro Leu
275 280 285

His Phe Asn Leu Gln Ala Ala Ser Ser Gln Gly Gly Gly Tyr Asp Met
290 295 300

Arg Arg Leu Leu Asp Gly Thr Val Val Ser Arg His Pro Glu Lys Ala
305 310 315 320

Val Thr Phe Val Glu Asn His Asp Thr Gln Pro 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 Thr Ser Pro Lys Glu Ile Pro Ser Leu Lys Asp Asn Ile
370 375 380

Glu Pro Ile Leu Lys Ala Arg Lys Glu Tyr Ala Tyr Gly Pro Gln His
385 390 395 400

Asp Tyr Ile Asp His Pro Asp Val Ile Gly Trp Thr Arg Glu Gly Asp
405 410 415

Ser Ser Ala Ala Lys Ser Gly Leu Ala Ala Leu Ile Thr Asp Gly Pro
420 425 430

Gly Gly Ser Lys Arg Met Tyr Ala Gly Leu Lys Asn Ala Gly Glu Thr
435 440 445

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

Asp Gly Trp Gly Glu Phe His Val Asn Asp Gly Ser Val Ser Ile Tyr
465 470 475 480

Val Gln Lys

<210> 7
<211> 1548
<212> DNA
<213> Bacillus stearothermophilus

<220>
<221> CDS
<222> (1)..(1548)

<400> 7

gcc gca ccg ttt aac ggc acc atg atg cag tat ttt gaa tgg tac ttg 48
Ala Ala Pro Phe Asn Gly Thr Met Met Gln Tyr Phe Glu Trp Tyr Leu
1 5 10 15

ccg gat gat ggc acg tta tgg acc aaa gtg gcc aat gaa gcc aac aac 96
Pro Asp Asp Gly Thr Leu Trp Thr Lys Val Ala Asn Glu Ala Asn Asn
20 25 30

tta tcc agc ctt ggc atc acc gct ctt tgg ctg ccg ccc gct tac aaa 144
Leu Ser Ser Leu Gly Ile Thr Ala Leu Trp Leu Pro Pro Ala Tyr Lys
35 40 45

gga aca agc cgc agc gac gta ggg tac gga gta tac gac ttg tat gac 192
Gly Thr Ser Arg Ser Asp Val Gly Tyr Gly Val Tyr Asp Leu Tyr Asp
50 55 60

ctc ggc gaa ttc aat caa aaa ggg acc gtc cgc aca aaa tac gga aca 240
Leu Gly Glu Phe Asn Gln Lys Gly Thr Val Arg Thr Lys Tyr Gly Thr
65 70 75 80

aaa gct caa tat ctt caa gcc att caa gcc gcc cac gcc gct gga atg 288
Lys Ala Gln Tyr Leu Gln Ala Ile Gln Ala Ala His Ala Ala Gly Met
85 90 95

caa gtg tac gcc gat gtc gtg ttc gac cat aaa ggc ggc gct gac ggc 336
Gln Val Tyr Ala Asp Val Val Phe Asp His Lys Gly Gly Ala Asp Gly
100 105 110

acg gaa tgg gtg gac gcc gtc gaa gtc aat ccg tcc gac cgc aac caa 384
Thr Glu Trp Val Asp Ala Val Glu Val Asn Pro Ser Asp Arg Asn Gln
115 120 125

gaa atc tcg ggc acc tat caa atc caa gca tgg acg aaa ttt gat ttt 432
Glu Ile Ser Gly Thr Tyr Gln Ile Gln Ala Trp Thr Lys Phe Asp Phe
130 135 140

ccc ggg cgg ggc aac acc tac tcc agc ttt aag tgg cgc tgg tac cat 480
Pro Gly Arg Gly Asn Thr Tyr Ser Ser Phe Lys Trp Arg Trp Tyr His
145 150 155 160

ttt gac ggc gtt gat tgg gac gaa agc cga aaa ttg agc cgc att tac 528
Phe Asp Gly Val Asp Trp Asp Glu Ser Arg Lys Leu Ser Arg Ile Tyr
165 170 175

aaa ttc cgc ggc atc ggc aaa gcg tgg gat tgg gaa gta gac acg gaa 576
Lys Phe Arg Gly Ile Gly Lys Ala Trp Asp Trp Glu Val Asp Thr Glu
180 185 190

aac gga aac tat gac tac tta atg tat gcc gac ctt gat atg gat cat 624
Asn Gly Asn Tyr Asp Tyr Leu Met Tyr Ala Asp Leu Asp Met Asp His
195 200 205

ccc gaa gtc gtg acc gag ctg aaa aac tgg ggg aaa tgg tat gtc aac 672
Pro Glu Val Val Thr Glu Leu Lys Asn Trp Gly Lys Trp Tyr Val Asn
210 215 220

aca acg aac att gat ggg ttc cgg ctt gat gcc gtc aag cat att aag 720
Thr Thr Asn Ile Asp Gly Phe Arg Leu Asp Ala Val Lys His Ile Lys
225 230 235 240

ttc agt ttt ttt cct gat tgg ttg tcg tat gtg cgt tct cag act ggc 768
Phe Ser Phe Phe Pro Asp Trp Leu Ser Tyr Val Arg Ser Gln Thr Gly
245 250 255

aag ccg cta ttt acc gtc ggg gaa tat tgg agc tat gac atc aac aag 816
Lys Pro Leu Phe Thr Val Gly Glu Tyr Trp Ser Tyr Asp Ile Asn Lys

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            260                 265                 270

ttg cac aat tac att acg aaa aca gac gga acg atg tct ttg ttt gat    864
Leu His Asn Tyr Ile Thr Lys Thr Asp Gly Thr Met Ser Leu Phe Asp
        275                 280                 285

gcc ccg tta cac aac aaa ttt tat acc gct tcc aaa tca ggg ggc gca    912
Ala Pro Leu His Asn Lys Phe Tyr Thr Ala Ser Lys Ser Gly Gly Ala
    290                 295                 300

ttt gat atg cgc acg tta atg acc aat act ctc atg aaa gat caa ccg    960
Phe Asp Met Arg Thr Leu Met Thr Asn Thr Leu Met Lys Asp Gln Pro
305                 310                 315                 320

aca ttg gcc gtc acc ttc gtt gat aat cat gac acc gaa ccc ggc caa   1008
Thr Leu Ala Val Thr Phe Val Asp Asn His Asp Thr Glu Pro Gly Gln
                325                 330                 335

gcg ctg cag tca tgg gtc gac cca tgg ttc aaa ccg ttg gct tac gcc   1056
Ala Leu Gln Ser Trp Val Asp Pro Trp Phe Lys Pro Leu Ala Tyr Ala
            340                 345                 350

ttt att cta act cgg cag gaa gga tac ccg tgc gtc ttt tat ggt gac   1104
Phe Ile Leu Thr Arg Gln Glu Gly Tyr Pro Cys Val Phe Tyr Gly Asp
        355                 360                 365

tat tat ggc att cca caa tat aac att cct tcg ctg aaa agc aaa atc   1152
Tyr Tyr Gly Ile Pro Gln Tyr Asn Ile Pro Ser Leu Lys Ser Lys Ile
    370                 375                 380

gat ccg ctc ctc atc gcg cgc agg gat tat gct tac gga acg caa cat   1200
Asp Pro Leu Leu Ile Ala Arg Arg Asp Tyr Ala Tyr Gly Thr Gln His
385                 390                 395                 400

gat tat ctt gat cac tcc gac atc atc ggg tgg aca agg gaa ggg ggc   1248
Asp Tyr Leu Asp His Ser Asp Ile Ile Gly Trp Thr Arg Glu Gly Gly
                405                 410                 415

act gaa aaa cca gga tcc gga ctg gcc gca ctg atc acc gat ggg ccg   1296
Thr Glu Lys Pro Gly Ser Gly Leu Ala Ala Leu Ile Thr Asp Gly Pro
            420                 425                 430

gga gga agc aaa tgg atg tac gtt ggc aaa caa cac gct gga aaa gtg   1344
Gly Gly Ser Lys Trp Met Tyr Val Gly Lys Gln His Ala Gly Lys Val
        435                 440                 445

ttc tat gac ctt acc ggc aac cgg agt gac acc gtc acc atc aac agt   1392
Phe Tyr Asp Leu Thr Gly Asn Arg Ser Asp Thr Val Thr Ile Asn Ser
    450                 455                 460

gat gga tgg ggg gaa ttc aaa gtc aat ggc ggt tcg gtt tcg gtt tgg   1440
Asp Gly Trp Gly Glu Phe Lys Val Asn Gly Gly Ser Val Ser Val Trp
465                 470                 475                 480

gtt cct aga aaa acg acc gtt tct acc atc gct cgg ccg atc aca acc   1488
Val Pro Arg Lys Thr Thr Val Ser Thr Ile Ala Arg Pro Ile Thr Thr
                485                 490                 495

cga ccg tgg act ggt gaa ttc gtc cgt tgg acc gaa cca cgg ttg gtg   1536
Arg Pro Trp Thr Gly Glu Phe Val Arg Trp Thr Glu Pro Arg Leu Val
            500                 505                 510

gca tgg cct tga                                                   1548
Ala Trp Pro
        515
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<210> 8
<211> 515
<212> PRT
<213> Bacillus stearothermophilus

<400> 8
Ala Ala Pro Phe Asn Gly Thr Met Met Gln Tyr Phe Glu Trp Tyr Leu
1   5   10   15

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

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 Gln Lys Gly Thr Val Arg Thr Lys Tyr Gly Thr
65   70   75   80

Lys Ala Gln Tyr Leu Gln Ala Ile Gln Ala Ala His Ala Ala Gly Met
                85   90   95

Gln Val Tyr Ala Asp Val Val Phe Asp His Lys Gly Gly Ala Asp Gly
            100   105   110

Thr Glu Trp Val Asp Ala Val Glu Val Asn Pro Ser Asp Arg Asn Gln
        115   120   125

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

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

Phe Asp Gly Val Asp Trp Asp Glu Ser Arg Lys Leu Ser Arg Ile Tyr
                165   170   175

Lys Phe Arg Gly Ile Gly Lys Ala Trp Asp Trp Glu Val Asp Thr Glu
            180   185   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 Asn 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 Tyr Val Arg Ser Gln Thr Gly
                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 Thr Lys Thr Asp 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 Ala
    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 Asp Asn His Asp Thr Glu Pro Gly Gln

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                    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 Ala Arg Arg Asp Tyr Ala Tyr Gly Thr Gln His
385                 390                 395                 400

Asp Tyr Leu Asp His Ser Asp Ile Ile Gly Trp Thr Arg Glu Gly Gly
                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 Gln 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 Gly Ser Val Ser Val Trp
465                 470                 475                 480

Val Pro Arg Lys Thr Thr Val Ser Thr Ile Ala Arg Pro Ile Thr Thr
                485                 490                 495

Arg Pro Trp Thr Gly Glu Phe Val Arg Trp Thr Glu Pro Arg Leu Val
            500                 505                 510

Ala Trp Pro
        515


<210> 9
<211> 31
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Primer

<400> 9
ggtcgtaggc accgtagccc caatccgctt g                                31


<210> 10
<211> 36
<212> DNA
<213> Artificial Sequence


<220>
<223> Description of Artificial Sequence: Primer

<400> 10
ggtcgtaggc accgtagccc caatcccatt ggctcg                           36


<210> 11
<211> 28
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<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Primer

<400> 11
ctgtgactgg tgagtactca accaagtc 28

<210> 12
<211> 31
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Primer

<400> 12
ggtcgtaggc accgtagccc tcatccgctt g 31

<210> 13
<211> 31
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Primer

<400> 13
ggtcgtaggc accgtagccc atatccgctt g 31

<210> 14
<211> 31
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Primer

<400> 14
ggtcgtaggc accgtagcca atatccgctt g 31

<210> 15
<211> 36
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Primer

<400> 15
gcagcatgga actgctyatg aagaggcacg tcaaac 36

<210> 16
<211> 30
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Primer

<400> 16

catagttgcc gaattcattg gaaacttccc 30

<210> 17
<211> 34
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Primer

<400> 17
catagttgcc gaattcaggg gaaacttccc aatc 34

<210> 18
<211> 41
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Primer

<400> 18
ccgcgccccg ggaaatcaaa ttttgtccag gctttaatta g 41

<210> 19
<211> 32
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Primer

<400> 19
caaaatggta ccaataccac ttaaaatcgc tg 32

<210> 20
<211> 29
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Primer

<400> 20
cttcccaatc ccaagtcttc ccttgaaac 29

<210> 21
<211> 36
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Primer

<400> 21
cttaatttct gctacgacgt caggatggtc ataatc 36

<210> 22
<211> 38
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Primer

<400> 22
cgcccaagtc attcgaccag tactcagcta ccgtaaac 38

<210> 23
<211> 29
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Primer

<400> 23
gccgttttca ttgtcgactt cccaatccc 29

<210> 24
<211> 35
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Primer

<400> 24
ggaatttcgc gctgactagt cccgtacata tcccc 35

<210> 25
<211> 36
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Primer

<400> 25
ggcaggaatt tcgcgacctt tcgtcccgta catatc 36

<210> 26
<211> 36
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Primer

<400> 26
cctcattctg cagcagcagc cgtaaatggc acgctg 36

<210> 27
<211> 38
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Primer

<400> 27
ccagacggca gtaataccga tatccgataa atgttccg 38

<210> 28
<211> 30
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Primer

<400> 28
cggatatcgg tattactgcc gtctggattc 30

<210> 29
<211> 21
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Primer

<400> 29
ctcgtcccaa tcggttccgt c 21

<210> 30
<211> 26
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Primer

<400> 30
gatgtatgcc gacttcgatt atgacc 26

<210> 31
<211> 30
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Primer

<400> 31
catagttgcc gaattcattg gaaacttccc 30

<210> 32
<211> 24
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Primer

<400> 32
ccgattgctg acgctgttat ttgc 24

<210> 33
<211> 25
<212> DNA
<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Primer

<400> 33
gccaagcgga taacggctac ggtgc 25

<210> 34
<211> 28
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Primer

<400> 34
gaacgagcca atcggacgtg ggctacgg 28

<210> 35
<211> 32
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Primer

<400> 35
ggaacgagcc aatcggataa cggctacggt gc 32

<210> 36
<211> 25
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Primer

<400> 36
gcatataagg gactgagcca agcgg 25

<210> 37
<211> 25
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Primer

<400> 37
caaccacaaa gccggcgctg atgcg 25

<210> 38
<211> 41
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Primer

<400> 38
gcatataagg gactgagcca atcggataac ggctacggtg c 41

<210> 39

<211> 28
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Primer

<400> 39
gaacgagccg atcggacgtg ggctacgg 28

<210> 40
<211> 28
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Primer

<400> 40
gaacgagcca aaacgacgtg ggctacgg 28

CLAIMS

1. A variant of a parent alpha-amylase, wherein the parent alpha-amylase has the amino acid sequence shown in SEQ ID NO: 4;

wherein the variant has at least 85% identity to SEQ ID NO: 4;

wherein the variant comprises one of the following mutations using the numbering in SEQ ID NO: 4:

V54N, A52S, A52S+V54N, A52S+V54N+T49L+G107A, A52S+V54N+T49L, G107A, Q51R, Q51R+A52S, A52N; T49F+G107A, T49V+G107A, T49D+G107A, T49Y+G107A, T49S+G107A, T49N+G107A, T49I+G107A, T49L+A52S+G107A, T49L+A52T+G107A, T49L+A52F+G107A, T49L+A52L+G107A, T49L+A52I+G107A, T49L+A52V+G107A; T49V, T49I, T49D, T49N, T49S, T49Y, T49F, T49W, T49M, T49E, T49Q, T49K, T49R, A52T, A52L, A52I, A52V, A52M, A52F, A52Y, A52W, V54M, G107V, G107I, G107L, and G107C;

and wherein the variant has alpha-amylase activity.

2. A variant of a parent alpha-amylase, wherein the parent alpha-amylase has the amino acid sequence shown in SEQ ID NO: 4;

wherein the variant has at least 85% identity to SEQ ID NO: 4;

wherein the variant comprises the following mutation using the numbering in SEQ ID NO: 4: T49X+A52X+V54N/I/L/Y/F/W+G107A;

and wherein the variant has alpha-amylase activity.

3. The variant of claims 1 or 2, further comprising the mutation G108A using the numbering in SEQ ID NO: 4.

4. A variant of a parent alpha-amylase, wherein the parent alpha-amylase has the amino acid sequence shown in SEQ ID NO: 4;

wherein the variant has at least 85% identity to SEQ ID NO: 4;

wherein the variant comprises one of the following mutations using the numbering in SEQ ID NO: 4:

T49L+G107A;

T49I+G107A;

T49L+G107A+V54I;

T49I+G107A+V54I;
A52S+V54N+T49L+G107A;
A52S+V54I+T49L+G107A;
A52S+T49L+G107A;
A52T+T49L+G107A;
A52S+V54N+T49I+G107A;
A52S+V54I+T49I+G107A;
A52S+T49I+G107A;
T49L+G108A;
T49I+G108A;
T49L+G108A+V54I; and
T49I+G108A+V54I;

and wherein the variant has alpha-amylase activity.

5. A variant of a parent hybrid alpha-amylase, wherein the parent hybrid 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. amyloliquefaciens* shown in SEQ ID NO: 6;

wherein the variant has at least 85% identity to SEQ ID NO: 4; and

wherein the variant comprises:

(i) one of the following mutations using the numbering in SEQ ID NO: 4:

V54N, A52S, A52S+V54N, A52S+V54N+T49L+G107A, A52S+V54N+T49L, G107A, Q51R, Q51R+A52S, A52N; T49F+G107A, T49V+G107A, T49D+G107A, T49Y+G107A, T49S+G107A, T49N+G107A, T49I+G107A, T49L+A52S+G107A, T49L+A52T+G107A, T49L+A52F+G107A, T49L+A52L+G107A, T49L+A52I+G107A, T49L+A52V+G107A; T49V, T49I, T49D, T49N, T49S, T49Y, T49F, T49W, T49M, T49E, T49Q, T49K, T49R, A52T, A52L, A52I, A52V, A52M, A52F, A52Y, A52W, V54M, G107V, G107I, G107L, and G107C; or

(ii) one of the following mutations using the numbering in SEQ ID NO: 4:

T49L+G107A;
T49I+G107A;
T49L+G107A+V54I;
T49I+G107A+V54I;
A52S+V54N+T49L+G107A;

A52S+V54I+T49L+G107A;
A52S+T49L+G107A;
A52T+T49L+G107A;
A52S+V54N+T49I+G107A;
A52S+V54I+T49I+G107A;
A52S+T49I+G107A;
T49L+G108A;
T49I+G108A;
T49L+G108A+V54I; and
T49I+G108A+V54I;
and wherein the variant has alpha-amylase activity.

6. The variant of claim 5, wherein the variant further comprises the following mutations: H156Y+A181T+N190F+A209V+Q264S using the numbering in SEQ ID NO: 4.

7. The variant of claim 5, wherein the variant further comprises the following mutations: H156Y+A181T+N190F+A209V+Q264S+I201F using the numbering of SEQ ID NO: 4.

8. A DNA construct comprising a DNA sequence encoding an alpha-amylase variant according to any one of claims 1-7.

9. A recombinant expression vector which carries a DNA construct according to claim 8.

10. A cell which is transformed with a DNA construct according to claim 8 or a vector according to 9.

11. A cell of claim 10, which is a microorganism of bacterial or fungal origin.

12. Use of an alpha-amylase variant of any one of claims 1-7 for starch liquefaction; for laundry, dish or hard surface cleaning; for ethanol production; or desizing of textiles, fabrics or garments.

His	His	Asn	Gly	Thr	Asn	Gly	Thr	Met	Met	Gln	Tyr	Phe	Glu	Trp	Tyr
1				5					10					15	
Leu	Pro	Asn	Asp	Gly	Asn	His	Trp	Asn	Arg	Leu	Arg	Asp	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	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

Fig. 1

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 Asn His Ser Ser Phe Lys Trp Arg Trp Tyr
145 150 155 160

His Phe Asp Gly Thr Asp Trp Asp Gln 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 Leu 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 Asn 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 Ala Ser Asn Ser Gly
290 295 300

Gly Tyr Tyr Asp Met Arg Asn Ile Leu Asn Gly Ser Val Val Gln 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 Gln 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

Fig. 1 (cont.)

370 375 380

Lys Ile Asp Pro Leu Leu Gln Ala Arg Gln Thr Phe Ala Tyr Gly Thr
385 390 395 400

Gln His Asp Tyr Phe Asp His His Asp 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

Gln Val Trp Arg Asp Ile Thr Gly Asn Arg Thr Gly Thr 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

Val Trp Val Lys Gln
485

Fig. 1 (cont.)

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	Arg	Gly	Ile	Thr	Ala	Ile	Trp	Ile	Pro	Pro	Ala	Trp
		35					40					45			
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	Ser	Gln	Leu	Glu	Ser	Ala	Ile	His	Ala	Leu	Lys	Asn	Asn	Gly
				85					90					95	

Fig. 2

Val Gln Val Tyr Gly Asp Val Val Met Asn His Lys Gly 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 Gly Asn Thr Tyr Ser Asp Phe Lys Trp Arg Trp Tyr
145 150 155 160

His Phe Asp Gly Val Asp Trp Asp Gln Ser Arg Gln Phe Gln 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 Trp 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 Asn 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
305 310 315 320

His Pro Met His Ala Val Thr Phe Val Asp Asn His Asp Ser Gln Pro
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 Gln 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 Gln Asn Phe Ala Tyr Gly Thr

Fig. 2 (cont.)

```
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 Gly Glu Lys Trp Met Tyr Val Gly Gln Asn 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
Asn Ala Asp Gly Trp Ala Asn Phe Ser Val Asn Gly Gly Ser Val Ser
465                 470                 475                 480
Ile Trp Val Lys Arg
                485
```

Fig. 2 (cont.)

His	His	Asn	Gly	Thr	Asn	Gly	Thr	Met	Met	Gln	Tyr	Phe	Glu	Trp	Tyr
1				5					10					15	
Leu	Pro	Asn	Asp	Gly	Asn	His	Trp	Asn	Arg	Leu	Asn	Ser	Asp	Ala	Ser
			20					25					30		
Asn	Leu	Lys	Ser	Lys	Gly	Ile	Thr	Ala	Val	Trp	Ile	Pro	Pro	Ala	Trp
		35					40					45			
Lys	Gly	Ala	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	Ser	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		
Ala	Thr	Glu	Met	Val	Arg	Ala	Val	Glu	Val	Asn	Pro	Asn	Asn	Arg	Asn
		115					120					125			
Gln	Glu	Val	Thr	Gly	Glu	Tyr	Thr	Ile	Glu	Ala	Trp	Thr	Arg	Phe	Asp
	130					135					140				
Phe	Pro	Gly	Arg	Gly	Asn	Thr	His	Ser	Ser	Phe	Lys	Trp	Arg	Trp	Tyr
145					150					155					160
His	Phe	Asp	Gly	Val	Asp	Trp	Asp	Gln	Ser	Arg	Arg	Leu	Asn	Asn	Arg
				165					170					175	
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	Trp	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	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		

Fig. 3

Gly Ala Ile Glu Asn Tyr Leu Gln 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 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 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 Gln 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 Gln Lys Tyr Ala Tyr Gly Lys
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 Ser Lys Trp Met Phe Val Gly Arg Asn Lys Ala Gly
        435   440   445

Asn Val Trp Ser Asp Ile Thr Gly Asn Arg Thr Gly Thr 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

Fig. 3 (cont.)