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New glycosyl hydrolases

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The present invention relates to new glycosyl hydrolases having amylolytic activity and to the nucleic acids coding for these glycosyl hydrolases, to a PCR-based method for identifying and isolating new glycosyl hydrolases from metagenomic DNA, and to various possible industrial applications thereof. Among these, washing and cleaning compositions with such enzymes, and corresponding methods and possible uses are of particular interest.

Glycosyl hydrolases represent a very diverse group of enzymes which hydrolyze glycosidic linkages between two or more carbohydrates, or between a carbohydrate and a non-carbohydrate function.

Glycosyl hydrolases of particular industrial and technical interest are the α-amylases (E.C. 3. 2. 1. 1) which hydrolyze α-1,4-glycosidic linkages, which are located in the interior of polymers, of starch and starch-like polymers, such as, for example, amylose, amylopectin or glycogen, to form dextrins and β-1,6-branched oligosaccharides. They are among the most important of all the enzymes utilized in industry. There are two reasons for this: firstly they are usually, like many substrate-degrading enzymes, released by microorganisms into the surrounding medium so that they can be obtained on the industrial scale with comparatively little effort by fermentation and purification from the culture medium. Secondly, amylases are required for a wide range of applications.

The principal industrial use of α-amylase is in the production of glucose syrup. Other uses are, for example, that as active components in washing and cleaning compositions, for the treatment of raw

materials in textile manufacture, for the production of adhesives or for the production of sugar-containing food products or food ingredients.

One example of an amylase which is employed particularly intensively in industry is the α-amylase from *Bacillus licheniformis*, which is supplied by Novozymes A/S, Bagsværd, Denmark, under the proprietary name Termamyl®. The amylase obtained from *B. subtilis* or *B. amyloliquefaciens* and disclosed in US patent US 1 227 374 is marketed by the same company under the name BAN®.

This amylase molecule, and its near relations, have been further developed in numerous inventions which were based on the object of optimizing, with the aid of various molecular biological modifications, their enzymatic properties for specific applications. Such optimizations may relate for example to the substrate specificities, the stability of the enzyme under various reaction conditions or the enzymatic activity itself. Examples of such optimizations which may be mentioned are the following property rights: EP 0410498 B1 for the sizing of textiles and WO 96/02633 A1 for the liquefaction of starch.

Since developments which consist merely of optimizations of only a few known starting enzymes are possibly restricted in the results which can be achieved, an intensive search is taking place in parallel thereto for comparable enzymes from other natural sources. Those which have been identified are starch-cleaving enzymes for example from *Pimelobacter*, *Pseudomonas* and *Thermus* for the manufacture of food products, cosmetics and drugs (EP 0 636 693 A2), ones of the same type from *Rhizobium*, *Arthrobacter*, *Brevibacterium* and *Micrococcus* (EP 0 628 630 A2), from

Pyrococcus (WO 94/19454 A2) and *Sulfolobus* for the liquefaction of starch at high temperatures, or strongly acidic reaction conditions (EP 0 727 485 A1 and WO 96/02633 A1). Amylases for use at alkaline pH values have been found in *Bacillus sp.* (WO 95/26397 A1 and WO 97/00324 A1). Because of their low sensitivity to washing compositions, other amylases from various *Bacilli* (EP 0 670 367 A1) are suitable for use in washing or cleaning compositions.

Further optimizations of enzymes isolated from natural sources for the respective area of application can be carried out for example by molecular biological methods (for example as in US 5171673 or WO 99/20768 A1) or by chemical modifications (DE 4013142 A1). The patent application WO 99/43793 A1, for example, describes a further development of the known Novamyl® α-amylase. Similarities in sequence between Novamyl® and known cyclodextrin glucanotransferases (CGTases) are utilized therein to construct, with the aid of molecular biology techniques, a multitude of related molecules. These comprise α-amylases with additional CGTase-specific consensus sequences (boxes) and functions or, conversely, CGTases with additional regions and functions typical of α-amylases, or chimeras of the two molecules. The point of this development is to optimize Novamyl® for these applications.

The new enzymes derived from the optimization of known glycosyl hydrolases and, in particular, amylases are, by their nature, limited in their properties because they represent local optima in the "fitness landscape" of glycosyl hydrolases. In order to find further, possibly even better, local optima it is necessary to start from other original enzymes which either themselves represent such a local optimum, or can be optimized with reasonable effort in the direction

thereof. However, the finding of new glycosyltransferases which may be such original enzymes requires, by the route of classical microbiological screening, the isolation of defined strains. However, more than 95% of all occurring microorganisms cannot be cultivated, so that the new glycosyl hydrolases occurring in these microorganisms, including the amylases, have not to date been accessible to characterization.

α-Amylases are typically monomeric enzymes with a molecular weight of about 55 kDa. The central domain has an α/β barrel structure (TIM barrel) which, according to the current state of knowledge, represents the most widespread protein-folding motif. The linear protein strand consists of in each case 8 α-helices and 8 β-pleated sheet segments (β sheets) which occur in alternating sequence and fold three-dimensionally in such a way that the β-pleated sheets are disposed in the form of a circle in parallel orientation and are surrounded by the α-helices on the outside.

The enzymatic activity is probably derived from amino acid residues which are located at the C-terminal end of the individual helices and β-pleated sheets.

For the example of the amylase known as Termamyl from *Bacillus licheniformis*, the amino acid residues are the aspartate 231 (general base catalyst) and glutamate 261 (general acid catalyst). The residue Asp 328 which was originally regarded as general acid catalyst is, according to recent investigations, attributed with an essential influence on the electrostatic conditions in the active site. Further residues such as, for example, His 210 are thought to be involved in the binding of the substrate or further factors such as, for example, Ca^{2+} ions.

Besides the actual amylases, further enzymes are included in the amylase superfamily, for example α-1,6-glucosidases, cyclodextrin glucanotransferases (CGTases), isoamylases, neopullulanases, glycogen debranching enzyme, dextran glucosidases and glycosyltransferases.

The substrate binding specificity, which differs widely in some cases, is brought about by protein loops which are inserted between particular structural elements and form relatively small independent domains.

Thus, a B domain exists and is disposed between β-pleated sheets 3 and helix 3. It comprises in the case of α-amylases, and α-1,6-glucosidases and cyclodextrin glucanotransferases (CGTases) closely related thereto, approximately 40 amino acids, but may also, as, for example, in the case of glucan-debranching enzymes, comprise 270 amino acids (Jespersen, H.M.; E.A. MacGregor, B. Henrissat, M. Sierks and B. Svensson: "Starch- and Glycogen-Debranching and Branching Enzymes", *J. of Protein Chemistry*, volume 12, pp. 791-805, 1993).

Further domains are disposed at the C terminus, such as the C domain in all enzymes and one or more further D or E domains (for example in CGTases). These are likewise attributed with involvement in substrate binding.

Despite this generally similar structure, the sequence homology of the linear protein strand varies widely, making it extremely difficult to define characteristic sequence segments as probes which could be used to search in an efficient manner for new amylases.

Structural elements responsible for the folding may also be formed without homology in the primary structure. Thus, the amylases from Bacillus licheniformis and Bacillus amyloliquefaciens are more than 80% identical. By contrast, the homology over all amylases of bacterial, animal and vegetable origin is less than 10% (Nakajima et al., *Appl. Microbiol. Biotechnol.*, volume 23, pp. 355-360 (1986)).

Further TIM barrel proteins are, despite an identical protein-folding structure, no longer homologous in any way.

Thus, proteins having little similarity in terms of homology may nevertheless display an identical or closely related enzymic activity as long as there is a similar protein-folding pattern and the appropriate topological disposition, which is necessary for the catalytic reaction, of the amino acid residues involved.

It can be stated in summary that there are great problems in the finding of new glycosyl hydrolases both due to the impossibility of cultivating many microorganisms which produce glycosyl hydrolases of potential interest, and due to the often only small sequence homology within the family of glycosyl hydrolases.

It has surprisingly been found that new glycosyl hydrolases can be characterized by screening DNA from environmental samples, so-called metagenomic DNA, and be provided in an industrially utilizable manner.

Mutually independent aspects of the present invention are therefore the new glycosyl hydrolases which are detailed in the sequence listing hereinafter under the

sequence identification numbers (Seq. Id.) 1, 2 and 3, glycosyl hydrolases which are substantially homologous thereto, and glycosyl hydrolases which can be obtained from those described by sequences 1 to 3 by conservative amino acid exchanges. Further aspects of the invention are nucleic acids (DNA or RNA) which code for one of the aforementioned glycosyl hydrolases.

An expression in the form "at least X%" hereinafter means "X% to 100% including the extreme values of X and 100 and all integral and non-integral percentages in between".

A protein means for the purposes of the present application a polymer which is composed of the natural amino acids, has a substantially linear structure, and assumes in most cases a three-dimensional structure to exercise its function. In the present application, the 19 proteinogenic naturally occurring L-amino acids are designated by the internationally used 1- and 3-letter codes.

An enzyme means for the purposes of the present application a protein which exercises a particular biochemical function. Glycosyl hydrolases means proteins which hydrolase glycosidic linkages. Preferred and industrially important glycosyl hydrolases are amylolytic proteins or enzymes having an amylolytic function, that is to say those which hydrolyze α-1,4-glycosidic linkages of polysaccharides, especially those linkages located in the interior of the polysaccharides. They are also referred to as α-1,4-amylases (E.C.3.2.1.1) or for short: α-amylases.

Numerous proteins are produced as so-called preproteins, i.e. together with a signal peptide. The latter then means the N-terminal part of the protein

whose function is usually to ensure exportation of the produced protein from the producing cell into the periplasm or the surrounding medium and/or correct folding thereof. Subsequently, the signal peptide is under natural conditions cleaved off the remaining protein by a signal peptidase, so that this protein exercises its actual catalytic activity without the initially present N-terminal amino acids.

For industrial applications, the mature peptides, i.e. the enzymes processed after production thereof, are preferred because of their enzymatic activity to the preproteins.

Proproteins are inactive precursors of proteins. The precursors thereof having a signal sequence are referred to as preproproteins.

Nucleic acids mean for the purposes of the present application the molecules which are naturally composed of nucleotides and serve as information carriers and which code for the linear amino acid sequence in proteins or enzymes. They may be in the form of a single strand, of a single strand complementary to the single strand, or of a double strand. For molecular biological operations, preference is given to the nucleic acid DNA, as the naturally more durable information carrier. In contrast, an RNA is produced to implement the invention in a natural environment such as, for example, in an expressing cell, which is why RNA molecules essential to the invention likewise represent embodiments of the present invention.

In the case of DNA, the sequences of the two complementary strands in all three possible reading frames in each case must be taken into account. Account must also be taken of the fact that different codon

triplets may code for the same amino acids, so that a particular amino acid sequence can be derived from a plurality of different nucleotide sequences which may exhibit only little identity (degeneracy of the genetic code). In addition, different organisms exhibit differences in the usage of these codons. For these reasons, both amino acid sequences and nucleotide sequences must be included in consideration of the scope of protection, and indicated nucleotide sequences are in each case to be regarded only as an example of coding for a particular amino acid sequence.

The information unit corresponding to a protein is also referred to as gene for the purposes of the present invention.

A skilled person is able by methods generally known nowadays, such as, for example, chemical synthesis or polymerase chain reaction (PCR) in conjunction with standard methods of molecular biology and/or protein chemistry, to prepare on the basis of known DNA and/or amino acid sequences the corresponding nucleic acids as far as the complete genes. Such methods are disclosed for example in the "Lexikon der Biochemie", Spektrum Akademischer Verlag, Berlin, 1999, volume 1, pp. 267-271 and volume 2, pp. 227-229.

Alterations in the nucleotide sequence like those which can be brought about for example by methods of molecular biology known per se are referred to as mutations. Depending on the nature of the alteration, known examples are deletion, insertion or substitution mutations or those in which different genes or parts of genes are fused together (shuffling); these are gene mutations. The relevant organisms are referred to as mutants. The proteins derived from mutated nucleic acids are referred to as variants. Thus, for example,

deletion, insertion, substitution mutations or fusions lead to deletion-, insertion-, substitution-mutated or fusion genes and, at the protein level, to corresponding deletion, insertion or substitution variants or fusion proteins.

Fragments mean all proteins or peptides which are smaller than natural proteins or those which correspond to completely translated genes and can for example also be obtained synthetically. They can be assigned to the relevant complete proteins on the basis of their amino acid sequences. They may, for example, adopt identical structures or exercise proteolytic activities or part activities such as, for example, complexation of a substrate. Fragments and deletion variants of starting proteins are in principle similar; whereas fragments tend to represent relatively small pieces, deletion mutants tend to lack only short regions and thus only individual part functions.

The fragments correspond at the nucleic acid level to the partial sequences.

Chimeric or hybrid proteins mean for the purposes of the present application proteins which are composed of elements which naturally originate from different polypeptide chains from the same organism or from different organisms. This procedure is also called shuffling or fusion mutagenesis. The point of such a fusion may be, for example, to bring about or to modify a particular enzymatic function with the aid of the protein portion which is fused on. It is in this connection immaterial for the purposes of the present invention whether such a chimeric protein consists of a single polypeptide chain or a plurality of subunits on which different functions may be distributed. To implement the latter alternative it is possible for

example for a single chimeric polypeptide chain to be broken down into a plurality thereof by a specific proteolytic cleavage post-translationally or only after a purification step.

Proteins obtained by insertion mutation mean variants which have been obtained by methods known per se through introduction of a nucleic acid fragment or protein fragment into the starting sequences. Because of their similarity in principle, they are to be assigned to the chimeric proteins. They differ from the latter only in the ratio of the size of the unaltered protein portion to the size of the complete protein. The proportion of foreign protein in such insertion-mutated proteins is less than in chimeric proteins.

Inversion mutagenesis, i.e. a partial inversion of sequence, can be regarded as a special form both of deletion and of insertion. The same applies to a regrouping, differing from the original amino acid sequence, of various parts of the molecule. It can be regarded as deletion variant, as insertion variant and as shuffling variant of the original protein.

Derivatives mean for the purposes of the present application proteins whose pure amino acid chain has been chemically modified. Such derivatizations may, for example, take place biologically in connection with protein biosynthesis by the host organism. Methods of molecular biology can be employed for this purpose. However, they can also be carried out chemically, for example by chemical transformation of a side chain of an amino acid or by covalent bonding of another compound to the protein. Such a compound may also be, for example, other proteins which are linked for example via bifunctional chemical compounds to proteins of the invention. Such modifications may, for example,

influence the substrate specificity or the strength of binding to the substrate or bring about a temporary blocking of the enzymatic activity if the substance which is coupled on is an inhibitor. This may be worthwhile for example for the period of storage. Derivatization likewise means covalent bonding to a macromolecular support.

Proteins may also be included in groups of immunologically related proteins via the reaction with an antiserum or a particular antibody. Those belonging to a group are distinguished by having the same antigenic determinant recognized by an antibody.

For the purposes of the present invention, all enzymes, proteins, fragments and derivatives are, unless they need to be explicitly referred to as such, included under the generic term of proteins.

Vectors mean for the purposes of the present invention elements which consist of nucleic acids and which comprise as characterizing nucleic acid region a gene of interest. They are able to establish the latter as a stable genetic element which replicates independently of the remaining genome in a species or a cell line over a plurality of generations or cell divisions. Vectors are, especially when used in bacteria, specific plasmids, that is to say circular genetic elements. In genetic engineering a distinction is made on the one hand between vectors which are used for storage and thus to a certain extent also for the genetic engineering operation, called cloning vectors, and on the other hand those which perform the function of establishing the gene of interest in the host cell, i.e. making expression of the relevant protein possible. These vectors are referred to as expression vectors.

The enzymatic activity of an enzyme under consideration can be deduced from the amino acid or nucleotide sequence by comparison with known enzymes which are deposited for example in generally accessible databases. This activity can be qualitatively or quantitatively modified by other regions of the protein which are not involved in the actual reaction. This might affect for example the enzyme stability, the activity, the reaction conditions or the substrate specificity.

Such a comparison takes place by correlating similar sequences in the nucleotide or amino acid sequence of the proteins under consideration with one another. This is called homologization. A tabular correlation of the relevant positions is referred to as alignment. In the analysis of nucleotide sequences in turn account must be taken of both complementary strands and all three possible reading frames in each case; likewise the degeneracy of the genetic code and the organism-specific codon usage. Alignments are now produced by computer programs, such as, for example, by the FASTA or BLAST algorithms; this procedure is described for example by D.J. Lipman and W.R. Pearson (1985) in *Science*, volume 227, pp. 1435-1441.

A compilation of all the positions which agree in the compared sequences is referred to as a consensus sequence.

Such a comparison also permits a statement to be made about the similarity or homology of the compared sequences with one another. This is expressed in percent identity, i.e. the proportion of identical nucleotides or amino acid residues at the same positions. A broader definition of homology includes

the conserved amino acid exchanges in this value. The term then used is percent similarity. Such statements can be made about complete proteins or genes or only about individual regions.

Production of an alignment is the first step in defining a sequence space. This hypothetical space includes all sequences which are to be derived by permutation in individual positions and which result by taking account of all the variations occurring in the relevant individual positions of the alignment. Every hypothetically possible protein molecule forms a point in this sequence space. For example, two amino acid sequences which, while being substantially identical, have two different amino acids only at two different positions in each case thus form the basis for a sequence space of four different amino acid sequences. A very large sequence space is obtained if further homologous sequences are found for each individual sequence in a space. Sequences of very low homology can also be recognized as belonging to a sequence space via such high homologies existing pairwise in each case.

Homologous regions of different proteins are those having the same functions which can be recognized by agreements in the primary amino acid sequence. This ranges as far as complete identities in very small regions, called boxes, which include only a few amino acids and usually exercise functions which are essential for the overall activity. Functions of the homologous regions mean very small partial functions of the function exercised by the complete protein, such as, for example, the formation of individual hydrogen bonds for complexation of a substrate or transition complex.

For the purposes of the present application, a distinction must be made between screening (hybridization screening or DNA screening) and activity assay. "Screening" of transformants generally means a detection reaction suitable for recognizing clones in which the desired transformation event has taken place. It usually aims, as, for example, in the familiar blue-white selection, at detection of a biochemical activity which the transformants have acquired or which is no longer present after recombination has taken place. This type of biochemical detection reaction is referred to for the purposes of the present application also as activity assay.

However, screening also designates the searching of a gene library with particular nucleic acids and the identification, possible thereby, of sufficiently similar nucleic acid sequences. This takes place for example via Southern or Northern blot hybridizations as are sufficiently well known from the prior art. This term includes for example also PCR-based methods of the invention for identifying and/or obtaining new genes from a collection of organisms or nucleic acids, which are characterized in that PCR primers having a variable 3′ region and a 5′ region with high homology to corresponding regions from known genes are used.

The performance of an enzyme means its activity in the industrial sector considered in each case. This is based on the actual enzymatic activity but also depends on further factors relevant to the particular process. These include, for example, stability, substrate binding, interaction with the material carrying the substrate or interactions with other ingredients, in particular synergies. Thus, for example, when investigating whether an enzyme is suitable for use in washing or cleaning compositions, its contribution to

the washing or cleaning performance of a composition formulated with further constituents will be considered. For various industrial applications it is possible for an enzyme to be developed further and optimized by techniques of molecular biology which are known per se, especially those mentioned above.

A first aspect of the present invention is a glycosyl hydrolase whose amino acid sequence agrees at least 75%, at least 80%, preferably at least 85%, in particular at least 90%, particularly preferably at least 95% and very particularly preferably 100% with the amino acid sequence indicated in Seq. 1.

A further aspect of the present invention is a glycosyl hydrolase the amino acid sequence of which is identical with the amino acid sequence set forth in Seq. 1 to at least 80%.

A further aspect of the present invention is a glycosyl hydrolase the amino acid sequence of which is identical with the amino acid sequence set forth in Seq. 1 to at least 85%, in particular at least 90%, particularly preferred at least 95% and exceptionally preferred to 100%.

A further aspect of the present invention is a glycosyl hydrolase whose amino acid sequence agrees at least 50%, at least 60%, preferably at least 70%, in particular at least 80%, particularly preferably at least 90% and very particularly preferably 100% with the amino acid sequence indicated in Seq. 2.

A further aspect of the present invention is a glycosyl hydrolase the amino acid sequence of which is identical with the amino acid sequence set forth in Seq. 2 to at least 50%.

A further aspect of the present invention is a glycosyl hydrolase the amino acid sequence of which is identical with the amino acid sequence set forth in Seq. 2 to at least 60%, preferably at least 70%, in particular at least 80%, particularly preferred at least 90% and exceptionally preferred to 100%.

A further aspect of the present invention is a glycosyl hydrolase whose amino acid sequence agrees at least 60%, at least 70%, preferably at least 80%, in particular at least 90%, particularly preferably at least 95% and very particularly preferably 100% with the amino acid sequence indicated in Seq. 3.

A further aspect of the present invention is a glycosyl hydrolase the amino acid sequence of which is identical with the amino acid sequence set forth in Seq. 3 to at least 60%.

A further aspect of the present invention is a glycosyl hydrolase the amino acid sequence of which is identical with the amino acid sequence set forth in Seq. 3 to at least 70%, preferably at least 80%, in particular at least 90%, particularly preferred at least 95% and exceptionally preferred to 100%.

A further aspect of the present invention is a nucleic acid coding for a glycosyl hydrolase whose amino acid sequence is at least 70%, preferably at least 80%, in particular at least 90%, particularly preferably at least 95% and very particularly preferably 100% identical to one of the amino acid sequences indicated in Seq. 1 to 3, or whose amino acid sequence comprises a part which is at least 70%, preferably at least 80%, in particular at least 90%, particularly preferably at

least 95% and very particularly preferably 100% identical to one of the amino acid sequences indicated in Seq. 1 to 3.

A further aspect of the present invention is a nucleic acid coding for a glycosyl hydrolase the amino acid sequence of which is identical with one of the amino acid sequences set forth in Seq. 1 to 3 to at least 80%, in particular at least 90%, particularly preferred at least 95% and exceptionally preferred to 100%.

A further aspect of the present invention is a nucleic acid which codes for a glycosyl hydrolase and whose nucleotide sequence agrees at least 75%, at least 80%, preferably at least 85%, in particular at least 90%, particularly preferably at least 95% and very particularly preferably 100% with the nucleotide sequence indicated in Seq. 4.

A further aspect of the present invention is a nucleic acid which codes for a glycosyl hydrolase and whose nucleotide sequence agrees at least 50%, at least 60%, preferably at least 70%, in particular at least 80%, particularly preferably at least 90% and very particularly preferably 100% with the nucleotide sequence indicated in Seq. 5.

A further aspect of the present invention is a nucleic acid which codes for a glycosyl hydrolase and whose nucleotide sequence agrees at least 60%, at least 70%, preferably at least 80%, in particular at least 90%, particularly preferably at least 95% and very particularly preferably 100% with the nucleotide sequence indicated in Seq. 6.

A further aspect of the present invention is a glycosyl hydrolase which is obtainable by one or more conservative amino acid exchanges from one of the abovementioned glycosyl hydrolases.

A further aspect of the present invention is a vector which comprises one of the nucleic acid regions of the invention.

A further aspect of the present invention is a host cell which expresses one of the proteins or derivatives of the invention or can be induced to the expression thereof, preferably using an expression vector of the invention.

A further aspect of the present invention is a method for identifying and isolating new glycosyl hydrolases, which is characterized in that

a) metagenomic DNA is obtained from various habitats,

b) the metagenomic DNA is subcloned into a suitable, in particular bacterial, cloning system, and

c) the gene library produced in this way is subjected to a screening for new glycosyl hydrolases.

In an important embodiment of the method of the invention, the biodiversity of the metagenomic DNA is determined by PCR typing using glycosyl hydrolase-specific PCR primers and subsequent sequencing, generating a multitude of gene fragments (PCR fragments) which cover one or more sequence spaces.

The glycosyl hydrolases of the invention have been provided by means of the method of the invention.

A habitat which is preferred according to the invention for obtaining metagenomic DNA is earth (soil). The soil samples are processed in a manner known to the skilled worker, for example dried and mechanically reduced in size. The sample is then chemically digested, and the DNA present in the sample extracted, for example as described in the publication by Zhou, J., Bruns, M.A., Tiedje, J.M. (1996): "DNA recovery from soils of diverse composition", *Appl. Environ. Microbiol.*, volume 62, pp. 316-322.

The screening in step c) is preferably carried out by selection for glycosyl hydrolase activity, for example by selection of clones which form a clear zone on starch-containing agar plates.

Cloning systems suitable for carrying out step b) are known to the skilled worker, for example *Escherichia*

coli and *Streptomyces lividans*, but also *Bacillus*; in addition yeasts and fungi, e.g. *Saccharomyces*, *Candida*, *Pichia*, *Aspergillus* and others.

The screening in step c) can, however, also be carried out by colony hybridization using probes based on the abovementioned PCR fragments. The probes may in this case be derived from a single gene fragment or else from a plurality, in the extreme case from an undifferentiated pool, of PCR fragments.

A minimum homology, which depends on the hybridization conditions, is necessary for a positive reaction. Screening a gene library with a probe which is derived from a single, previously known enzyme gene greatly restricts the breadth of diversity of the positive clones to be found. In order to be able to find the genes which are actually present in the gene library, including those less closely related, it is worthwhile to use a multitude of probes whose composition does not need to be characterized in detail but which encompass a part which is as large as possible of the sequence space.

Selection of suitable probes for embracing a sequence space which is as large as possible takes place as described in DE-A-101 31 441.8-41 or PCT/EP02/06842, which is incorporated herein by reference.

This is followed where appropriate by analysis of the sequence of individual clones found, checking of the open reading frame (ORF) for completeness, and determination of sequence homology.

Individual, overexpressed clones can be biochemically characterized and investigated for their stability in respect of, for example, pH, temperature and

formulation components typical of washing and cleaning compositions.

Glycosyl hydrolase-specific PCR primers which can be employed in the method of the invention are those which bind to highly conserved regions of the nucleic acids coding for glycosyl hydrolases. The primer binding sites should additionally flank a region which codes for a protein segment having as few as possible loops of different lengths.

Suitable glycosyl hydrolase-specific PCR primers are especially those which bind to nucleic acid regions which code for those regions of the glycosy hydrolases which are grouped in direct vicinity around the catalytically active residues. Thus, for example, for the regions at the C-terminal end of β-pleated sheet 3, of β-pleated sheet 4 and of β-pleated sheet 7.

Glycosyl hydrolase-specific PCR primers which can be employed particularly preferably in the method of the invention are those listed in Table 1, with very particular preference for the primer pairs GEX 24/26 and GEX 23/25.

A number of PCR fragments covering a sequence space of glycosyl hydrolases has been provided with the aid of the method of the invention.

50 DNA sequences have been provided by means of the primer pair GEX 24/26 and are listed in the sequence listing under IDs 57 to 106. The amino acid sequences corresponding to these DNA sequences are listed in the sequence listing under IDs 7 to 56.

99 DNA sequences have been provided by means of the primer pairs GEX 23/25, GEX 36/38 and 210-211/25 and

are listed in the sequence listing under IDs 206 to 304. Since the sequences produced with the three primer combinations cover essentially the same DNA segment, they are treated together below and, where appropriate, referred to as GEX23/25 for simplicity. The amino acid sequences corresponding to these DNA sequences are listed in the sequence listing under IDs 107 to 205.

Production of sequence alignments of the PCR fragments resulted in consensus sequences which represent this sequence space or part-spaces thereof.

Further aspects of the present invention are therefore glycosyl hydrolases which are to be regarded as representatives of the sequence spaces represented in consensus sequences A and B or of the sequence spaces represented in consensus sequences D and C.

Consensus sequences A, D, B and C are depicted in Figures 5 to 8 and in the sequence listing under SEQ ID NO. 305 to 308.

There is only very little variance in the consensus sequences shown, in positions 1-8 and 116-123 (A), 1-8, 114-121 (D), 1-12, 102-109 (B) and 1-12, 72-78 (C). This emerges from the PCR-based method which is described hereinafter and in the examples and which was employed to identify this sequence: these partial sequences correspond to the DNA regions onto which the primers employed for the amplification have bound. Depending on the stringency of the conditions under which this binding takes place, it is also possible for different nucleotide sequences to be bound and amplified; thus, with the comparatively low selectivity at these positions, the DNA obtained as product does not agree completely with the template. For this reason, protection is sought in particular for the

inner partial region of the consensus sequences which corresponds to the position between the sequence segments indicated above.

Comparison of the 149 amino acid sequences derived from the PCR fragments with the entries in the enzyme database GenBank (National Center for Biotechnology Information NCBI, National Institutes of Health, Bethesda, MD, USA) confirms that they are all partial sequences of glycosyl hydrolases.

The result of this comparison is shown in Table 2. Also indicated therein in each case are the database entries most similar to the partial sequences found, and the degree of homology between these two. Comparison with the sequences deposited in the database shows that the most similar proteins have levels of homology of 95.1% (primer pair 23/25) identity, and 91% (primer pair 24/26).

These amino acid sequences 107 to 205 define a sequence space which represents a part-space of the sequence space described by the consensus sequences with SEQ ID NO. 307 and 308. It is included in the preferred scope of protection. Thus, every point in this sequence space represents a partial sequence of the invention.

In other words, all glycosyl hydrolases whose amino acid sequences can be homologized in a partial region with the consensus sequences with SEQ ID NO. 307 and 308, and have in this region only amino acids which are located at the appropriate position in one of these two sequences, are claimed. In this sense, they can be traced back in any position of this part to one of these sequences.

Further aspects of the present invention are therefore glycosyl hydrolases whose amino acid sequences comprise a part which is 96% and increasingly preferably 97%, 98%, 99%, 99.25%, 99.5%, 99.75% or particularly preferably 100% identical to one of these consensus sequences with SEQ ID NO. 307 (B) and 308 (C), or can be traced back in any homologous position directly to one of these sequences. This applies, as described above, in particular over the partial region corresponding to positions 13 to 101 of consensus sequence B, or 13 to 71 of consensus sequence C.

Further aspects of the present invention are therefore glycosyl hydrolases whose amino acid sequences comprise a part which is 92% and increasingly preferably 93%, 94%, 95%, 96%, 97%, 98%, 99% or particularly preferably 100% identical to one of these consensus sequences with SEQ ID NO. 305 (A) and 306 (D), or can be traced back in any homologous position directly to one of these sequences. This applies, as described above, in particular over the partial region corresponding to positions 9 to 115 of consensus sequence A or 9 to 113 of consensus sequence D.

A further specific aspect of the invention is a glycosyl hydrolase whose amino acid sequence comprises a part which is at least 92%, in particular at least 94%, preferentially at least 96%, preferably at least 99%, particularly preferably 100% identical to one of the amino acid sequences indicated under SEQ ID NO. 7 to 56, in particular over the partial region corresponding to positions 9 to 113 according to the consensus sequence of SEQ ID NO. 306.

A further specific aspect of the invention is a glycosyl hydrolase whose amino acid sequence comprises a part which is at least 92%, in particular at least

94%, preferentially at least 96%, preferably at least 99%, particularly preferably 100% identical to one of the amino acid sequences indicated under SEQ ID NO. 7 to 56, in particular over the partial region corresponding to positions 9 to 115 according to the consensus sequence of SEQ ID NO. 305.

A further specific aspect of the invention is a glycosyl hydrolase whose amino acid sequence comprises a part which is 96%, in particular at least 97%, preferentially at least 98%, preferably at least 99%, particularly preferably 100% identical to one of the amino acid sequences indicated under SEQ ID NO. 107 to 205, in particular over the partial region corresponding to positions 13 to 71 according to the consensus sequence of SEQ ID NO. 308.

A further specific aspect of the invention is a glycosyl hydrolase whose amino acid sequence comprises a part which is 96%, in particular at least 97%, preferentially at least 98%, preferably at least 99%, particularly preferably 100% identical to one of the amino acid sequences indicated under SEQ ID NO. 107 to 205, in particular over the partial region corresponding to positions 13 to 101 according to the consensus sequence of SEQ ID NO. 307.

A further specific aspect of the invention is a glycosyl hydrolase which is obtainable by one or more conservative amino acid exchanges from a glycosyl hydrolase of the invention.

A further specific aspect of the invention is a glycosyl hydrolase which can be obtained by derivatization, fragmentation, deletion mutation or insertion mutation of a glycosyl hydrolase as claimed in any of claims 1 to 6, or 14 to 21.

A further specific aspect of the invention is a nucleic acid coding for a glycosyl hydrolase whose amino acid sequence comprises a part which is at least 96%, in particular at least 97%, preferentially at least 98%, preferably at least 99%, particularly preferably 100% identical to the consensus sequence of SEQ ID NO. 308, in particular over the partial region corresponding to positions 13 to 71.

A further specific aspect of the invention is a nucleic acid coding for a glycosyl hydrolase whose amino acid sequence comprises a part which is at least 96%, in particular at least 97%, preferentially at least 98%, preferably at least 99%, particularly preferably 100% identical to the consensus sequence of SEQ ID NO. 307, in particular over the partial region corresponding to positions 13 to 101.

A further specific aspect of the invention is a nucleic acid coding for a glycosyl hydrolase whose amino acid sequence comprises a part which is at least 92%, in particular at least 94%, preferentially at least 96%, preferably at least 99%, particularly preferably 100% identical to the consensus sequence of SEQ ID NO. 305, in particular over the partial region corresponding to positions 9 to 115.

A further specific aspect of the invention is a nucleic acid coding for a glycosyl hydrolase whose amino acid sequence comprises a part which is at least 92%, in particular at least 94%, preferentially at least 96%, preferably at least 99%, particularly preferably 100% identical to the consensus sequence of SEQ ID NO. 306, in particular over the partial region corresponding to positions 9 to 113.

A further specific aspect of the invention is a nucleic acid coding for a glycosyl hydrolase whose amino acid sequence comprises a part which is at least 92%, in particular at least 94%, preferentially at least 96%, preferably at least 99%, particularly preferably 100% identical to one of the amino acid sequences indicated under SEQ ID NO. 7 to 56, in particular over the partial region corresponding to positions 9 to 113 according to the consensus sequence of SEQ ID NO. 306.

A further specific aspect of the invention is a nucleic acid coding for a glycosyl hydrolase whose amino acid sequence comprises a part which is at least 92%, in particular at least 94%, preferentially at least 96%, preferably at least 99%, particularly preferably 100% identical to one of the amino acid sequences indicated under SEQ ID NO. 7 to 56, in particular over the partial region corresponding to positions 9 to 115 according to the consensus sequence of SEQ ID NO. 305.

A further specific aspect of the invention is a nucleic acid coding for a glycosyl hydrolase whose amino acid sequence comprises a part which is 96%, in particular at least 97%, preferentially at least 98%, preferably at least 99%, particularly preferably 100% identical to one of the amino acid sequences indicated under SEQ ID NO. 107 to 205, in particular over the partial region corresponding to positions 13 to 71 according to the consensus sequence of SEQ ID NO. 308.

A further specific aspect of the invention is a nucleic acid coding for a glycosyl hydrolase whose amino acid sequence comprises a part which is 96%, in particular at least 97%, preferentially at least 98%, preferably at least 99%, particularly preferably 100% identical to one of the amino acid sequences indicated under SEQ ID NO. 107 to 205, in particular over the partial region

corresponding to positions 13 to 101 according to the consensus sequence of SEQ ID NO. 307.

A further specific aspect of the invention is a nucleic acid coding for a glycosyl hydrolase of the invention.

A further specific aspect of the invention is a nucleic acid which codes for a glycosyl hydrolase and whose sequence is at least 80%, preferably at least 90%, particularly preferably 100% identical to one of the nucleotide sequence indicated under SEQ ID NO. 57 to 106, or 206 to 304, preferably in the positions which are not specified by the PCR primer sequences.

A further specific aspect of the invention is an oligonucleotide, in particular a PCR primer, having one of the sequences indicated in SEQ ID NO. 309 to 316.

Further aspects of the invention are described in claims 37 to 68.

A further aspect of the invention is the use of a nucleic acid or oligonucleotide of the invention for identifying and/or isolating a novel glycosyl hydrolase.

A further aspect of the invention is the use of a glycosyl hydrolase of the invention for fusion or linkage to another protein, in particular for developing a novel enzyme.

A further aspect of the invention is the use of a nucleic acid of the invention for fusion to another nucleic acid, in particular to develop a novel enzyme.

Nucleic acids which code for the variants possible according to the consensus sequence can be produced by

generally known methods. For example, the "Lexikon der Biochemie", Spektrum Akademischer Verlag, Berlin, 1999, presents in volume 1, pp. 267-271, methods for *de novo* synthesis of DNA and in volume 2, pp. 227-229, the polymerase chain reaction (PCR). The sequences can be specified by the generally known coding system for amino acids, where appropriate in a codon usage characteristic of particular genera.

It is possible to employ for this purpose all conventional and expedient methods as disclosed for example in the manual of Fritsch, Sambrook and Maniatis "Molecular cloning: a laboratory manual", *Cold Spring Harbor Laboratory Press*, New York, 1989. It is also

possible to use a PCR to introduce individual base exchanges into DNA.

The glycosyl hydrolases of the invention can be further optimized for example by chemical modification, directed evolution or sequence shuffling of the enzymes or enzyme fragments to give novel proteins or fusion proteins (hybrids, chimeric proteins). Methods known for this are, for example, the methods of directed evolution of enzymes like "In vivo homologous recombination" (Zhang, Y.M., Buchholz, F., Muyrers, J.P.P., Stewart, A.F. (1998): "A new logic for DNA engineering using recombination in *Escherichia coli*", *Nat. Genetics*, volume 20, pp. 123-128), "Staggered extension process StEP" (Zhao, H., Giver, L., Shao, Z., Affholter, J.A., Arnold, F.H. (1998): "Molecular evolution by staggered extension process (StEP) in vitro recombination", *Nat. Biotechnol.*, volume 16, pp. 258-261), "Random priming recombination" (Shao, Z., Zhao, H., Giver, L., Arnold, F.H. (1998): "Random-priming in vitro recombination: an effective tool for directional evolution", *Nucleic Acids Res.*, volume 26, pp. 681-683), "DNA shuffling" (Stemmer, W.P.C. (1994): "Rapid evolution of a protein in vitro by DNA shuffling", *Nature*, volume 370, pp. 389-391) and "Molecular breeding" (Ness, J.E., Welch, M., Giver, L., Bueno, M., Cherry, J.R., Borchert, T.V., Stemmer, W.P.C., Minshull, J. (1999): "DNA shuffling of subgenomic sequences of subtilisin", *Nat. Biotechnol.*, volume 17, pp. 893-896).

The glycosyl hydrolases of the invention can be protected in particular during storage by stabilizers for example from denaturation, decomposition or inactivation, for example through physical effects, oxidation or proteolysis. In many cases, complementary

or mutually enhancing combinations of stabilizers are also used.

One group of stabilizers are reversible protease inhibitors such as, for example, benzamidine hydrochloride and leupeptin, borax, boric acids, boronic acids, salts or esters thereof, peptide aldehydes or purely peptide inhibitors such as ovomucoid or specific subtilisin inhibitors. Further familiar enzyme stabilizers are amino alcohols such as mono-, di-, triethanol- and -propanolamine, aliphatic carboxylic acids up to C_{12}, dicarboxylic acids, lower aliphatic alcohols, but especially polyols such as, for example, glycerol, ethylene glycol, propylene glycol or sorbitol. Calcium salts are likewise used, such as, for example, calcium acetate or calcium formate, magnesium salts, a wide variety of polymers such as, for example, lignin, cellulose ethers, polyamides or water-soluble vinyl copolymers, in order to stabilize the enzyme preparation in particular against physical effects or pH variations. Reducing agents and antioxidants such as, for example, sodium sulfite or reducing sugars increase the stability of the proteins to oxidative decomposition.

Numerous possible applications established in industry for glycosyl hydrolases are listed in manuals such as, for example, the book "Industrial enzymes and their applications" by H. Uhlig, published by Wiley, New York, 1998. The following compilation is not to be understood as a definitive list, but represents a selection of the possible industrial uses. Should it emerge that individual proteins lying outside the range of similarity are suitable, because of their enzymatic, in particular amylolytic, properties for the additional applications not expressly claimed herein, these are

hereby included in the scope of protection of the present invention.

A further aspect of the invention are washing and cleaning compositions which are characterized in that they comprise a glycosyl hydrolase of the invention or a derivative thereof.

A further aspect of the invention is the use of a glycosyl hydrolase of the invention, preferably in a washing or cleaning composition of the invention, for cleaning textiles or hard surfaces.

One important area of use of glycosyl hydrolases and, in particular, amylases is that as active component in washing or cleaning compositions for cleaning textiles or solid surfaces, such as, for example, crockery, floors or utensils. In these applications, the amylolytic activity serves to break down by hydrolysis, or detach from the substrate, carbohydrate-containing contaminations and especially those based on starch. In such cases, they can be used alone, in suitable media or else in washing or cleaning compositions. The conditions to be chosen for this, such as, for example, temperature, pH, ionic strength, redox conditions or mechanical effects, should be optimized for the particular cleaning problem, i.e. in relation to the soiling and the substrate. Thus, usual temperatures for washing and cleaning compositions are in ranges from 10°C for manual compositions via 40°C and 60°C up to 95° for machine compositions or for industrial applications. The ingredients of the relevant compositions are preferably also matched to one another. Since the temperature can usually be adjusted

continuously in modern washing and dishwashing machines, all intermediate temperatures are also included. The other conditions can likewise be designed very specifically for the particular cleaning purpose via the other components of the compositions listed below.

Preferred compositions of the invention are distinguished by the washing or cleaning performance of this composition being improved by adding an amylolytic enzyme of the invention, compared with the formulation without this enzyme, under any of the conditions definable in this way. To this extent, the amylolytic proteins incorporated into compositions of the invention are preferably those able to improve the washing and/or cleaning performance of a washing or cleaning composition.

Further preferred compositions are distinguished by the amylolytic enzymes and the other components acting synergistically to remove the contaminations. This takes place for example by the hydrolysis products of the amylolytic proteins being solubilized by other components of the compositions, such as, for example, surfactants. A protein of the invention can be used both in compositions for large-scale consumers or industrial users and in products for the private consumer, and all presentations which are expedient and/or established in the art also represent embodiments of the present invention.

The washing or cleaning compositions of the invention mean all conceivable types of cleaning compositions, both concentrates and compositions to be used undiluted; for use on the commercial scale, in the washing machine or in hand washing or cleaning. They include, for example, washing compositions for textiles, carpets, or natural fibers, for which the term washing composition is used according to the present invention. They also include, for example, dishwashing compositions for dishwashing machines or manual dishwashing compositions or cleaners for hard surfaces such as metal, glass, porcelain, ceramic, tiles, stone, painted surfaces, plastics, wood or

leather; the term cleaning composition is used according to the present invention for these. Every type of washing or cleaning composition represents an embodiment of the present invention as long as a protein of the invention has been added thereto.

Embodiments of the present invention include all presentations established in the art and/or all expedient presentations of the compositions of the invention. These include, for example, compositions which are solid, in powder form, liquid, in gel form or pasty, where appropriate also composed of a plurality of phases, compressed or uncompressed; the following also belong thereto, for example: extrudates, granules, tablets or pouches, packaged both in large containers and in portions.

The glycosyl hydrolases, in particular amylases, are combined in compositions of the invention for example with one or more of the following ingredients: nonionic, anionic and/or cationic surfactants, bleaches, bleach activators, bleach catalysts, builders and/or cobuilders, solvents, thickeners, sequestrants, electrolytes, optical brighteners, antiredeposition agents, corrosion inhibitors, especially silver protectants, soil-release agents, color transfer inhibitors, foam inhibitors, abrasives, dyes, fragranches, antimicrobial agents, UV stabilizers, enzymes such as, for example, proteases, (where appropriate other) amylases, lipases, cellulases, hemicellulases or oxidases, stabilizers, especially enzyme stabilizers, and other components known in the art.

Preferred compositions are characterized in that they comprise from 0.000001 percent by weight to 5% by weight, and increasingly preferably 0.00001 to 4% by

weight, 0.0001 to 3% by weight, 0.001 to 2% by weight or 0.01 to 1% by weight, of the amylolytic protein or derivative.

The nonionic surfactants used are preferably alkoxylated, advantageously ethoxylated, in particular primary alcohols having preferably from 8 to 18 carbon atoms and, on average, from 1 to 12 mol of ethylene oxide (EO) per mole of alcohol, in which the alcohol radical can be linear or, preferably, methyl-branched in the 2-position or can comprise linear and methyl-branched radicals in a mixture as are customarily present in oxo alcohol radicals. Particular preference is, however, given to alcohol ethoxylates containing linear radicals of alcohols of native origin having from 12 to 18 carbon atoms, for example from coconut, palm, tallow fatty or oleyl alcohol, and, on average, from 2 to 8 EO per mole of alcohol. Preferred ethoxylated alcohols include, for example, C_{12-14}-alcohols having 3 EO or 4 EO, C_{9-11}-alcohol having 7 EO, C_{13-15}-alcohols having 3 EO, 5 EO, 7 EO or 8 EO, C_{12-18}-alcohols having 3 EO, 5 EO or 7 EO, and mixtures of these, such as mixtures of C_{12-14}-alcohol having 3 EO and C_{12-18}-alcohol having 5 EO. The degrees of ethoxylation given are statistical averages which may be an integer or a fraction for a specific product. Preferred alcohol ethoxylates have a narrowed homolog distribution (narrow range ethoxylates, NRE). In addition to these nonionic surfactants, fatty alcohols having more than 12 EO can also be used. Examples thereof are tallow fatty alcohol having 14 EO, 25 EO, 30 EO or 40 EO.

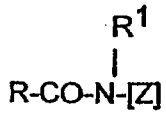
A further class of preferably used nonionic surfactants which are used either as the sole nonionic surfactant or in combination with other nonionic surfactants are alkoxylated, preferably ethoxylated or ethoxylated and propoxylated, fatty acid alkyl esters, preferably

having from 1 to 4 carbon atoms in the alkyl chain, in particular fatty acid methyl esters.

A further class of nonionic surfactants which can advantageously be used are the alkyl polyglycosides (APG). Alkyl polyglycosides which may be used satisfy the general formula $RO(G)_z$, in which R is a linear or branched, in particular methyl-branched in the 2-position, saturated or unsaturated, aliphatic radical having from 8 to 22, preferably from 12 to 18 carbon atoms, and G is the symbol which stands for a glycose unit having 5 or 6 carbon atoms, preferably for glucose. The degree of glycosylation z is here between 1.0 and 4.0, preferably between 1.0 and 2.0 and in particular between 1.1 and 1.4. Preference is given to using linear alkyl polyglucosides, i.e. alkyl polyglycosides in which the polyglycosyl radical is a glucose radical, and the alkyl radical is an n-alkyl radical.

Nonionic surfactants of the amine oxide type, for example N-cocoalkyl-N,N-dimethylamine oxide and N-tallow alkyl-N,N-dihydroxyethylamine oxide, and of the fatty acid alkanolamides may also be suitable. The proportion of these nonionic surfactants is preferably no more than that of the ethoxylated fatty alcohols, in particular no more than half thereof.

Further suitable surfactants are polyhydroxy fatty acid amides of the formula (II)

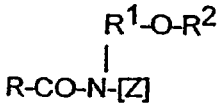


(II)

in which RCO is an aliphatic acyl radical having from 6 to 22 carbon atoms, R^1 is hydrogen, an alkyl or

hydroxyalkyl radical having from 1 to 4 carbon atoms and [Z] is a linear or branched polyhydroxyalkyl radical having from 3 to 10 carbon atoms and from 3 to 10 hydroxyl groups. The polyhydroxy fatty acid amides are known substances which can usually be obtained by reductive amination of a reducing sugar with ammonia, an alkylamine or an alkanolamine and subsequent acylation with a fatty acid, a fatty acid alkyl ester or a fatty acid chloride.

The group of polyhydroxy fatty acid amides also includes compounds of the formula (III)



(III)

in which R is a linear or branched alkyl or alkenyl radical having from 7 to 12 carbon atoms, R^1 is a linear, branched or cyclic alkyl radical or an aryl radical having from 2 to 8 carbon atoms, and R^2 is a linear, branched or cyclic alkyl radical or an aryl radical or an oxyalkyl radical having from 1 to 8 carbon atoms, where C_{1-4}-alkyl or phenyl radicals are preferred, and [Z] is a linear polyhydroxyalkyl radical whose alkyl chain is substituted with at least two hydroxyl groups, or alkoxylated, preferably ethoxylated or propoxylated, derivatives of this radical.

[Z] is preferably obtained by reductive amination of a reducing sugar, for example glucose, fructose, maltose, lactose, galactose, mannose or xylose. The N-alkoxy- or N-aryloxy-substituted compounds may be converted, for example, by reaction with fatty acid methyl esters in the presence of an alkoxide as catalyst, into the desired polyhydroxy fatty acid amides.

The anionic surfactants used are, for example, those of the sulfonate and sulfate type. Suitable surfactants of the sulfonate type are preferably C_{9-13}-alkylbenzenesulfonates, olefinsulfonates, i.e. mixtures of alkene- and hydroxyalkanesulfonates, and disulfonates, as obtained, for example, from C_{12-18}-monoolefins having a terminal or internal double bond by sulfonation with gaseous sulfur trioxide and subsequent alkaline or acidic hydrolysis of the sulfonation products. Also suitable are alkanesulfonates which are obtained from C_{12-18}-alkanes, for example, by sulfochlorination or sulfoxidation with subsequent hydrolysis or neutralization. Likewise suitable are also the esters of α-sulfo fatty acids (estersulfonates), for example the α-sulfonated methyl esters of hydrogenated coconut, palm kernel or tallow fatty acids.

Further suitable anionic surfactants are sulfated fatty acid glycerol esters. Fatty acid glycerol esters mean the mono-, di- and triesters, and mixtures thereof, as are obtained during the preparation by esterification of a monoglycerol with from 1 to 3 mol of fatty acid or during the transesterification of triglycerides with from 0.3 to 2 mol of glycerol. Preferred sulfated fatty acid glycerol esters are here the sulfation products of saturated fatty acids having from 6 to 22 carbon atoms, for example of caproic acid, caprylic acid, capric acid, myristic acid, lauric acid, palmitic acid, stearic acid or behenic acid.

Preferred alk(en)yl sulfates are the alkali metal, and in particular the sodium, salts of sulfuric monoesters of C_{12}-C_{18}-fatty alcohols, for example of coconut fatty alcohol, tallow fatty alcohol, lauryl, myristyl, cetyl or stearyl alcohol or of C_{10}-C_{20}-oxo alcohols and those monoesters of secondary alcohols of these chain lengths. Further preferred are alk(en)yl sulfates of

said chain length which comprise a synthetic, petrochemical-based straight-chain alkyl radical and have analogous degradation behavior to the equivalent compounds based on fatty chemical raw materials. From a washing performance viewpoint, preference is given to C_{12}-C_{16}-alkyl sulfates and C_{12}-C_{15}-alkyl sulfates, and C_{14}-C_{15}-alkyl sulfates. 2,3-Alkyl sulfates are also suitable anionic surfactants.

The sulfuric monoesters of straight-chain or branched C_{7-21}-alcohols ethoxylated with from 1 to 6 mol of ethylene oxide, such as 2-methyl-branched C_{9-11}-alcohols having, on average, 3.5 mol of ethylene oxide (EO) or C_{12-18}-fatty alcohols having from 1 to 4 EO, are also suitable. Owing to their high foaming behavior, they are used in cleaning compositions only in relatively small amounts, for example in amounts up to 5% by weight, usually from 1 to 5% by weight.

Further suitable anionic surfactants are also the salts of alkylsulfosuccinic acid, which are also referred to as sulfosuccinates or as sulfosuccinic esters and which are monoesters and/or diesters of sulfosuccinic acid with alcohols, preferably fatty alcohols and, in particular, ethoxylated fatty alcohols. Preferred sulfosuccinates contain C_{8-18}-fatty alcohol radicals or mixtures thereof. Particularly preferred sulfosuccinates contain a fatty alcohol radical derived from ethoxylated fatty alcohols, which are themselves nonionic surfactants (see above for description). In this connection, sulfosuccinates whose fatty alcohol radicals are derived from ethoxylated fatty alcohols having a narrowed homolog distribution are, in turn, particularly preferred. Likewise, it is also possible to use alk(en)ylsuccinic acid having preferably from 8 to 18 carbon atoms in the alk(en)yl chain or salts thereof.

Further suitable anionic surfactants are, in particular, soaps. Saturated fatty acid soaps such as the salts of lauric acid, myristic acid, palmitic acid, stearic acid, hydrogenated erucic acid and behenic acid, and, in particular, soap mixtures derived from natural fatty acids, for example coconut, palm kernel or tallow fatty acids, are suitable.

The anionic surfactants including soaps may be present in the form of their sodium, potassium or ammonium salts, and as soluble salts of organic bases such as mono-, di- or triethanolamine. The anionic surfactants are preferably in the form of their sodium or potassium salts, in particular in the form of the sodium salts.

The surfactants may be present in the cleaning compositions or washing compositions of the invention in an overall amount of from preferably 5% by weight to 50% by weight, in particular from 8% by weight to 30% by weight, based on the finished agent.

Compositions of the invention may contain bleaches. Of the compounds which serve as bleaches and produce H_2O_2 in water, sodium percarbonate, sodium perborate tetrahydrate and sodium perborate monohydrate are of particular importance. Other bleaches which can be used are, for example, peroxopyrophosphates, citrate perhydrates and H_2O_2-producing peracidic salts or peracids, such as persulfates or persulfuric acid. Also useful is the urea peroxohydrate percarbamide which can be described by the formula $H_2N-CO-NH_2 \cdot H_2O_2$. In particular when used for cleaning hard surfaces, for example for machine dishwashing, the agents, if desired, may also contain bleaches from the group of organic bleaches, although the use thereof is possible in principle also in agents for washing textiles.

Typical organic bleaches are diacyl peroxides such as, for example, dibenzoyl peroxide. Further typical organic bleaches are the peroxy acids, specific examples being alkyl peroxy acids and aryl peroxy acids. Preferred representatives are peroxy benzoic acid and its ring-substituted derivatives, such as alkylperoxybenzoic acids, but also peroxy-α-naphthoic acid and magnesium monoperphthalate, the aliphatic or substituted aliphatic peroxy acids such as peroxylauric acid, peroxystearic acid, ε-phthalimidoperoxycaproic acid (phthalimidoperoxyhexanoic acid, PAP), o-carboxybenzamidoperoxycaproic acid, N-nonenylamidoperadipic acid and N-nonenylamidopersuccinates, and aliphatic and araliphatic peroxydicarboxylic acids such as 1,12-diperoxycarboxylic acid, 1,9-diperoxyazelaic acid, diperoxysebacic acid, diperoxybrassylic acid, diperoxyphthalic acids, 2-decyldiperoxybutane-1,4-dioic acid, N,N-terephthaloyldi(6-aminopercaproic acid) may be used.

The bleach content of the agents may be from 1 to 40% by weight and, in particular, from 10 to 20% by weight, using advantageously perborate monohydrate or percarbonate. A synergistic use of amylase with percarbonate or of amylase with percarboxylic acid is disclosed by the applications WO 99/63036 and WO 99/63037.

In order to achieve improved bleaching action in cases of washing at temperatures of 60°C and below, and in particular in the case of laundry pretreatment, the compositions may also include bleach activators. Bleach activators which can be used are compounds which, under perhydrolysis conditions, give aliphatic peroxocarboxylic acids having preferably from 1 to 10 carbon atoms, in particular from 2 to 4 carbon atoms, and/or substituted or unsubstituted perbenzoic acid.

Substances which carry O- and/or N-acyl groups of said number of carbon atoms and/or substituted or unsubstituted benzoyl groups are suitable. Preference is given to polyacylated alkylenediamines, in particular tetraacetylethylenediamine (TAED), acylated triazine derivatives, in particular 1,5-diacetyl-2,4-dioxohexahydro-1,3,5-triazine (DADHT), acylated glycoluriles, in particular 1,3,4,6-tetraacetylglycoluril (TAGU), N-acylimides, in particular N-nonanoylsuccinimide (NOSI), acylated phenolsulfonates, in particular *n*-nonanoyl- or isononanoyloxybenzenesulfonate (*n*- or iso-NOBS), acylated hydroxycarboxylic acids such as triethyl O-acetylcitrate (TEOC), carboxylic anhydrides, in particular phthalic anhydride, isatoic anhydride and/or succinic anhydride, carboxamides such as N-methyldiacetamide, glycolide, acylated polyhydric alcohols, in particular triacetin, ethylene glycol diacetate, isopropenyl acetate, 2,5-diacetoxy-2,5-dihydrofuran and the enol esters disclosed in German patent applications DE 196 16 693 and DE 196 16 767, and acetylated sorbitol and mannitol, or mixtures thereof described in European patent application EP 0 525 239 (SORMAN), acylated sugar derivatives, in particular pentaacetylglucose (PAG), pentaacetylfructose, tetraacetylxylose and octaacetyllactose, and acetylated, optionally N-alkylated glucamine or gluconolactone, triazole or triazole derivatives and/or particulate caprolactams and/or caprolactam derivatives, preferably N-acylated lactams, for example N-benzoylcaprolactam and N-acetylcaprolactam, which are disclosed in international patent applications WO 94/27970, WO 94/28102, WO 94/28103, WO 95/00626, WO 95/14759 and WO 95/17498. The hydrophilically substituted acyl acetals disclosed in German patent application DE 196 16 769 and the acyl lactams described in German patent application DE 196 16 770

and in international patent application WO 95/14075 are likewise used with preference. It is also possible to use the combinations of conventional bleach activators disclosed in German patent application DE 44 43 177. Nitrile derivatives such as cyanopyridines, nitrile quats, e.g. N-alkylammonium acetonitriles, and/or cyanamide derivatives may also be used. Preferred bleach activators are sodium 4-(octanoyloxy)benzenesulfonate, *n*-nonanoyl- or isononanoyloxybenzenesulfonate (*n*- or iso-NOBS), undecenoyloxybenzenesulfonate (UDOBS), sodium dodecanoyloxybenzenesulfonate (DOBS), decanoyloxybenzoic acid (DOBA, OBC 10) and/or dodecanoyloxybenzenesulfonate (OBS 12), and N-methylmorpholinium acetonitrile (MMA). Such bleach activators may be present in the customary quantitative range from 0.01 to 20% by weight, preferably in amounts from 0.1 to 15% by weight, in particular 1% by weight to 10% by weight, based on the total composition.

In addition to the conventional bleach activators or instead of them, it is also possible for "bleach catalysts" to be present. These substances are bleach-enhancing transition metal salts or transition metal complexes such as, for example, Mn, Fe, Co, Ru or Mo salen complexes or carbonyl complexes. Mn, Fe, Co, Ru, Mo, Ti, V and Cu complexes containing N-containing tripod ligands, and Co, Fe, Cu and Ru amine complexes are also suitable as bleach catalysts, preference being given to using those compounds described in DE 197 09 284 A1. Acetonitrile derivatives, according to WO 99/63038, and bleach-activating transition metal complex compounds, according to WO 99/63041, are capable of developing a bleach-activating action in combination with amylases.

Compositions of the invention usually contain one or more builders, in particular zeolites, silicates, carbonates, organic cobuilders and, where no ecological reasons oppose their use, also phosphates. The latter are the preferred builders for use in particular in cleaning compositions for machine dishwashing.

Compounds which may be mentioned here are crystalline, layered sodium silicates of the general formula $NaMSi_xO_{2x+1} \cdot yH_2O$, where M is sodium or hydrogen, x is a number from 1.6 to 4, preferably from 1.9 to 4.0, and y is a number from 0 to 20, and preferred values for x are 2, 3 or 4. Crystalline phyllosilicates of this kind are described, for example, in European patent application EP 0 164 514. Preferred crystalline phyllosilicates of the formula indicated are those where M is sodium and x adopts the values 2 or 3. In particular, both β- and δ-sodium disilicates $Na_2Si_2O_5 \cdot yH_2O$ are preferred. Compounds of this kind are sold, for example, under the name SKS® (Clariant). Thus, SKS-6® is primarily a δ-sodium disilicate having the formula $Na_2Si_2O_5 \cdot yH_2O$, and SKS-7® is primarily the β-sodium disilicate. Reacting the δ-sodium disilicate with acids (for example citric acid or carbonic acid) gives kanemite, $NaHSi_2O_5 \cdot yH_2O$, sold under the names SKS-9® and, respectively, SKS-10® (Clariant). It may also be advantageous to use chemical modifications of said phyllosilicates. The alkalinity of the phyllosilicates, for example, can thus be suitably influenced. Phyllosilicates doped with phosphate or with carbonate have, compared to the δ-sodium disilicate, altered crystal morphologies, dissolve more rapidly and display an increased calcium binding ability, compared to δ-sodium disilicate. Thus, phyllosilicates of the general empirical formula $xNa_2O \cdot ySiO_2 \cdot zP_2O_5$ where the x-to-y ratio corresponds to a number from 0.35 to 0.6, the x-to-z ratio to a number

from 1.75 to 1200 and the y-to-z ratio to a number from 4 to 2800 are described in patent application DE 196 01 063. The solubility of the phyllosilicates may also be increased by using particularly finely granulated phyllosilicates. It is also possible to use compounds of the crystalline phyllosilicates with other ingredients. Compounds which may be mentioned here are in particular those with cellulose derivatives which have advantageous disintegrating action and are used in particular in washing composition tablets, and those with polycarboxylates, for example citric acid, or polymeric polycarboxylates, for example copolymers of acrylic acid.

It is also possible to use amorphous sodium silicates having an $Na_2O:SiO_2$ modulus of from 1:2 to 1:3.3, preferably from 1:2 to 1:2.8, and in particular from 1:2 to 1:2.6, which have delayed dissolution and secondary washing composition properties. The dissolution delay relative to conventional amorphous sodium silicates can have been induced by various means, for example by surface treatment, compounding, compaction/compression or by overdrying. Within the scope of this invention, the term "amorphous" also means "X-ray amorphous". This means that in X-ray diffraction experiments the silicates do not give the sharp X-ray reflections typical of crystalline substances, but instead, at best, one or more maxima of the scattered X-ray radiation, which have a width of several degree units of the diffraction angle. However, even particularly good builder properties will very likely result if, in electron diffraction experiments, the silicate particles give poorly defined or even sharp diffraction maxima. This is to be interpreted to the effect that the products have microcrystalline regions with a size from 10 to a few hundred nm, preference being given to values up to at most 50 nm

and in particular up to at most 20 nm. Particular preference is given to compressed/compacted amorphous silicates, compounded amorphous silicates and overdried X-ray amorphous silicates.

A finely crystalline, synthetic zeolite containing bonded water, which may be used where appropriate, is preferably zeolite A and/or P. As zeolite P, zeolite MAP® (commercial product from Crosfield) is particularly preferred. However, zeolite X and mixtures of A, X and/or P are also suitable. A product which is commercially available and can be used with preference within the scope of the present invention is, for example, also a cocrystal of zeolite X and zeolite A (approx. 80% by weight zeolite X), which is sold by CONDEA Augusta S.p.A. under the trade name VEGOBOND AX® and can be described by the formula

$$nNa_2O \cdot (1-n)K_2O \cdot Al_2O_3 \cdot (2-2.5)SiO_2 \cdot (3.5-5.5)H_2O.$$

Suitable zeolites have an average particle size of less than 10 µm (volume distribution; measurement method: Coulter counter) and preferably contain from 18 to 22% by weight, in particular from 20 to 22% by weight, of bonded water.

Use of the generally known phosphates as builder substances is of course also possible, provided such a use should not be avoided for ecological reasons. Among the multiplicity of commercially available phosphates, the alkali metal phosphates are the most important in the washing and cleaning compositions industry, with pentasodium or pentapotassium triphosphate (sodium or potassium tripolyphosphate) being particularly preferred.

In this connection, alkali metal phosphates is the collective term for the alkali metal (in particular sodium and potassium) salts of the various phosphoric acids, it being possible to differentiate between metaphosphoric acids $(HPO_3)_n$ and orthophosphoric acid H_3PO_4 as well as higher molecular weight representatives. The phosphates combine several advantages: they act as alkali carriers, prevent lime deposits on machine parts and lime incrustations in fabrics and, moreover, contribute to the cleaning performance.

Sodium dihydrogenphosphate, NaH_2PO_4, exists as dihydrate (density 1.91 gcm^{-3}, melting point 60°C) and as monohydrate (density 2.04 gcm^{-3}). Both salts are white powders which are very readily soluble in water and which lose their water of crystallization upon heating and at 200°C convert to the weakly acidic diphosphate (disodium hydrogendiphosphate, $Na_2H_2P_2O_7$), at a higher temperature to sodium trimetaphosphate ($Na_3P_3O_9$) and Maddrell's salt (see below). NaH_2PO_4 is acidic; it forms when phosphoric acid is adjusted to a pH of 4.5 using sodium hydroxide solution and the slurry is sprayed. Potassium dihydrogenphosphate (primary or monobasic potassium phosphate, potassium biphosphate, KDP), KH_2PO_4, is a white salt of density 2.33 gcm^{-3}, has a melting point of 253°C [decomposition with the formation of potassium polyphosphate $(KPO_3)_x$] and is readily soluble in water.

Disodium hydrogenphosphate (secondary sodium phosphate), Na_2HPO_4, is a colorless crystalline salt which is very readily soluble in water. It exists in anhydrous form and with 2 mol (density 2.066 gcm^{-3}, loss of water at 95°C), 7 mol (density 1.68 gcm^{-3}, melting point 48°C with loss of 5 H_2O), and 12 mol (density 1.52 gcm^{-3}, melting point 35°C with loss of 5 H_2O) of

water, becomes anhydrous at 100°C and upon more vigorous heating converts to the diphosphate $Na_4P_2O_7$. Disodium hydrogenphosphate is prepared by neutralizing phosphoric acid with sodium carbonate solution using phenolphthalein as indicator. Dipotassium hydrogenphosphate (secondary or dibasic potassium phosphate), K_2HPO_4, is an amorphous, white salt which is readily soluble in water.

Trisodium phosphate, tertiary sodium phosphate, Na_3PO_4, are colorless crystals which, in the form of the dodecahydrate, have a density of 1.62 gcm^{-3} and a melting point of 73-76°C (decomposition), in the form of the decahydrate (corresponding to 19-20% P_2O_5) have a melting point of 100°C and in anhydrous form (corresponding to 39-40% P_2O_5) have a density of 2.536 gcm^{-3}. Trisodium phosphate is readily soluble in water with an alkaline reaction and is prepared by evaporating a solution of exactly 1 mol of disodium phosphate and 1 mol of NaOH. Tripotassium phosphate (tertiary or tribasic potassium phosphate), K_3PO_4, is a white, deliquescent granular powder of density 2.56 gcm^{-3}, has a melting point of 1340°C and is readily soluble in water with an alkaline reaction. It is produced, for example, during the heating of Thomas slag with carbon and potassium sulfate. Despite the higher price, the more readily soluble, and therefore highly effective, potassium phosphates are often preferred over corresponding sodium compounds in the cleaning compositions industry.

Tetrasodium diphosphate (sodium pyrophosphate), $Na_4P_2O_7$, exists in anhydrous form (density 2.534 gcm^{-3}, melting point 988°, also 880° quoted) and as decahydrate (density 1.815-1.836 gcm^{-3}, melting point 94° with loss of water). Both substances are colorless crystals which dissolve in water with an alkaline reaction. $Na_4P_2O_7$ is

formed during the heating of disodium phosphate to >200°C or by reacting phosphoric acid with sodium carbonate in a stoichiometric ratio and dewatering the solution by spraying. The decahydrate complexes heavy metal salts and hardness constituents and thus reduces the water hardness. Potassium diphosphate (potassium pyrophosphate), $K_4P_2O_7$, exists in the form of the trihydrate and is a colorless, hygroscopic powder of density 2.33 gcm^{-3}, which is soluble in water, the pH of the 1% strength solution at 25°C being 10.4.

Condensation of NaH_2PO_4 and KH_2PO_4 results in higher molecular weight sodium phosphates and potassium phosphates, respectively, amongst which cyclic representatives, the sodium and potassium metaphosphates, respectively, and chain-like types, the sodium and potassium polyphosphates, respectively, can be differentiated. Particularly for the latter, a multiplicity of names are in use: melt or thermal phosphates, Graham's salt, Kurrol's and Maddrell's salt. All higher sodium and potassium phosphates are together referred to as condensed phosphates.

The industrially important pentasodium triphosphate, $Na_5P_3O_{10}$ (sodium tripolyphosphate), is a nonhygroscopic, white, water-soluble salt which is anhydrous or crystallizes with 6 H_2O and is of the general formula $NaO-[P(O)(ONa)-O]_n-Na$ where n=3. In 100 g of water, about 17 g of the salt which is free of water of crystallization dissolve at room temperature, approx. 20 g dissolve at 60°, and about 32 g dissolve at 100°; if the solution is heated at 100° for two hours, about 8% of orthophosphate and 15% of diphosphate form due to hydrolysis. In the preparation of pentasodium triphosphate, phosphoric acid is reacted with sodium carbonate solution or sodium hydroxide solution in a stoichiometric ratio, and the solution is dewatered by

spraying. Similarly to Graham's salt and sodium diphosphate, pentasodium triphosphate dissolves many insoluble metal compounds (including lime soaps, etc.). Pentapotassium triphosphate, $K_5P_3O_{10}$ (potassium tripolyphosphate), is available commercially, for example, in the form of a 50% strength by weight solution (>23% P_2O_5, 25% K_2O). The potassium polyphosphates are used widely in the washing compositions and cleaning compositions industry. In addition, sodium potassium tripolyphosphates also exist which can likewise be used within the scope of the present invention. These form, for example, when sodium trimetaphosphate is hydrolyzed with KOH:

$$(NaPO_3)_3 + 2\ KOH \rightarrow Na_3K_2P_3O_{10} + H_2O$$

According to the invention, these can be used in exactly the same way as sodium tripolyphosphate, potassium tripolyphosphate or mixtures of these two; mixtures of sodium tripolyphosphate and sodium potassium tripolyphosphate or mixtures of potassium tripolyphosphate and sodium potassium tripolyphosphate or mixtures of sodium tripolyphosphate and potassium tripolyphosphate and sodium potassium tripolyphosphate can also be used according to the invention.

Organic cobuilders which can be used in the washing compositions and cleaning compositions of the invention are, in particular, polycarboxylates or polycarboxylic acids, polymeric polycarboxylates, polyaspartic acid, polyacetals, optionally oxidized dextrins, further organic cobuilders (see below), and phosphonates. These classes of substance are described below.

Useable organic builder substances are, for example, the polycarboxylic acids usable in the form of their sodium salts, the term polycarboxylic acids meaning

those carboxylic acids which carry more than one acid function. Examples of these are citric acid, adipic acid, succinic acid, glutaric acid, malic acid, tartaric acid, maleic acid, fumaric acid, sugar acids, aminocarboxylic acids, nitrilotriacetic acid (NTA), as long as such a use is not to be avoided for ecological reasons, and mixtures thereof. Preferred salts are the salts of the polycarboxylic acids such as citric acid, adipic acid, succinic acid, glutaric acid, tartaric acid, sugar acids, and mixtures thereof.

It is also possible to use the acids per se. In addition to their builder action, the acids typically also have the property of an acidifying component and thus also serve to establish a lower and milder pH of washing or cleaning compositions, if the pH resulting from the mixture of the remaining components is not desired. Particular mention should be made here of system-compatible and environmentally safe acids such as citric acid, acetic acid, tartaric acid, malic acid, lactic acid, glycolic acid, succinic acid, glutaric acid, adipic acid, gluconic acid and any mixtures thereof. However, mineral acids, in particular sulfuric acid, or bases, in particular ammonium or alkali metal hydroxides, may also serve as pH regulators. The agents of the invention contain such regulators in amounts of preferably not more than 20% by weight, in particular from 1.2% by weight to 17% by weight.

Suitable builders are also polymeric polycarboxylates; these are, for example, the alkali metal salts of polyacrylic acid or of polymethacrylic acid, for example those having a relative molecular mass of from 500 to 70 000 g/mol.

The molar masses given for polymeric polycarboxylates are, for the purposes of this specification, weight-

average molar masses, M_W, of the respective acid form, always determined by means of gel permeation chromatography (GPC), using a UV detector. The measurement was made against an external polyacrylic acid standard which, owing to its structural similarity to the polymers studied, provides realistic molecular weight values. These figures differ considerably from the molecular weight values obtained using polystyrenesulfonic acids as the standard. The molar masses measured against polystyrenesulfonic acids are usually considerably higher than the molar masses given in this specification.

Suitable polymers are, in particular, polyacrylates which preferably have a molecular mass of from 2000 to 20 000 g/mol. Owing to their superior solubility, preference in this group may be given in turn to the short-chain polyacrylates which have molar masses of from 2000 to 10 000 g/mol, and particularly preferably from 3000 to 5000 g/mol.

Also suitable are copolymeric polycarboxylates, in particular those of acrylic acid with methacrylic acid and of acrylic acid or methacrylic acid with maleic acid. Copolymers which have proven to be particularly suitable are those of acrylic acid with maleic acid which contain from 50 to 90% by weight of acrylic acid and from 50 to 10% by weight of maleic acid. Their relative molecular mass, based on free acids, is generally from 2000 to 70 000 g/mol, preferably from 20 000 to 50 000 g/mol and in particular from 30 000 to 40 000 g/mol. The (co)polymeric polycarboxylates may be used either as powders or as aqueous solution. The (co)polymeric polycarboxylates may be from 0.5 to 20% by weight, in particular 1 to 10% by weight of the content of the compositions.

To improve the solubility in water, the polymers may also contain allylsulfonic acids such as, for example, allyloxybenzenesulfonic acid and methallylsulfonic acid as monomers.

Particular preference is also given to biodegradable polymers of more than two different monomer units, for example those which contain, as monomers, salts of acrylic acid and of maleic acid, and vinyl alcohol or vinyl alcohol derivatives, or those which contain, as monomers, salts of acrylic acid and of 2-alkylallylsulfonic acid, and sugar derivatives.

Further preferred copolymers are those which preferably have, as monomers, acrolein and acrylic acid/acrylic acid salts or acrolein and vinyl acetate.

Further preferred builder substances which may be mentioned are also polymeric aminodicarboxylic acids, their salts or their precursor substances. Particular preference is given to polyaspartic acids or salts and derivatives thereof.

Further suitable builder substances are polyacetals which can be obtained by reacting dialdehydes with polyolcarboxylic acids having from 5 to 7 carbon atoms and at least 3 hydroxyl groups. Preferred polyacetals are obtained from dialdehydes such as glyoxal, glutaraldehyde, terephthalaldehyde and mixtures thereof and from polyolcarboxylic acids such as gluconic acid and/or glucoheptonic acid.

Further suitable organic builder substances are dextrins, for example oligomers or polymers of carbohydrates, which can be obtained by partial hydrolysis of starches. The hydrolysis can be carried out by customary processes, for example acid-catalyzed

or enzyme-catalyzed processes. The hydrolysis products preferably have average molar masses in the range from 400 to 500 000 g/mol. Preference is given here to a polysaccharide having a dextrose equivalent (DE) in the range from 0.5 to 40, in particular from 2 to 30, where DE is a common measure of the reducing action of a polysaccharide compared with dextrose which has a DE of 100. It is possible to use both maltodextrins having a DE between 3 and 20 and dried glucose syrups having a DE between 20 and 37, and also "yellow dextrins" and "white dextrins" with higher molar masses in the range from 2000 to 30 000 g/mol.

The oxidized derivatives of such dextrins are their reaction products with oxidizing agents which are able to oxidize at least one alcohol function of the saccharide ring to the carboxylic acid function. Particularly preferred organic builders for compositions of the invention are oxidized starches and derivatives thereof of the applications EP 472 042, WO 97/25399 and EP 755 944, respectively.

Oxydisuccinates and other derivatives of disuccinates, preferably ethylenediamine disuccinate, are also further suitable cobuilders. Here, ethylenediamine N,N'-disuccinate (EDDS) is preferably used in the form of its sodium or magnesium salts. In this connection, further preference is also given to glycerol disuccinates and glycerol trisuccinates. Suitable use amounts in zeolite-containing and/or silicate-containing formulations are between 3 and 15% by weight.

Further organic cobuilders which can be used are, for example, acetylated hydroxycarboxylic acids or salts thereof, which may also be present, where appropriate, in lactone form and which contain at least 4 carbon

atoms and at least one hydroxy group and at most two acid groups.

A further class of substance having cobuilder properties is the phosphonates. These are, in particular, hydroxyalkane- and aminoalkanephosphonates. Among the hydroxyalkanephosphonates, 1-hydroxyethane-1,1-diphosphonate (HEDP) is of particular importance as a cobuilder. It is preferably used as sodium salt, the disodium salt being neutral and the tetrasodium salt being alkaline (pH 9). Suitable aminoalkanephosphonates are preferably ethylenediaminetetramethylenephosphonate (EDTMP), diethylenetriaminepentamethylenephosphonate (DTPMP) and higher homologs thereof. They are preferably used in the form of the neutral sodium salts, for example as the hexasodium salt of EDTMP or as the hepta- and octasodium salt of DTPMP. Here, preference is given to using HEDP as builder from the class of phosphonates. In addition, the aminoalkane-phosphonates have a marked heavy metal-binding capacity. Accordingly, particularly if the agents also contain bleaches, it may be preferable to use aminoalkanephosphonates, in particular DTPMP, or mixtures of said phosphonates.

In addition, all compounds which are able to form complexes with alkaline earth metal ions can be used as cobuilders.

The agents of the invention may contain builder substances, where appropriate in amounts of up to 90% by weight, and preferably contain them in amounts of up to 75% by weight. Washing compositions of the invention have builder contents of, in particular, from 5% by weight to 50% by weight. In inventive compositions for cleaning hard surfaces, in particular for machine cleaning of dishes, the builder substance content is in

particular from 5% by weight to 88% by weight, with preferably no water-insoluble builder materials being used in such compositions. A preferred embodiment of inventive compositions for, in particular, machine cleaning of dishes contains from 20% by weight to 40% by weight water-soluble organic builders, in particular alkali metal citrate, from 5% by weight to 15% by weight alkali metal carbonate and from 20% by weight to 40% by weight alkali metal disilicate.

Solvents which may be used in the liquid to gelatinous compositions of washing compositions and cleaning compositions are, for example, from the group of monohydric or polyhydric alcohols, alkanolamines or glycol ethers, as long as they are miscible with water in the given concentration range. Preferably, the solvents are selected from ethanol, n- or i-propanol, butanols, ethylene glycol methyl ether, ethylene glycol ethyl ether, ethylene glycol propyl ether, ethylene glycol mono-n-butyl ether, diethylene glycol methyl ether, diethylene glycol ethyl ether, propylene glycol methyl, ethyl or propyl ether, dipropylene glycol monomethyl or monoethyl ether, diisopropylene glycol monomethyl or monoethyl ether, methoxy, ethoxy or butoxy triglycol, 1-butoxyethoxy-2-propanol, 3-methyl-3-methoxybutanol, propylene glycol t-butyl ether, and mixtures of these solvents.

Solvents may be used in the liquid to gelatinous washing compositions and cleaning compositions of the invention in amounts of between 0.1 and 20% by weight, but preferably below 15% by weight, and in particular below 10% by weight.

To adjust the viscosity, one or more thickeners or thickening systems may be added to the compositions of the invention. These high molecular weight substances

which are also called swell(ing) agents usually soak up the liquids and swell in the process, converting ultimately into viscous, true or colloidal solutions.

Suitable thickeners are inorganic or polymeric organic compounds. Inorganic thickeners include, for example, polysilicic acids, clay minerals, such as montmorillonites, zeolites, silicas and bentonites. The organic thickeners are from the groups of natural polymers, modified natural polymers and completely synthetic polymers. Such natural polymers are, for example, agar-agar, carrageen, tragacanth, gum arabic, alginates, pectins, polyoses, guar flour, carob seed flour, starch, dextrins, gelatin and casein. Modified natural substances which are used as thickeners are primarily from the group of modified starches and celluloses. Examples which may be mentioned here are carboxymethylcellulose and other cellulose ethers, hydroxyethylcellulose and hydroxypropylcellulose, and carob flour ether. Completely synthetic thickeners are polymers such as polyacrylic and polymethacrylic compounds, vinyl polymers, polycarboxylic acids, polyethers, polyimines, polyamides and polyurethanes.

The thickeners may be present in an amount up to 5% by weight, preferably from 0.05 to 2% by weight, and particularly preferably from 0.1 to 1.5% by weight, based on the finished composition.

The washing and cleaning composition of the invention may, where appropriate, comprise, as further customary ingredients, sequestering agents, electrolytes and further excipients.

Textile washing compositions of the invention may contain, as optical brighteners, derivatives of diaminostilbenedisulfonic acid or alkali metal salts

thereof. Suitable are, for example, salts of 4,4'-bis(2-anilino-4-morpholino-1,3,5-triazinyl-6-amino)-stilbene-2,2'-disulfonic acid or similarly constructed compounds which carry a diethanolamino group, a methylamino group, an anilino group or a 2-methoxyethylamino group instead of the morpholino group. In addition, brighteners of the substituted diphenylstyryl type may be present, for example the alkali metal salts of 4,4'-bis(2-sulfostyryl)diphenyl, 4,4'-bis(4-chloro-3-sulfostyryl)diphenyl, or 4-(4-chlorostyryl)-4'-(2-sulfostyryl)diphenyl. Mixtures of the abovementioned optical brighteners may also be used.

Antiredeposition agents have the function of keeping the soil detached from the textile fiber in suspension in the liquor. Suitable for this purpose are water-soluble colloids, usually organic in nature, for example starch, size, gelatin, salts of ethercarboxylic acids or ethersulfonic acids of starch or of cellulose, or salts of acidic sulfuric esters of cellulose or of starch. Water-soluble polyamides containing acidic groups are also suitable for this purpose. Furthermore, starch derivatives other than those mentioned above may be used, for example aldehyde starches. Preference is given to cellulose ethers such as carboxymethyl-cellulose (Na salt), methylcellulose, hydroxyalkyl-cellulose and mixed ethers such as methyl-hydroxyethylcellulose, methylhydroxypropylcellulose, methylcarboxymethylcellulose, and mixtures thereof, for example in amounts of from 0.1 to 5% by weight, based on the compositions.

In order to protect against silver corrosion, silver corrosion inhibitors may be used in dishwashing cleaning compositions of the invention. Such inhibitors are known in the prior art, for example benzotriazoles,

iron(III) chloride or $CoSO_4$. As disclosed by, for example, European patent EP 0 736 084 B1, silver corrosion inhibitors which are particularly suitable for being used together with enzymes are manganese, titanium, zirconium, hafnium, vanadium, cobalt, or cerium salts and/or complexes in which the specified metals are present in any of the oxidation states II, III, IV, V or VI. Examples of such compounds are $MnSO_4$, V_2O_5, V_2O_4, VO_2, $TiOSO_4$, K_2TiF_6, K_2ZrF_6, $Co(NO_3)_2$, $Co(NO_3)_3$, and mixtures thereof.

Soil-release active ingredients or soil repellents are usually polymers which, when used in a washing composition, impart soil-repellent properties to the laundry fiber and/or assist the ability of the other washing composition ingredients to detach soil. A comparable effect can also be observed with their use in cleaning compositions for hard surfaces.

Soil-release active ingredients which are particularly effective and have been known for a long time are copolyesters having dicarboxylic acid, alkylene glycol and polyalkylene glycol units. Examples thereof are copolymers or mixed polymers of polyethylene terephthalate and polyoxyethylene glycol (DT 16 17 141, and, respectively, DT 22 00 911). German laid-open specification DT 22 53 063 discloses acidic agents containing, inter alia, a copolymer of a dibasic carboxylic acid and an alkylene or cycloalkylene polyglycol. German documents DE 28 57 292 and DE 33 24 258 and European patent EP 0 253 567 describe polymers of ethylene terephthalate and polyethylene oxide terephthalate and the use thereof in washing compositions. European patent EP 066 944 relates to compositions containing a copolyester of ethylene glycol, polyethylene glycol, aromatic dicarboxylic acid and sulfonated aromatic dicarboxylic acid in particular

molar ratios. European patent EP 0 185 427 discloses methyl or ethyl group end-capped polyesters having ethylene and/or propylene terephthalate and polyethylene oxide terephthalate units, and washing compositions containing such a soil-release polymer. European patent EP 0 241 984 discloses a polyester which contains, in addition to oxyethylene groups and terephthalic acid units, also substituted ethylene units and glycerol units. European patent EP 0 241 985 discloses polyesters which contain, in addition to oxyethylene groups and terephthalic acid units, 1,2-propylene, 1,2-butylene and/or 3-methoxy-1,2-propylene groups, and glycerol units and which are end-group-capped with C_1- to C_4-alkyl groups. European patent application EP 0 272 033 discloses polyesters having polypropylene terephthalate and polyoxyethylene terephthalate units, which are at least partially end-group-capped by C_{1-4}-alkyl or acyl radicals. European patent EP 0 274 907 describes sulfoethyl end-group-capped terephthalate-containing soil-release polyesters. According to European patent application EP 0 357 280, sulfonation of unsaturated end groups produces soil-release polyesters having terephthalate, alkylene glycol and poly-C_{2-4}-glycol units. International patent application WO 95/32232 relates to acidic, aromatic polyesters capable of detaching soil. International patent application WO 97/31085 discloses nonpolymeric soil-repellent active ingredients for materials made of cotton, which have a plurality of functional units: a first unit which may be cationic, for example, is able to adsorb to the cotton surface by means of electrostatic interaction, and a second unit which is hydrophobic is responsible for the active ingredient remaining at the water/cotton interface.

The color transfer inhibitors suitable for use in laundry washing compositions of the invention include,

in particular, polyvinylpyrrolidones, polyvinylimidazoles, polymeric N-oxides such as poly(vinylpyridine N-oxide) and copolymers of vinylpyrrolidone with vinylimidazole.

For use in machine cleaning processes, it may be of advantage to add foam inhibitors to the agents. Examples of suitable foam inhibitors are soaps of natural or synthetic origin having a high proportion of C_{18}-C_{24} fatty acids. Examples of suitable nonsurfactant-type foam inhibitors are organopolysiloxanes and their mixtures with microfine, optionally silanized silica and also paraffins, waxes, microcrystalline waxes, and mixtures thereof with silanized silica or bis-stearyl-ethylenediamide. With advantages, use is also made of mixtures of different foam inhibitors, for example mixtures of silicones, paraffins or waxes. The foam inhibitors, in particular those containing silicone and/or paraffin, are preferably bound to a granular, water-soluble or dispersible support substance. Particular preference is given here to mixtures of paraffins and bis-stearylethylenediamides.

A cleaning composition of the invention for hard surfaces may, in addition, contain ingredients with abrasive action, in particular from the group comprising quartz flours, wood flours, polymer flours, chalks and glass microbeads, and mixtures thereof. Abrasives are present in the cleaning compositions of the invention preferably at not more than 20% by weight, in particular from 5% by weight to 15% by weight.

Dyes and fragrances are added to washing compositions and cleaning compositions in order to improve the esthetic appeal of the products and to provide the consumer, in addition to washing and cleaning

performance, with a visually and sensorially "typical and unmistakable" product. As perfume oils and/or fragrances it is possible to use individual odorant compounds, for example the synthetic products of the ester, ether, aldehyde, ketone, alcohol and hydrocarbon types. Odorant compounds of the ester type are, for example, benzyl acetate, phenoxyethyl isobutyrate, p-tert-butylcyclohexyl acetate, linalyl acetate, dimethylbenzylcarbinyl acetate, phenylethyl acetate, linalyl benzoate, benzyl formate, ethyl methylphenyl glycinate, allylcyclohexyl propionate, styrallyl propionate and benzyl salicylate. The ethers include, for example, benzyl ethyl ether; the aldehydes include, for example, the linear alkanals having 8-18 carbon atoms, citral, citronellal, citronellyloxyacetaldehyde, cyclamenaldehyde, hydroxycitronellal, lilial and bourgeonal; the ketones include, for example, the ionones, α-isomethylionone and methyl cedryl ketone; the alcohols include anethol, citronellol, eugenol, geraniol, linalool, phenylethyl alcohol, and terpineol; the hydrocarbons include primarily the terpenes such as limonene and pinene. Preference, however, is given to the use of mixtures of different odorants which together produce an appealing fragrance note. Such perfume oils may also contain natural odorant mixtures, as are obtainable from plant sources, for example pine oil, citrus oil, jasmine oil, patchouli oil, rose oil or ylang-ylang oil. Likewise suitable are muscatel, sage oil, camomile oil, clove oil, balm oil, mint oil, cinnamon leaf oil, lime blossom oil, juniper berry oil, vetiver oil, olibanum oil, galbanum oil and labdanum oil, and also orange blossom oil, neroli oil, orangepeel oil and sandalwood oil. The dye content of washing compositions and cleaning compositions is usually less than 0.01% by weight, while fragrances may make up up to 2% by weight of the overall formulation.

The fragrances may be incorporated directly into the washing compositions and cleaning compositions; however, it may also be advantageous to apply the fragrances to carriers which enhance the adhesion of the perfume to the material to be cleaned and, by means of slower fragrance release, ensure long-lasting fragrance, in particular of treated textiles. Materials which have become established as such carriers are, for example, cyclodextrins, it being possible, in addition, for the cyclodextrin-perfume complexes also to be coated with further auxiliaries. Another preferred carrier for fragances is the described zeolite X which can also absorb fragrances instead of or in a mixture with surfactants. Preference is therefore given to washing compositions and cleaning compositions which contain the described zeolite X and fragrances which, preferably, are at least partially absorbed on the zeolite.

Preferred dyes whose selection is by no means difficult for the skilled worker have high storage stability and insensitivity to the other ingredients of the compositions and to light, and also have no pronounced affinity for textile fibers, so as not to stain them.

To control microorganisms, washing compositions or cleaning compositions may contain antimicrobial agents. Depending on antimicrobial spectrum and mechanism of action, a distinction is made here between bacteriostatics and bactericides, fungistatics and fungicides, etc. Examples of important substances from these groups are benzalkonium chlorides, alkylaryl-sulfonates, halophenols and phenylmercury acetate. The terms antimicrobial action and antimicrobial agent have, within the teaching of the invention, the meaning common in the art, which is described, for example, by *K.H. Wallhäußer* in "Praxis der Sterilisation,

Desinfektion - Konservierung: Keimidentifizierung - Betriebshygiene" (5th Edition - Stuttgart; New York: Thieme, 1995), it being possible to use all of the substances having antimicrobial action described there. Suitable antimicrobial agents are preferably selected from the groups of alcohols, amines, aldehydes, antimicrobial acids or their salts, carboxylic esters, acid amides, phenols, phenol derivatives, diphenyls, diphenylalkanes, urea derivatives, oxygen acetals, nitrogen acetals and also oxygen and nitrogen formals, benzamidines, isothioazolines, phthalimide derivatives, pyridine derivatives, antimicrobial surfactant compounds, guanidines, antimicrobial amphoteric compounds, quinolines, 1,2-dibromo-2,4-dicyanobutane, iodo-2-propylbutyl carbamate, iodine, iodophors, peroxo compounds, halogen compounds, and any mixtures of the above.

The antimicrobial agent may be selected from ethanol, n-propanol, isopropanol, 1,3-butanediol, phenoxyethanol, 1,2-propylene glycol, glycerol, undecylenic acid, benzoic acid, salicylic acid, dihydracetic acid, o-phenylphenol, N-methylmorpholino-acetonitrile (MMA), 2-benzyl-4-chlorophenol, 2,2'-methylenebis(6-bromo-4-chlorophenol), 4,4'-dichloro-2'-hydroxydiphenyl ether (dichlosan), 2,4,4'-trichloro-2'-hydroxydiphenyl ether (trichlosan), chlorohexidine, N-(4-chlorophenyl)-N-(3,4-dichlorophenyl)urea, N,N'-(1,10-decanediyldi-1-pyridinyl-4-ylidene)-bis(1-octanamine) dihydrochloride, N,N'-bis(4-chlorophenyl)-3,12-diimino-2,4,11,13-tetraazatetradecanediimideamide, glucoprotamines, antimicrobial surface-active quaternary compounds, guanidines including the bi- and polyguanidines, such as, for example, 1,6-bis(2-ethylhexylbiguanidohexane) dihydrochloride, 1,6-di-(N_1,N_1'-phenyldiguanido-N_5,N_5')hexane tetrahydrochloride, 1,6-di-(N_1,N_1'-phenyl-

N_1,N_1-methyldiguanido-N_5,N_5')hexane dihydrochloride, 1,6-di-(N_1,N_1'-o-chlorophenyldiguanido-N_5,N_5')hexane dihydrochloride, 1,6-di-(N_1,N_1'-2,6-dichlorophenyldiguanido-N_5,N_5')hexane dihydrochloride, 1,6-di-[N_1,N_1'-beta-(p-methoxyphenyl)diguanido-N_5,N_5']hexane dihydrochloride, 1,6-di-(N_1,N_1'-alpha-methyl-beta-phenyldiguanido-N_5,N_5')hexane dihydrochloride, 1,6-di-(N_1,N_1'-p-nitrophenyldiguanido-N_5,N_5')hexane dihydrochloride, omega:omega-di-(N_1,N_1'-phenyldiguanido-N_5,N_5')-di-n-propyl ether dihydrochloride, omega:omega'-di-(N_1,N_1'-p-chlorophenyldiguanido-N_5,N_5')-di-n-propyl ether tetrahydrochloride, 1,6-di-(N_1,N_1'-2,4-dichlorophenyldiguanido-N_5,N_5')hexane tetrahydrochloride, 1,6-di-(N_1,N_1'-p-methylphenyldiguanido-N_5,N_5')hexane dihydrochloride, 1,6-di-(N_1,N_1'-2,4,5-trichlorophenyldiguanido-N_5,N_5')hexane tetrahydrochloride, 1,6-di-[N_1,N_1'-alpha-(p-chlorophenyl)ethyldiguanido-N_5,N_5']-hexane dihydrochloride, omega:omega-di-(N_1,N_1'-p-chlorophenyldiguanido-N_5,N_5')m-xylene dihydrochloride, 1,12-di-(N_1,N_1'-p-chlorophenyldiguanido-N_5,N_5')dodecane dihydrochloride, 1,10-di-(N_1,N_1'-phenyldiguanido-N_5,N_5')decane tetrahydrochloride, 1,12-di-(N_1,N_1'-phenyldiguanido-N_5,N_5')dodecane tetrahydrochloride, 1,6-di-(N_1,N_1'-o-chlorophenyldiguanido-N_5,N_5')hexane dihydrochloride, 1,6-di-(N_1,N_1'-o-chlorophenyldiguanido-N_5,N_5')hexane tetrahydrochloride, ethylenebis(1-tolylbiguanide), ethylenebis(p-tolylbiguanide), ethylenebis(3,5-dimethylphenylbiguanide), ethylenebis(p-tert-amylphenylbiguanide), ethylenebis(nonylphenylbiguanide), ethylenebis(phenylbiguanide), ethylenebis(N-butylphenylbiguanide), ethylenebis(2,5-diethoxyphenylbiguanide), ethylenebis(2,4-dimethylphenylbiguanide), ethylenebis(o-diphenylbiguanide), ethylenebis(mixed amyl naphthylbiguanide), N-butylethylenebis(phenylbiguanide), trimethylenebis(o-tolylbiguanide), N-butyltrimethylbis(phenylbiguanide) and the corresponding

salts such as acetates, gluconates, hydrochlorides, hydrobromides, citrates, bisulfites, fluorides, polymaleates, N-cocoalkylsarcosinates, phosphites, hypophosphites, perfluorooctanoates, silicates, sorbates, salicylates, maleates, tartrates, fumarates, ethylenediaminetetraacetates, iminodiacetates, cinnamates, thiocyanates, arginates, pyromellitates, tetracarboxybutyrates, benzoates, glutarates, monofluorophosphates, perfluoropropionates, and any mixtures thereof. Also suitable are halogenated xylene and cresol derivatives, such as p-chlorometacresol or p-chlorometaxylene, and natural antimicrobial agents of plant origin (for example from spices or herbs), animal origin and microbial origin. Preference may be given to using antimicrobial surface-active quaternary compounds, a natural antimicrobial agent of plant origin and/or a natural antimicrobial agent of animal origin, most preferably at least one natural antimicrobial agent of plant origin from the group comprising caffeine, theobromine and theophylline and essential oils such as eugenol, thymol and geraniol, and/or at least one natural antimicrobial agent of animal origin from the group comprising enzymes such as milk protein, lysozyme and lactoperoxidase, and/or at least one antimicrobial surface-active quaternary compound having an ammonium, sulfonium, phosphonium, iodonium or arsonium group, peroxo compounds and chlorine compounds. It is also possible to use substances of microbial origin, the "bacteriocines".

The quaternary ammonium compounds (QACs) which are suitable as antimicrobial agents have the general formula $(R^1)(R^2)(R^3)(R^4)\ N^+\ X^-$ where R^1 to R^4 are identical or different C_1-C_{22}-alkyl radicals, C_7-C_{28}-aralkyl radicals or heterocyclic radicals, where two, or in the case of an aromatic incorporation such as in pyridine, even three radicals, together with the

nitrogen atom, form the heterocycle, for example a pyridinium or imidazolinium compound, and X^- are halide ions, sulfate ions, hydroxide ions or similar anions. For optimal antimicrobial action, at least one of the radicals preferably has a chain length of from 8 to 18, in particular 12 to 16, carbon atoms.

QACs can be prepared by reacting tertiary amines with alkylating agents such as, for example, methyl chloride, benzyl chloride, dimethyl sulfate, dodecyl bromide, or else ethylene oxide. The alkylation of tertiary amines having one long alkyl radical and two methyl groups proceeds particularly readily, and the quaternization of tertiary amines having two long radicals and one methyl group can also be carried out with the aid of methyl chloride under mild conditions. Amines which have three long alkyl radicals or hydroxy-substituted alkyl radicals have low reactivity and are preferably quaternized using dimethyl sulfate.

Examples of suitable QACs are benzalkonium chloride (N-alkyl-N,N-dimethylbenzylammonium chloride, CAS No. 8001-54-5), benzalkone B (*m,p*-dichlorobenzyldimethyl-C12-alkylammonium chloride, CAS No. 58390-78-6), benzoxonium chloride (benzyldodecylbis(2-hydroxy-ethyl)ammonium chloride), cetrimonium bromide (N-hexadecyl-N,N-trimethylammonium bromide, CAS No. 57-09-0), benzetonium chloride (N,N-dimethyl-N-[2-[2-[*p*-(1,1,3,3-tetramethylbutyl)phenoxy]ethoxy]ethyl]-benzylammonium chloride, CAS No. 121-54-0), dialkyldimethylammonium chlorides such as di-*n*-decyldimethylammonium chloride (CAS No. 7173-51-5-5), didecyldimethylammonium bromide (CAS No. 2390-68-3), dioctyldimethylammonium chloride, 1-cetylpyridinium chloride (CAS No. 123-03-5) and thiazoline iodide (CAS No. 15764-48-1), and mixtures thereof. Particularly preferred QACs are the benzalkonium chlorides having

C_8-C_{18}-alkyl radials, in particular C_{12}-C_{14}-alkyl-benzyldimethylammonium chloride.

Benzalkonium halides and/or substituted benzalkonium halides are commercially available, for example, as Barquat® ex Lonza, Marquat® ex Mason, Variquat® ex Witco/Sherex and Hyamine® ex Lonza, and Bardac® ex Lonza. Further commercially available antimicrobial agents are N-(3-chloroallyl)hexaminium chloride such as Dowicide® and Dowicil® ex Dow, benzethonium chloride such as Hyamine® 1622 ex Rohm & Haas, methylbenzethonium chloride such as Hyamine® 10X ex Rohm & Haas, cetylpyridinium chloride such as cepacol chloride ex Merrell Labs.

The antimicrobial agents are used in amounts of from 0.0001% by weight to 1% by weight, preferably from 0.001% by weight to 0.8% by weight, particularly preferably from 0.005% by weight to 0.3% by weight, and in particular from 0.01 to 0.2% by weight.

The compositions may contain UV absorbers which attach to the treated textiles and improve the light stability of the fibers and/or the light stability of other formulation constituents. UV absorbers mean organic substances (light protection filters) which are able to absorb ultraviolet radiation and to emit the absorbed energy again in the form of radiation of longer wavelength, for example heat.

Compounds which have these desired properties are, for example, the compounds which are active via radiationless deactivation and derivatives of benzophenone having substituents in position(s) 2 and/or 4. Furthermore, also suitable are substituted benzotriazoles, acrylates which are phenyl-substituted in position 3 (cinnamic acid derivatives, with or

without cyano groups in position 2), salicylates, organic Ni complexes and natural substances such as umbelliferone and the endogenous urocanic acid. Of particular importance are biphenyl and especially stilbene derivatives, as described, for example, in EP 0728749 A and commercially available as Tinosorb® FD or Tinosorb® FR ex Ciba. UV-B absorbers which may be mentioned are: 3-benzylidenecamphor or 3-benzylidenenorcamphor and derivatives thereof, for example 3-(4-methylbenzylidene)camphor, as described in EP 0693471 B1; 4-aminobenzoic acid derivatives, preferably 2-ethylhexyl 4-(dimethylamino)benzoate, 2-octyl 4-(dimethylamino)benzoate and amyl 4-(dimethylamino)benzoate; esters of cinnamic acid, preferably 2-ethylhexyl 4-methoxycinnamate, propyl 4-methoxycinnamate, isoamyl 4-methoxycinnamate, 2-ethylhexyl 2-cyano-3,3-phenylcinnamate (octocrylenes); esters of salicylic acid, preferably 2-ethylhexyl salicylate, 4-isopropylbenzyl salicylate, homomenthyl salicylate; derivatives of benzophenone, preferably 2-hydroxy-4-methoxybenzophenone, 2-hydroxy-4-methoxy-4'-methylbenzophenone, 2,2'-dihydroxy-4-methoxybenzophenone; esters of benzalmalonic acid, preferably di-2-ethylhexyl 4-methoxybenzmalonate; triazine derivatives such as, for example, 2,4,6-trianilino-(p-carbo-2'-ethyl-1'-hexyloxy)-1,3,5-triazine and octyltriazone, as described in EP 0818450 A1, or dioctylbutamidotriazones (Uvasorb® HEB); propane-1,3-diones such as, for example, 1-(4-tert-butylphenyl)-3-(4'-methoxyphenyl)-propane-1,3-dione; ketotricyclo(5.2.1.0)decane derivatives, as described in EP 0694521 B1. Further suitable are 2-phenylbenzimidazole-5-sulfonic acid and its alkali metal, alkaline earth metal, ammonium, alkylammonium, alkanolammonium and glucammonium salts; sulfonic acid derivatives of benzophenones, preferably 2-hydroxy-4-methoxybenzophenone-5-sulfonic acid and its salts; sulfonic acid derivatives of

3-benzylidenecamphor, such as, for example, 4-(2-oxo-3-bornylidenemethyl)benzenesulfonic acid and 2-methyl-5-(2-oxo-3-bornylidene)sulfonic acid and salts thereof.

Suitable typical UV-A filters are, in particular, derivatives of benzoylmethane, such as, for example, 1-(4'-tert-butylphenyl)-3-(4'-methoxyphenyl)propane-1,3-dione, 4-tert-butyl-4'-methoxydibenzoylmethane (Parsol 1789), 1-phenyl-3-(4'-isopropylphenyl)propane-1,3-dione, and enamine compounds, as described in DE 19712033 A1 (BASF). The UV-A and UV-B filters may of course also be used in mixtures. In addition to said soluble substances, insoluble light protection pigments, namely finely dispersed, preferably nanoized, metal oxides or salts, are also suitable for this purpose. Examples of suitable metal oxides are, in particular, zinc oxide and titanium dioxide and also oxides of iron, zirconium, silicon, manganese, aluminum and cerium, and mixtures thereof. Salts which may be used are silicates (talc), barium sulfate or zinc stearate. The oxides and salts are already used in the form of the pigments for skin-care and skin-protective emulsions and decorative cosmetics. The particles here should have an average diameter of less than 100 nm, preferably between 5 and 50 nm, and in particular between 15 and 30 nm. They can have a spherical shape, but it is also possible to use particles which have an ellipsoidal shape or a shape deviating in some other way from the spherical form. The pigments may also be surface-treated, i.e. hydrophilicized or hydrophobicized. Typical examples are coated titanium dioxides such as, for example, titanium dioxide T 805 (Degussa) or Eusolex® T2000 (Merck); suitable hydrophobic coating agents are here preferably silicones and, particularly preferably, trialkoxyoctylsilanes or simethicones. Preference is given to using micronized zinc oxide. Further suitable

UV light protection filters can be found in the review by P. Finkel in *SÖFW-Journal*, volume 122 (1996), p. 543.

The UV absorbers are usually used in amounts of from 0.01% by weight to 5% by weight, preferably from 0.03% by weight to 1% by weight.

Enzymes such as proteases, amylases, lipases or cellulases have been used for decades as active components in washing compositions and cleaning compositions. Their particular contribution to the washing and, respectively, cleaning performance of the composition in question is, in the case of protease, the ability to break down proteinaceous soilings, in the case of amylase, the breaking-down of starch-containing soilings, and, in the case of lipase, fat-cleaving activity. Cellulases are preferably used in washing compositions, in particular due to their contribution to the secondary washing performance of a washing composition and due to their fiber action on textiles. The particular hydrolysis products are attacked, dissolved, emulsified or suspended by the other washing or cleaning composition components or are, due to their greater solubility, washed away with the wash liquor, advantageously resulting in synergistic effects between the enzymes and the other components.

Compositions of the invention may, in addition to the proteins essential to the invention, comprise other amylolytic enzymes, in particular α-amylases. These may also include enzymes established for use in washing and cleaning compositions. Examples of commercially available amylases are BAN®, Termamyl®, Purastar®, Amylase-LT®, Maxamyl®, Duramyl® and/or Purafect® OxAm. This is appropriate when the various enzymes are able

to supplement one another. Such a supplementation may take place for example in respect of regulation, for example by mutual activation or by inactivation. It may result for example from at least one part, which is not homologous to the known α-amylases, of the enzyme essential to the invention having an effect on the amylolytic activities which are not essential to the invention. The joint use may, however, also be worthwhile because of different substrate specificities. Both are embodiments of the present invention.

Especially with chemically diverse soilings it may be advantageous to employ amylolytic enzymes in washing and cleaning compositions together with other enzymes having washing and/or cleaning activity. Thus, washing or cleaning compositions which, besides a protein of the invention, are characterized by further enzymes in addition represent preferred embodiments of the present invention.

Besides further amylases, these include for example proteases, but also lipases, cutinases, esterases, pullulanases, cellulases, hemicellulases and/or xylanases, and mixtures thereof. Particular preference is given to proteases, lipases, β-glucanases and/or cellulases. Further enzymes extend the cleaning performance of corresponding compositions by their respective enzymatic performance. These include, for example, oxidoreductases or peroxidases as components of enzymatic bleaching systems, for example laccases (WO 00/39306), β-glucanases (WO 99/06515 and WO 99/06516) or pectinolytic enzymes (WO 00/42145), which are employed in particular in specialty washing compositions.

Examples of commercially available enzymes for use in compositions of the invention are proteases such as Subtilisin BPN', Properase®, BLAP®, Optimase®, Opticlean®, Maxatase®, Maxacal®, Mazapem®, Alcalase®, Esperase®, Savinase®, Durazym®, Everlase®, and/or Purafect®G or Purafect® OxP and lipases such as Lipolase®, Lipomax®, Lumafast® and/or Lipozym®.

The protease activity in such compositions can be determined by the method described in *Tenside*, volume 7 (1970), pp. 125-132. They are accordingly indicated in PU (protease units). The protease activity of preferred compositions may be up to 1 500 000 protease units per gram of preparation.

Concerning the obtaining of the enzymes which can be used, those primarily suitable are obtained from microorganisms such as bacteria or fungi, for example from *Bacillus subtilis, B. licheniformis, Streptomyces griseus, Humicola lanuginosa, H. insolens, Pseudomonas pseudoalcaligenes* or *P. cepacia*, in particular the enzyme mixtures naturally produced by these strains, or mixtures with those from other strains. They are obtained in a known manner by fermentation processes from suitable microorganisms which are described for example in German laid-open specifications DE 19 40 488 and DE 21 21 397, US patents US 3 623 957 and US 4 264 738, European patent application EP 006 638, and the international patent application WO 91/02792.

These enzymes which are additionally used where appropriate can also be, as described for example in European patent EP 0 564 476 or in the international patent application WO 94/23005, adsorbed onto carriers and/or embedded in enveloping substances in order to protect them from premature inactivation. They are present in washing compositions preferably in amounts

of up to 10% by weight, in particular from 0.2% by weight to 2% by weight, particularly preferably employing enzymes stabilized against oxidative degradation, as disclosed for example in international patent applications WO 94/18314.

A protein of the invention can be protected, especially during storage, by stabilizers for example from denaturation, decomposition or inactivation, for example by physical effects, oxidation or proteolytic cleavage. Inhibition of proteolysis is particularly critical for proteins obtained from microorganisms because most microorganisms secrete various proteases as digestive enzymes into the surrounding media. These may cause considerable harm to the proteins of interest during the subsequent purification stages. Proteins essential to the invention may also be associated with proteases in washing and cleaning compositions, and therefore require special protection.

Compositions of the invention may also comprise stabilizers for this purpose. One group of stabilizers are reversible protease inhibitors which dissociate off on dilution of the composition in the wash liquor. Benzamidine hydrochloride and leupeptin are established for this purpose. Borax, boric acids, boronic acids or salts or esters thereof are frequenly used, and among them especially derivatives having aromatic groups, for example ortho-, meta- or para-substituted phenylboronic acids, especially 4-formylphenylboronic acid, or the salts or esters of said compounds. Peptide aldehydes, i.e. oligopeptides with reduced C terminus, especially those composed of 2-50 monomers, are also employed for reversible inhibition of proteases in washing and cleaning compositions. Reversible protease inhibitors which are peptides include inter alia ovomucoid. Specific, reversible peptide inhibitors for the

protease subtilisin are also suitable for use in protease-containing compositions, and corresponding fusion proteins composed of protease and inhibitor.

Further enzyme stabilizers are amino alcohols such as mono-, di-, triethanol- and -propanolamine and mixtures thereof, aliphatic carboxylic acids up to C_{12}, such as, for example, succinic acid, other dicarboxylic acids or salts of said acids. End-capped fatty amide alkoxylates are also suitable for this purpose. Certain organic acids employed as builders are able, as disclosed in WO 97/18287, additionally to stabilize a contained enzyme.

Lower aliphatic alcohols, but especially polyols such as, for example, glycerol, ethylene glycol, propylene glycol or sorbitol, are further frequently employed enzyme stabilizers. Diglycerol phosphate also protects from denaturation by physical effects. Calcium and/or magnesium salts are likewise employed, such as, for example, calcium acetate or calcium formate.

Polyamide oligomers or polymeric compounds such as lignin, water-soluble vinyl copolymers or cellulose ethers, acrylic polymers and/or polyamides stabilize the enzyme preparation inter alia against physical effects or pH variations. Polyamine N-oxide-containing polymers act simultaneously as enzyme stabilizers and color transfer inhibitors. Other polymeric stabilizers are linear C_8-C_{18} polyoxyalkylenes. Alkyl polyglycosides can also stabilize the enzymatic components of the composition of the invention and are preferably able additionally to enhance their performance. Crosslinked N-containing compounds preferably perform a dual function as soil release agents and as enzyme stabilizers. Hydrophobic, nonionic polymer has a stabilizing effect, preferably mixed with other

stabilizers, on a cellulase which is present where appropriate and preferably also on the enzyme of the invention.

Reducing agents and antioxidants increase the stability of the enzymes against oxidative decomposition; sulfur-containing reducing agents for example are commonly used for this purpose. Other examples are sodium sulfite and reducing sugars.

Combinations of stabilizers in particular are employed, for example a combination of polyols, boric acid and/or borax, the combination of boric acid or borate, reducing salts and succinic acid or other dicarboxylic acids or the combination of boric acid or borate with polyols or polyamino compounds and with reducing salts. The effect of peptide aldehyde stabilizers is beneficially enhanced by combination with boric acid and/or boric acid derivatives and polyols, and even further by additional use of calcium ions.

Compositions having stabilized enzymic activities represent preferred embodiments of the present invention. Particular preferance is given to those with enzymes stabilized in a plurality of the ways described.

Compositions of the invention are characterized in that they consist of more than one phase, for example in order to release the contained active substances separately from one another in time or space. Possibilities in this connection are phases in different states of aggregation, but especially phases in the same states of aggregation.

Compositions of the invention which are composed of more than one solid component can be produced in a

simple manner by mixing various solid components, especially powders, granules or extrudates with various ingredients and/or different release characteristics, with one another in overall loose form. Solid compositions of the invention which are composed of one or more phases can be produced in a known manner, for example by spray drying or granulation, in which cases the enzymes and possibly other thermally sensitive ingredients such as, for example, bleaches are, where appropriate, added separately later. Compositions of the invention with increased bulk density, in particular in the range from 650 g/l to 950 g/l, are produced preferably in the process disclosed in European patent EP 0 486 592 and having an extrusion step. A further preferred production with the aid of a granulation process is described in European patent EP 0 642 576.

Proteins can be employed for solid compositions for example in dried, granulated, encapsulated or encapsulated and additionally dried form. They can be added separately, i.e. as independent phase, or with other components together in the same phase, with or without compaction. If microencapsulated enzymes are to be processed in solid form, the water can be removed from the aqueous solutions resulting from the processing by methods known in the art, such as spray drying, centrifugation or by resolubilization. The particles obtained in this way normally have a particle size between 50 and 200 µm.

The encapsulated form is appropriate for protecting the enzymes from other constituents such as, for example, bleaches, or for making controlled release possible. Depending on the size of these capsules, a distinction is made between milli-, micro- and nanocapsules, with microcapsules being particularly preferred for enzymes.

Capsules of this type are disclosed for example in the patent applications WO 97/24177 and DE 199 18 267. A further possible encapsulation method is to encapsulate the enzymes suitable for use in washing or cleaning compositions in starch, or a starch derivative, starting from a mixture of the enzyme solution with a solution or suspension of starch or the starch derivative. Such an encapsulation method is described in the German application DE 199 56 382.

It is also possible for at least two solid phases to be present connected together. Thus, one possibility is to provide a solid composition of the invention in the compression or compaction to tablets. Such tablets may be single phase or multiphase. This presentation thus also makes it possible to introduce a solid composition of the invention with two phases. Compositions of the invention in tablet form, which may be single phase or multiphase, single color or multicolor, and/or may consist of one more layers, are produced by preferably mixing all the constituents - where appropriate each of one layer - together in a mixture and compressing the mixture using conventional tablet presses, for example eccentric presses or rotary presses, with compressive forces in the range from about 50 to 100 kN/cm^2, preferably at 60 to 70 kN/cm^2. It may be advantageous, especially in the case of multilayer tablets, for at least one layer to be precompressed. This is preferably carried out with compressive forces between 5 and 20 kN/cm^2, in particular at 10 to 15 kN/cm^2. A tablet produced in this way preferably has a weight of from 10 g to 50 g, in particular from 15 g to 40 g. The three-dimensional shape of the tablets is as desired and may be circular, oval or angular, with intermediate shapes also being possible.

It is particularly advantageous in multiphase compositions for at least one of the phases to comprise an amylase-sensitive material, in particular starch, or be at least partially surrounded or coated thereby. In this way, this phase is mechanically stabilized and/or protected from external influences and, at the same time, attacked by an amylase which is active in the wash liquor, so that release of the ingredients is facilitated.

Likewise preferred compositions of the invention are characterized in that they are overall in liquid, gel or pasty form. The contained proteins, preferably a protein of the invention, are added to such compositions preferably starting from a protein isolation carried out in accordance with the state of the art and preparation in concentrated aqueous or nonaqueous solution, for example in liquid form, for example as solution, suspension or emulsion, but also in gel form or encapsulated or as dry powder. Washing or cleaning compositions of the invention of this type in the form of solutions in conventional solvents are usually produced by simply mixing the ingredients which can be put undiluted or as solution into an automatic mixer.

One embodiment of the present invention are such compositions which are liquid, in gel form or pasty and to which a protein which is essential to the invention and/or one of the other contained proteins and/or one of the other contained ingredients has been added encapsulated, preferably in the form of microcapsules. Particular preference is given among these to those with capsules made of amylase-sensitive material. Such a joint use of amylase-sensitive materials and the amylolytic enzyme which is essential to the invention in a washing or cleaning composition may show

synergistic effects, for example in such a way that the starch-cleaving enzyme assists the cleavage of the microcapsules and thus controls the process of release of the encapsulated ingredients, so that release thereof takes place not during storage and/or not at the start of the cleaning process but only at a particular time. Complex washing and cleaning composition systems with a wide variety of ingredients and a wide variety of capsule types may be based on this mechanism and represent particularly preferred embodiments of the present invention.

There is a comparable effect when the ingredients of the washing or cleaning composition are distributed to at least two different phases, for example two or more solid phases which are connected together of a washing or cleaning composition in tablet form, or different granules within the same composition in powder form. Two-phase or multiphase cleaners for use both in machine dishwashers and in washing compositions are state of the art. The activity of an amylolytic enzyme in a phase which has been activated earlier is a precondition for activation of a later phase if the latter is surrounded by an amylase-sensitive envelope or coating, or the amylase-sensitive material represents an integral constituent of the solid phase, and on partial or complete hydrolysis thereof the relevant phase disintegrates. The use of the enzyme which is essential to the invention for this purpose thus represents a preferred embodiment of the present invention.

The ingredients of washing and cleaning compositions are suitably capable of mutual assistance in their performance. The synergistic use of amylase and color transfer inhibitors to enhance the cleaning performance is disclosed for example in the application

WO 99/63035. It is also known that polymers which can simultaneously be employed as cobuilders, such as alkyl polyglycosides, are able to stabilize and enhance the activity and stability of contained enzymes, for example from the application WO 98/45396. It is thus preferred for a protein or derivative of the invention, preferably having amyloytic activity, to be modified, in particular stabilized and/or enhanced in its contribution to the washing or cleaning performance of the composition, by one of the other constituents listed above. Correspondingly adjusted formulations for compositions of the invention thus represent particularly preferred embodiments of the present invention.

In accordance with the previous statements, methods of the invention for cleaning textiles or hard surfaces can be improved by using in at least one of the method steps a protein or derivative of the invention, preferably having amylolytic activity. The methods are then according to the invention and thus embodiments of the present invention.

A further aspect of the present invention is a method for cleaning textile or hard surfaces, characterized in that a glycosyl hydrolase of invention is used in at least one of the method steps, preferably in a composition of the invention, particularly preferably in machines.

Such methods are preferably characterized in that a composition as described above is employed in at least one of the method steps.

Such methods are particularly preferably characterized in that the protein or derivative of the invention, preferably having amylolytic activity, is employed in machines, for example in usual domestic dishwashers or domestic washing machines, preferentially from 0.01 mg to 400 mg, preferably from 0.02 mg to 200 mg, particularly preferably from 0.02 mg to 100 mg, of the enzyme of the invention in the method step characterized by this enzyme.

The concentrations resulting therefrom are beneficially from 0.0005 to 20 mg per l, preferably 0.005 to 10 mg per l, particularly preferably 0.005 to 8 mg of the amylolytic protein per l of wash liquor. The protein concentration can be determined with the aid of known methods, for example the BCA method (bicinchoninic acid; 2,2'-biquinolyl-4,4'-dicarboxylic acid) or the biuret method (A.G. Gornall, C.S. Bardawill and M.M. David, *J. Biol. Chem.* 177 (1948), pp. 751-766).

In accordance with previous statements, the use of a protein or derivative of the invention, preferably having amylolytic activity, alone or together with at least one other active substance which has cleaning activity or assists the cleaning effect for cleaning textiles or hard surfaces represents an embodiment of the present invention.

This preferably takes place by the use of a composition of the invention.

The composition of the invention or a protein or derivative of the invention, preferably having amylolytic activity, is preferably employed in the ranges of amounts indicated above, so that the concentrations indicated above of the amylolytic protein beneficially result in the wash liquor. This dosage can be undertaken by the manufacturer of the composition or by the final user, depending on the cleaning problem.

A further possibility for using a protein or derivative of the invention, preferably having amylolytic activity, represents the for activation of its own or other phases when it is provided alone or together with at least one other active substance which has cleaning

activity or assists the cleaning effect in a washing or cleaning composition consisting of more than one phase.

A further possibility for using a protein or derivative of the invention, preferably having amylolytic activity, represents the for release of the ingredients from the capsules when it is provided alone or together with at least one other active substance which has cleaning activity or assists the cleaning effect in a washing or cleaning composition having encapsulated ingredients.

A further embodiment represents the use of a protein or derivative of the invention for the treatment of raw materials or intermediate products in textile manufacture, in particular for desizing cotton.

Raw materials and intermediate products of textile manufacture, for example for those based on cotton, are finished with starch during their manufacture and further processing in order to make better processing possible. This method, which is applied to yarns, to intermediate products and to textiles, is called sizing. Amylolytic proteins of the invention are suitable for removing the sizes, i.e. the starch-containing protective layer (desizing).

A further embodiment is represented by methods for liquefaction of starch, in particular for ethanol production, which are characterized in that the protein or derivative of the invention is employed therein.

For liquefaction of starch, starch swollen in water or buffer is incubated with amylolytic enzymes, whereby the polysaccharide is cleaved into smaller constituents, finally predominantly into maltose. Enzymes of the invention are preferably employed for

such a process or a part step thereof if they can be fitted well into a corresponding production process because of their biochemical properties. This may be the case for example if they are to be introduced into a step in addition to other enzymes which require the same reaction conditions. Amylolytic proteins of the invention are particularly preferred when interest centers precisely on the products they themselves produce. Liquefaction of starch may also represent one step in a multistage process for obtaining ethanol or downstream products derived therefrom, for example acetic acid.

A further embodiment is represented by the use of a protein or derivative of the invention for producing linear and/or short-chain oligosaccharides.

Because of their enzymatic activity, amylolytic proteins of the invention produce from starch-like polymers, after a relatively short incubation time, predominantly relatively high molecular weight oligosaccharides such as, for example, maltohexose, maltoheptose or maltooctose. After a longer incubation time, the proportion of lower oligosaccharides such as, for example, maltose or maltotriose increases among the reaction products. If there is particular interest in certain reaction products, corresponding variants of proteins of the invention can be used and/or the reaction conditions can be designed appropriately. This is attractive especially when the interest is not in pure compounds but in mixtures of similar compounds, such as, for example, in the production of solutions, suspensions or gels merely with certain physical properties.

A further embodiment is represented by the use of a protein or derivative of the invention for hydrolyzing cyclodextrins.

Cyclodextrins are α-1,4-glycosidically linked, cyclic oligosaccharides, of which the greatest economic importance is possessed by the α-, β- or γ-cyclodextrins composed of 6, 7 or 8 glucose monomers (or cyclohexa-, -hepta- or -octa-amyloses). They are able to form inclusion compounds with hydrophobic guest molecules such as, for example, fragrances, flavorings or pharmaceutical active substances, from which the guest molecules can be released again as required. Such inclusion compounds are important, depending on the area of use of the ingredients, for example for producing foodstuffs, for pharmacy or for cosmetics, for example in corresponding products for the final consumer. The release of ingredients from cyclodextrins thus represents a possible use of proteins of the invention.

A further embodiment is represented by the use of a protein or derivative of the invention for releasing low molecular weight compounds from polysaccharide carriers or cyclodextrins.

Because of their enzymatic activity, amylolytic proteins of the invention are also able to release low molecular weight compounds from other α-1,4-glycosidically linked polysaccharides. This may, as for the cyclodextrins, take place at the molecular level and in larger systems such as, for example, ingredients encapsulated in the form of microcapsules. For example, starch is a material established in the art for encapsulating compounds such as, for example, enzymes, which are to be introduced in defined amounts into reaction mixtures, during storage. The controlled

release process from such capsules can be assisted by amylolytic enzymes of the invention.

A further aspect is represented by the use of a protein or derivative of the invention for producing food products and/or food ingredients.

The use of a protein or derivative of the invention for producing animal feed and/or animal feed ingredients likewise represents a further aspect of the invention.

Wherever starch or carbohydrates derived from starch play a role as ingredient of food products or animal feeds, an amylolytic activity can be used to produce these articles. It increases the proportion of mono- or oligomers compared with the polymeric sugar, which may benefit for example the taste, the digestability or the consistency of the food product. This may be necessary for the production of certain animal feeds, but also for example in the production of fruit juices, wine or other food products if the proportion of polymeric sugars is to be reduced and that of sweet and/or more readily soluble sugars is to be increased. The possible use for liquefaction of starch and/or ethanol production mentioned above can be regarded as industrial variant of this principle.

In addition, amylases also counteract the loss of taste, known as staling, of bakery products (antistaling effect). It is suitable for this purpose to add them to the dough before baking. Preferred embodiments of the present invention are therefore those in which proteins of the invention are used for manufacturing bakery products.

A further aspect is represented by the use of a protein or derivative of the invention for breaking down starch-containing adhesive bonds.

Likewise, temporary bonding methods which are characterized in that a protein or derivative of the invention is employed therein represent a further aspect of the invention.

Besides other natural products, starch has also been used for centuries as binder in paper manufacturing and the gluing of papers and boards. This relates for example to prints and books. Over a long period of time, such papers may with unfavorable effects such as, for example, moisture become wrinkled or break, which may lead to complete destruction thereof. In the restoration of such papers and boards it may be necessary to break down the adhesive layers and this can be considerably facilitated through use of an amylolytic protein of the invention.

Vegetable polymers such as starch or cellulose and water-soluble derivatives thereof are used inter alia as adhesives or pastes. For this purpose, they must first swell in water and, after application to the item for gluing, dry, whereby the latter is fixed on the substrate. The enzyme of the invention can be added to such an aqueous suspension in order to influence the adhesive properties of the resulting paste. However, instead of or in addition to this function, it can also be added to the paste in order to remain inactively on the item for gluing after drying for a long time, for example some years. Deliberate alteration of the environmental conditions, for example by humidification, can then be used at a later time to activate the enzyme and thus bring about breakdown of the paste. In this way, the glued item can more easily

be detached again from the substrate. In this process, the enzyme of the invention acts, because of its amylolytic activity, as separating agent in a temporary bonding process or as so-called "switch" for detaching the glued item.

The following examples illustrate the invention without, however, restricting it thereto:

Example 1: Isolation of metagenomic DNA from microbial habitats

Obtaining soil samples

The soil samples were obtained from various locations in Germany (inter alia) and initially processed mechanically while dry (screening through 4 mm and 1 mm sieves).

DNA isolation from soil

For DNA preparation, 5 g of soil were slurried by addition of 13.5 ml of extraction buffer (100 mM Tris-HCl, pH 8.0; 100 mM EDTA, pH 8.0; 100 mM sodium phosphate, pH 8.0; 1.5 M NaCl; 1% hexadecyltrimethyl-ammonium bromide). The suspension was shock-frozen 3 times with addition of liquid nitrogen (N_2), ground in a mortar and boiled in a microwave. After being transferred into a 50 ml screw-cap vessel and adding 1.5 ml of lysozyme solution (50 mg/ml), the suspension was incubated at 37°C for 30 min and inverted at intervals of 5 min. Addition of 200 µl of a proteinase K solution (10 mg/ml) was followed by incubation at 37°C for 30 minutes with inversion as before. This was followed by treatment with SDS (by addition of 3 ml of a 10% solution) at 65°C for two hours (inversion of the vessel every 20 min). After centrifugation (6000×g, 10 min) and slurrying with 4.5 ml of extraction buffer plus 1 ml of 10% SDS, the sediment was again extracted and incubated at 65°C for

10 min and centrifuged as before. The combined supernatants from the two centrifugations were mixed with 1 volume of phenol/chloroform (1:1) and centrifuged as before. The DNA was precipitated from the upper aqueous phase by addition of 0.6 volume of isopropanol and incubation at room temperature for one hour and centrifugation at 16 000xg for 20 minutes. The DNA pellet was washed with 70% ethanol and dried in air and then dissolved in 200 µl of TE buffer (100 mM Tris-HCl pH 8.0; 1 mM EDTA pH 8.0).

The high molecular weight genomic DNA was finally separated from dissolved inhibitory humic acids by preparative gel electrophoresis (0.7% agarose) and subsequent extraction (QIAex II Gel Extraction Kit; from Qiagen, Hilden).

Example 2: Setting up the expression gene libraries and screening for amylolytic activity

Expression cloning in the heterologous host organism *Escherischia coli* was chosen for the cloning and isolation of complete α-amylase genes from metagenome. Since heterologous recognition of the promoters of unknown donor organisms cannot be assumed in the host organism *E. coli*, use was made of an *E. coli* promoter which is inducible in a known manner with IPTG (isopropyl thiogalactoside), the β-galactosidase promoter of the lac operon (*lac* promoter) on the pUC18 plasmid vector (GenBank NCBI/NIH, Bethesda, Acc.-No. L08752; Fig. 1). It is thus possible in host strains with a *lac*Iq genotype (for example JM109) to induce expression of the genes under the control of this promoter by adding IPTG.

The *E. coli* strains JM 109, DH 10B and DH 12S proved to be suitable systems for cloning the metagenome-derived α-amylases and activity-dependent detection thereof.

This is because, although they have the periplasmic enzyme encoded by *malS* (Freundlieb, S., Boos, W. (1986): "Alpha-amylase of Escherichia coli, mapping and cloning of the structural gene, malS, and identification of its product as a periplasmic protein"; *J. Biol. Chem.* 261 (6), pp. 2946-2953), they showed negligible release of amylase under the experimental conditions.

Purified metagenomic DNA from soil was partially cleaved with restriction enzymes, preferably *Sau*3AI, and the fragment size range from preferably 8 to 12 kb was fractionated and isolated by gel electrophoresis. These DNA fragments were ligated into suitable vectors, preferably pUC18 plasmid vector linearized with the restriction enzyme *Bam*HI (Fig. 1).

To establish the optimal restriction parameters for the preparative partial digestion of metagenomic DNA, firstly restriction kinetics were carried out using about 4 µg of DNA and restriction enzyme (*Sau*3AI) in concentrations of from 0.3 to 0.4 U per µg of DNA in 1 × buffer where appropriate with bovine serum album (BSA) in accordance with the manufacturer's instructions, in a total volume of 20 µl. The mixtures were for this purpose initially equilibrated at 37°C without enzyme. Then the respective reaction was started by adding restriction endonuclease. At fixed intervals between 0 and 10 min, in each case 2 µl of reaction mixture were added to 1 × stop buffer (6X: 10 mM Tris, pH 7.0; 20% glycerol; 0.1% SDS) on ice and analyzed on a 0.7% agarose gel. In this way, the optimal restriction time for a partial digestion was established individually for each DNA preparation.

The preparative partial digestion took place with the absolutely identical procedure as for the restriction kinetics in 15 to 20 individual mixtures. After the reaction was stopped by adding 1 × stop buffer, the

mixture was fractionated by electrophoresis on a 0.7% agarose gel, the region of the gel with DNA 8-12 kb in size was cut out and it was isolated by electroelution in dialysis tubes at 4°C for two hours. The DNA was precipitated with 1/10 volume of 3 M sodium acetate and 2.5 times the volume of ethanol, and taken up in a smaller volume and was, for further removal of smaller fragments, subjected to a second gel electrophoresis with subsequent electroelution and renewed concentration.

Ligation with the plasmid vector pUC18 took place in a total volume of 20 µl using 100 ng of *Bam*HI-linearized pUC18 vector, which had been dephosphorylated with CIAP (alkaline phosphatase from calf thymus) in accordance with the manufacturer's instructions, and 800 ng of partially cleaved genomic DNA, and an appropriate amount of ligase (600 NEB units in this case) in 1 × ligase buffer at 16°C overnight.

Transformation of competent *E. coli* DH12S cells (Gibco Life Technologies, Karlsruhe) took place by electrotransformation. For this purpose, 1 µl of ligation mixture and 25 µl of cells were mixed, incubated in an electroporation cuvette on ice for 1 min and treated in the electroporator (BTX® ECM630, Genetronics Inc. San Diego, USA) in accordance with the manufacturer's instructions. After immediate transfer into 1 ml of SOC medium (2% Bacto tryptone; 0.5% yeast extract; 10 mM NaCl; 2.5 mM KCl; pH 7.0, adjusted with NaOH; autoclaved; supplemented with 10 mM $MgSO_4$ and $MgCl_2$, and 20 mM D(+)glucose), a recovery period of 1 h at 37°C followed before plating.

To examine the quality of the gene library, the number of primary transformants generated in total, and the number of insert-harboring clones was determined by

blue/white selection in a test plating. For this purpose, 1 and 10 µl portions of the ligation mixture were plated out on LB medium with ampicillin, IPTG, X-Gal (as described above) and incubated at 37°C overnight. To confirm the actually cloned insert sizes, the plasmids were isolated from at least 10 white colonies of the test plating, and a suitable restriction digestion was carried out with subsequent size analysis by gel electrophoresis.

In the screening of the gene libraries, the ability of individual recombinant *E. coli* clones in metagenomic libraries to degrade starch was determined. For this purpose, LB agar with 1% soluble starch (from Merck, Darmstadt, Order No. #1252) on which clones able to degrade starch can be identified by zones of degradation was used. Initial storage of the plates at 4°C for two to four weeks until cloudy was necessary for this, storage degradation of the starch was visible as clearing around the active colonies on the plates. For use, IPTG (100 mM) was added to the plates to induce the promoter and ampicillin (100 µg/ml) was added to apply the selection pressure to the transformants.

Appropriate for the titer of the library generated in each case, a defined volume of the transformation mixture was plated out uniformly by means of glass beads with about 10 000 colonies per plate (14 cm diameter) (primary plating). After incubation at 37°C for 16 hours, the plates were incubated at 28°C for a further 24-48 h for amylase expression and release. The incubation at 28°C was usually indispensible for visible degradation of starch. It is possible that improved protein folding and a permeabilization of the outer cell membrane are involved in this (Stathopoulos, C., Georgiou, G., Earhart, C.F. (1996):

"Characterization of *Escherichia coli* expressing an Lpp'*Omp*A(46-159)*Pho*A fusion protein localized in the outer membrane"; *Appl. Microbiol. Biotechnol.*, 45 (1-2), 112-119). Thus, release of the α-amylase produced in each case was associated therewith, and it was unnecessary additionally to carry out cell lysis to detect unexported cytoplasmic amylases. After isolation of the colonies from the zone regions of the primary plating by renewed (secondary) plating on the same starch plates it was possible to select amylase-producing single colonies on the basis of renewed zone formation. For photodocumentation purposes after securing the clones, staining of the starch plates with 50% lugol solution (from Merck) proved suitable for unambiguous visualization of starch-free regions (degradation zones).

The plasmid DNA of active clones obtained after minipreparation (kit from Qiagen, Hilden, Germany, used in accordance with the manufacturer's instructions) was examined for the size of the insert in a restriction analysis with *Eco* RI or *Sac*I / *Hin*d III and then sequenced. In this case, the insert-flanking M13 primers (M13 forward: 5'-GTAAAACGACGGCCAG-3'; M13 reverse: 5'-CAGGAAACAGCTATGAC-3') were initially used in order finally to obtain the complete sequence by so-called primer walking as known in the art.

Example 3: Characterization of the metagenomic pool by PCR

To estimate the content of gene sequences for glycosyl hydrolases in metagenomic DNA from soil, a DNA amplification was carried out by the polymerase chain reaction (PCR). This was carried out using HotStar *Taq* polymerase (from Qiagen, Hilden) and the glycosylhydrolase-specific primers (Table 1). For this

purpose, 0.1 µl portions of the metagenomic DNA isolated by the above method were employed in the presence of 200 µM dNTPs (in each case dATP, dTTP, dCTP, dGTP), 20 pmol of each primer of a pair (forward-/reverse), 2.5 U HotStar *Taq*-polymerase (Qiagen, Hilden) and 1 × PCR buffer in a total volume of 50 µl. Fragments of sizes 150 to 300 bp were amplified in the following cycles: 1 cycle at 95°C for 15 min, 35 cycles at 95°C for 30 s each, 30 s at 57°C (GEX036/038) or 60°C (other primer pairs) and 30 s at 72°C; 1 cycle at 72°C for 7 min.

Using the primer pairs GEX024/026 and GEX023/025, in each case unique PCR products were amplified (respectively 300 bp and 280 bp; Fig. 4). Amplification in these cases was frequently possible only after 1:10 dilution of the metagenomic DNA. This was presumably due to inhibitory components in the DNA preparations which became inactive by dilution below a critical threshold. With primer pair GEX036/038, PCR fragments (150 and 300 bp) were obtained only after reamplification using 4 µl of the original reaction.

All PCR fragments firstly underwent intermediate cloning using the TOPO TA kit (Invitrogen, Groningen NL) in accordance with the manufacturer's instructions into the pCR2.1-TOPO® vector for the sequencing. The ligation products were used for transformation into *E.coli*-TOP® 10F' (Invitrogen, Groningen NL). After blue/white selection of recombinant clones on LB medium with ampicillin (100 µg/ml), IPTG (100 mM) and X-Gal (40 mg/ml), the plasmid DNA of white clones was isolated after minipreparation and examined in a restriction analysis with *Eco*RI for the content of insert DNA. In this case, differences in the insert sizes are evident, especially in the clones from the PCR with GEX024/026 and 036/038 (Fig. 4), and reflect

the molecular diversity of the amplified glycosyl hydrolase sequences in the metagenome.

Sequence analysis of plasmids with insert DNA took place routinely on an ABI PRISM® 310 (from Perkin Elmer, Weiterstadt) in accordance with the manufacturer's instructions using AmpliTaq-FS-Big Dye® Terminator kit (from Perkin Elmer, Weiterstadt) and employing 200-500 ng of plasmid DNA, 10 pmol of primers (TopoF: 5'-GCTCGGATCCACTAGTAACG-3' and TopoR: 5'-CTCTAGATGCATGCTCGAG-3'), 4 µl of premix in a total volume of 20 µl.

The resulting DNA sequences were conceptionally transcribed into an amino acid sequence, compared via the BlastX, BlastN and BlastP algorithms (Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol 1990 Oct 5; 215(3): 403-10) using the FASTA program (Pearson WR, Lipman DJ. (1988) Improved tools for biological sequence comparison. Proc Natl Acad Sci USA Apr; 85(8): 2444-8) with the entries present in the sequence database GenBank (Rel 122.0) and identified in this way as partial sequences of α-amylases and other glycosyl hydrolases (see Table 2).

Parts of the constant primer regions which were unclear owing to a late onset of sequencing reading definition, or unexpectedly differed from the synthetically presented sequence, were corrected only at the amino acid sequence level and thus entered in the corresponding sequence lists. It is therefore possible for DNA sequences in these constant primer regions to differ from the conceptionally translated amino acid sequences.

The amino acid sequences are listed in the sequence listing under numbers SEQ ID No. 7 to 56 and SEQ ID No. 107 to 205 and included in the consensus sequences A to D.

Example 4: Sequence analysis of the amylase genes found

A clone HA 11 which indicated amylase activity by zone formation in the expression screening described above was completely sequenced. Sequence analysis revealed the existence of two open reading frames, one of which, ORF1, was identifiable as amylase gene by homology comparisons. The derived protein sequence is shown in Seq_ID 1. The result of a Fasta3 search revealed an amylase from Bacillus sp. as the most similar enzyme with an AA homology of 72.3%. This amylase showed in the carboxy-terminal region of the protein sequence extensive homologies with an experimentally verified new type of starch-binding domain in the most similar known sequence (Sumitani, J.; Tottori, T., Kawaguchi, T. and Arai, M.; Biochem J 350, 477-484, (2000)).
A second reading frame, ORF 2, with a homology of 46%, based on the complete available gene sequence, to the most similar gene (bifunctional amylase-dextrinase) from Streptomyces coelicolor A3 (Redenbach, M; Kieser, H.M.; Depanaite, D.; Eichner, A, Cullum J; Kinashi, H. and Hopwood, D.A., Mol. Microbiol 21, 77-96 (1996)) was only incompletely present in the genomic clone. A stop codon at the 3′ end was missing, so that the actual gene extends beyond the known cloned sequence.

The amylase gene from HA 11 was amplified from the isolated genomic clone by PCR and use of suitable primer oligonucleotides and was cloned in the *E. coli-Streptomyces* shuttle vector pEX601. After expression in the heterologous host *Streptomyces lividans* TK 24, the

enzyme obtained in the culture supernatant underwent biochemical characterization.

The amylase domain of the bifunctional ORF 2 was separately cloned in analogy to the amylase and was expressed in *S. lividans* TK 24. Analysis of the supernatants revealed unambiguously an amylase activity measurable by the DNSA assay. The part, cloned and expressed in this way, of the bifunctional ORF 2 is called HA 13, and the protein sequence is indicated in Seq_ID 3.

Example 5: Biochemical characterization of the expressed genes

In view of the diverse areas of application of α-amylases, the enzymatic properties which may be the basis for application-based selection of enzymes were characterized. The enzymes can for this purpose be prepared by cloning and heterologous expression of the gene sequences in accordance with Example 4. The amylolytic activity was determined by means of a modified dinitrosalicylic acid assay (DNSA assay; Hofstetter, F. & E. Borel (1951); Helv. Chim. Acta, 34, 2133-2139)). This entails the hydrolysis of a complex starch substrate being determined on the basis of the increase in reducing ends of the polysaccharide, with the absorption at 540 nm of the unreacted DNSA reagent serving as measure of the hydrolytic activity.

To carry out the assay, 25 µl portions of substrate solution (1% soluble starch Merck analytical grade, in 180 mM Tris-maleate buffer, pH 8.6, or in 180 mM of the appropriate assay buffer) were introduced into the sample and blank wells of a 96-well plate for PCR thermocycler (0.2 ml "thin-wall plate" #3416, Molecular BioProducts MßP, San Diego, USA) and preincubated at

50°C, or the particular assay temperature, in a thermocycler block for 5 min. After addition of 20 µl of enzyme solution to each well, substrate conversion took place at 50°C or the particular assay temperature for 15 min. After addition of 65 µl of DNSA reagent (8.8 g of dinitrosalicylic acid, 250 g of potassium sodium tartrate, 6.3 g of sodium disulfite, 334 ml of 4.5% (w/v) NaOH, 915 ml of H_2O) to the sample and blank wells, and addition of 20 µl of enzyme solution to the blank wells, the assay plate was immediately transferred into a thermoblock preheated to 100°C, and incubated at 100°C for 20 min. The samples were measured by transferring 60 µl portions of the assay mixture into 200 µl of H_2O which had been introduced into a 96-well measuring plate (PS-Microplate, 96 well #655101, Greiner) subsequently determining the absorption at 540 nm using a microtiter plate spectral photometer (Spectramax 190, Molecular Devices, Sunnivale USA) with H_2O as reference. Three measurements were carried out in each case; evaluation took place by subtracting the average of the 3 blank wells from the average of the 3 sample wells.

Temperature profile

The temperature profile was determined by carrying out the conversion by the procedure described above at various temperatures with 100 mM Tris-maleate, pH 8.6. The alpha-amylase HA11 (SEQ ID No. 1) was found to have maximum activity at 50.4°C. Temperatures above 60° led to losses of activity of this enzyme (see Table 3).
The maximum for the amylase domain HA13 was found to be 55.5°C (see Table 4).

Temperature stability of the amylase domain HA13

After preincubation in the temperature gradient from 50°C to 69.7°C for 15 min, substrate conversion and the DNSA assay took place as described above at 50°C in Tris-HCl, pH 8.6. Table 5 shows that the stability declines markedly above 60°C.

Stability to pH variations

To determine the activity of the α-amylase amylases under various pH conditions, the starch substrate solution was made up in the pH range from 4 to 12 using the buffer suitable for the particular pH. The buffer concentration was adjusted in each case to 180 mM so that a concentration of 100 mM resulted after dilution under assay conditions. The amylolytic activity at the respective pH values was determined as indicated above at 50°C. The respective buffers and the relative activity values for the two amylases (HA 11 and HA 13) are to be found in Table 6. It is evident that both amylases exhibit the maximum in the range pH 5 to 6.5, and that amylase HA 13 is distinguished by a broad maximum and still has considerable remaining activity even at 12.

Stability to surfactants:

The surfactant stability was determined by adjusting a final concentration of SDS between 0 and 1%. The substrate conversion took place with 100 mM Tris-HCl pH 8.6 at 50°C for 15 min (Table 7 = HA11; Tab. 8=HA13).

Effect of bleaches

The effect of bleaches was determined from the action of up to 0.7% H_2O_2 in 100 mM glycine-NaOH pH 9.5. Preincubation in the presence of H_2O_2 at room temperature under appropriate conditions was followed by further incubation at 50°C for 15 min. The substrate conversion was then measured in the DNSA assay. (Table 9: HA 11; Table 10: HA13)

Effect of complexing agents:

The effect of complexing agents was investigated by adjusting a concentration of 2 mM $CaCl_2$ or 1 mM EDTA in 100 mM Tris-HCl pH 8.6 at 50°C for 15 min. (Table 11 = HA11; Table 12 = HA13)

Example 6: Investigation of the washing performance

The washing performance of prepared amylase sample was determined in miniwash tests using standardized test soilings. These consisted of starch immobilized on fabric, and a color indicator (EMPA 162, starch on blended polyester/cotton fabric and CFT PCS 26, cornstarch on cotton)

For this purpose, 19.6 cm^2 fabric samples were incubated in 20 ml of wash liquor with and without added bleach. The amount of enzyme employed in each case was that able to generate from soluble starch, at 37°C and pH 6.5, per minute an amount of reducing sugars equivalent to 12 µmol of glucose. The fabric samples were incubated at 40°C and 80 rpm for 90 minutes. They were then rinsed with H_2O, dried and the color (L value) was determined using a Minolta surface photometer. The lightening as the measure of

the detachment of starch and thus the washing performance was then determined by forming the difference from the untreated fabric sample. The results are shown in Table 13.

It is evident from this that the washing performance of HA 11 (SEQ ID No. 1) on use of the same enzymic activity was greatest in each case. Termamyl® was weaker, but in each case better than BAN®. These enzymes showed a decrease in the presence of a bleach-containing formulation. HA13 (SEQ ID No. 3) by contrast proved to be relatively insensitive to bleach, so that the washing performance in the bleach-free formulation was below that of the other enzymes but was comparable with Termamyl in the bleach-containing one.

List of figures:

Figure 1: Diagrammatic representation of the plasmid vector pUC18 (GenBank Acc. L08752) used for the expression gene libraries. The vector was linearized with *Bam*HI for the uptake of *Sau*3AI-digested metagenomic DNA.
ORI: original of replication; *lac*I: gene for *lac* repressor; *lac*Z-alpha: gene for alpha peptide of beta-galactosidase; ampicillinR: beta-lactamase.

Figure 2: Analysis of *Sau*3AI restriction kinetics of metagenomic DNA from soil to establish the optimal restriction time at a specified enzyme concentration. From a complete mixture of 20 µl with 0.3 U of *Sau*3A1 per µg of DNA, 2 µl portions were transferred after 0, 1, 2, 3, 4, 5, 6, 8 and 10 min to 1× stop buffer and fractionated on a 0.7% agarose gel (lanes 1-9 from left to right). Markers (M): 1 kb DNA ladder (in kb from top to bottom: 10; 8; 6; 5; 4; 3.5; 3; 2.5; 2; 1.5; 1. Lane 7 representative of restriction for 5 min here shows optimal partial digestion in the size range 8-12 kb.

Figure 3: Activity assay to detect amylase in a metagenomic expression gene library. Lugol-stained LB agar plates with 1% soluble starch. Amylase-expressing recombinant *E. coli* clones exhibit a zone of starch degradation around the colonies, which appears pale after lugol staining.

A) Example of a "parent plate" (primary plating) of a zone-forming clone from the complete plating of a plasmid expression gene library after transformation of *E. coli*.

B) Isolated colonies after replating (secondary plating) from a starch zone region of a parent plate. Individual colonies with and without zone are evident. The plasmids from colonies with zone were analyzed and their insert DNA was sequenced.

Left: lugol-stained plate with pale zones of degradation

Right: clarification zones of active clones on cloudy starch without Lugol stain

Figure 4: Size analysis of the PCR reaction products with primers GEX023/025, GEX024/026 and GEX036/038 on soil metagenomic DNA.

Example of the size analysis of the PCR reaction products from soil S196 (A) and soil S131 (C). The PCR was carried out with the amylase-specific primers GEX024/026 (A, lane 1), GEX023/025 (A, lane 2) and GEX036/038 (C, lane 1), and the reaction products were fractionated (1/10 of the reaction volume) in a 2% agarose gel. A PCR with GEX024/026 on genomic DNA from *Streptomyces sp.* strain B124 (C, lane 2) took place in parallel as positive control. See text (above) for PCR conditions.

Markers (M): 100 bp DNA ladder (in bp from top to bottom: 3000, 2000, 1500, 1200, 1031, 900, 800, 700, 600, 500, 400, 300, 200, 100).
A 300 bp band is amplified with the primer pair GEX024/026, and a 280 bp fragment with the primer pair GEX023/025, whereas 2 products 300 and 150 bp in size are amplified with GEX036/038 only after reamplification of the original PCR mixture. All the PCR products were cloned, and the DNA of up to 18 clones was fractionated after excision of the inserts by EcoRI restriction on a 2% agarose gel (B). The clones for the PCR product GEX023/025 show insert DNA sizes of about 280 bp throughout (B, left), the fragment sizes for the clones from the PCR of GEX024/026 vary between 300 and 350 bp (B, right). The clones from the PCR of GEX036/038 show fragments around 300 bp and 150 bp (D).

Figure 5: Consensus sequence -A- and amino acid exchanges of 50 glycosyl hydrolases from soil microorganisms. The consensus sequence is based on an alignment with Clustal X Version 1.64b (standard settings). Amino acid positions 1-8 and 116-123 are in part specified by the PCR primer sequences (GEX024/026).

Figure 6: Consensus sequence -D- (subsequence space to A) and amino acid exchanges of 42 amylases from soil microorganisms. The consensus sequence is based on an alignment with Clustal X Version 1.64b (standard settings). Amino acid positions 1-8 and 114-121 are in part specified by the PCR primer sequences (GEX024/026).

Figure 7: Consensus sequence -B- and amino acid exchanges of 99 glycosyl hydrolases from soil microorganisms. The consensus sequence is based on an

alignment with Clustal X Version 1.64b (standard settings). Amino acid positions 1-12 and 102-109 are in part specified by the PCR primer sequences (GEX023/210/211/036 and GEX25/038).

Figure 8: Consensus sequence -C- (subsequence space to B) and amino acid exchanges of 10 amylases from soil microorganisms. The consensus sequence is based on an alignment with Clustal X Version 1.64b (standard settings). Amino acid positions 1-12 and 72-78 are in part specified by the PCR primer sequences (GEX023/210/211 and GEX25).

Figure 9: Diagrammatic representation of the plasmid shuttle vector pEX601 used for the expression of the metagenomic amylases in *Streptomyces lividans* TK24.
The vector was produced by inserting the ermE/MCS/terminator cassette (BgIII/EcoRI) from pWHM680pro into the BamHI/EcoRI-digested vector pWHM601. The BamHI recognition sequence present in the multicloning site was used to clone the amylase genes. Both pWHM601 and pWHM680pro originate from the laboratory of Dr C.R. Hutchinson (University of Wisconsin, pers. communication).
Expression of inserted genes takes place starting from the constitutive *ermE* promoter.
The indicated sizes of the vector and of the elements contained therein are estimates. SCP2* replicon origin of replication in streptomyces; *ermE*, *ermE* up promoter of *S. erythrea*; apramycinR apramycin resistance gene.
Expression of the amylase genes in *Streptomyces lividans* can also be achieved with other suitable shuttle vectors such as, for example, pAX5a (Faß S.H., Engels, J.W. 1996; Influence of Specific Signal Peptide Mutations on the Expression and Secretion of the alpha-Amylase Inhibitor Tendamistat in *Streptomyces lividans*. J. Biol. Chem. 271, Number 25, 15244-15252).

Table 1: PCR primers for amplification of partial sequences of metagenomic DNA sequences coding for glycosyl hydrolases.

Symbols for degenerate base positions (mixtures of different nucleotides): R=AG; Y=CT; M=AC; K=GT; W=AT; S=CG; B=CGT; D=AGT; H=ACT; V=ACG; N=ACGT

Name	Sequence (5'-3')	Orientation
GEX 023	GGTCTACGCCGACGTCGTSWWCAACCA	forward
GEX 024	CGTCGACGGCTTCCGSATCGACRC	forward
GEX 025	GGCGTCGATGCGGAASCCGTC	reverse
GEX 026	GCTCGGTGTCGTGGTTGTCSACGWA	reverse
GEX 036	GTACGCCGACGCCGTNWTHAAYCA	forward
GEX 038	GGCGGCGTCGATCCKRAANCCRTC	reverse
GEX 210	CGACGTGGTGTTCAACCAYYTNGGNCC	forward
GEX 211	GCCGACGTGGTGTTCAAYCAYYTNGG	forward

Table 2: List of the 149 single sequences with the identities to their closest relations in GenBank (NCBI; Release 121.0); determined using the FASTA program on Feb. 2, 2001.

Primer	SEQ ID No. as in Seq. listing	Closest database hit	Gene/product	Identity (%)	Organism
23/25	107	AB003697	oligo-1,6-glucosidase	74,5	Bacillus flavocaldarius
23/25	108	AB015615	isoamylase	65	Bacillus flavocaldarius
23/25	109	AB015615	isoamylase	61,7	Orysa sativum
23/25	110	AB015615	isoamylase	66,2	Orysa sativum
23/25	111	AB015615	isoamylase	64,2	Orysa sativum
23/25	112	AB015615	isoamylase	68,8	Orysa sativum
23/25	113	AB031392	glycogen debranching enzyme	77,8	Arthrobacter sp.
23/25	114	AB031392	glycogen debranching enzyme	63	Arthrobacter sp.
23/25	115	AB031392	glycogen debranching enzyme	80,2	Arthrobacter sp.

23/25	116	AB031392	glycogen debranching enzyme	77,8	Arthrobacter sp.
23/25	117	AB031392	glycogen debranching enzyme	79	Arthrobacter sp.
23/25	118	AB031392	glycogen debranching enzyme	77,8	Arthrobacter sp.
23/25	119	AB031392	glycogen debranching enzyme	81,5	Arthrobacter sp.
23/25	120	AB031392	glycogen debranching enzyme	76,5	Arthrobacter sp.
23/25	121	AB031392	glycogen debranching enzyme	86,4	Arthrobacter sp.
23/25	122	AB031392	glycogen debranching enzyme	86,8	Arthrobacter sp.
23/25	123	AB031392	glycogen debranching enzyme	80,2	Arthrobacter sp.
23/25	124	AE001888	glycogen operon protein GlgX	76,5	Deinococcus radiodurans
23/25	125	AE001888	glycogen operon protein GlgX	37,8	Deinococcus radiodurans
23/25	126	AE001888	glycogen operon protein GlgX	72,8	Deinococcus radiodurans
23/25	127	AE001888	glycogen operon protein GlgX	77,8	Deinococcus radiodurans
23/25	128	AE001888	glycogen operon protein GlgX	69,1	Deinococcus radiodurans
23/25	129	AE001888	glycogen operon protein GlgX	76,5	Deinococcus radiodurans
23/25	130	AE001888	glycogen operon protein GlgX	76,5	Deinococcus radiodurans
23/25	131	AE001888	glycogen operon protein GlgX	71,6	Deinococcus radiodurans
23/25	132	AE001888	glycogen operon protein GlgX	77,8	Deinococcus radioduransv
23/25	133	AE001888	glycogen operon protein GlgX	77,8	Deinococcus radiodurans
23/25	134	AE001905	maltooligosyltrehalose trehalohydrolase	45,6	Deinococcus radiodurans
23/25	135	AE001905	maltooligosyltrehalose trehalohydrolase	50,7	Deinococcus radiodurans
23/25	136	AE004643	probable glycosyl hydrolase	38,9	Pseudomonas aeruginosa
23/25	137	AE004643	probable glycosyl hydrolase	53,8	Pseudomonas aeruginosa
23/25	138	AF002109	putative isoamylase	71,2	Arabidopsis thaliana
23/25	139	AF002109	putative isoamylase	62	Arabidopsis thaliana
23/25	140	AF002109	putative isoamylase	60,8	Arabidopsis thaliana
23/25	141	AF002109	putative isoamylase	62	Arabidopsis thaliana

23/25	142	AF002109	putative isoamylase	64,6	Arabidopsis thaliana
23/25	143	AF002109	putative isoamylase	65,4	Arabidopsis thaliana
23/25	144	AF002109	putative isoamylase	60,8	Arabidopsis thaliana
23/25	145	AF002109	putative isoamylase	62	Arabidopsis thaliana
23/25	146	AF142590	isoamylase 1	66,7	Triticum aestivum
23/25	147	AF142590	isoamylase 1	53,2	Triticum aestivum
23/25	148	AF201335	maltooligosyl trehalose trehalohydrolase	47,1	Sulfolobus shibatae
23/25	149	AJ001206	putative glycogen debranching enzyme	82,7	Streptomyces coelicolor
23/25	150	AJ133789	cyclomaltodextrinase	91	Alicyclobacillus acidocaldarius
23/25	151	AJ133789	cyclomaltodextrinase	93,3	Alicyclobacillus acidocaldarius
23/25	152	AJ133789	cyclomaltodextrinase	92,1	Alicyclobacillus acidocaldarius
23/25	153	AJ291603	glycogen debranching enzyme	70,4	Rhizobium tropici
23/25	154	AJ291603	glycogen debranching enzyme	44,4	Rhizobium tropici
23/25	155	AJ291603	glycogen debranching enzyme	41,9	Rhizobium tropici
23/25	156	AL157916	putative glycosyl hydrolase	60	Streptomyces coelicolor
23/25	157	AL355752	secreted alpha-amylase/dextrinase	93,5	Streptomyces coelicolor
23/25	158	AL356932	glycogen debranching enzyme	79	Streptomyces coelicolor
23/25	159	AL356932	putative glycogen debranching enzyme	95,1	Streptomyces coelicolor
23/25	160	AL512975	glycogen operon protein GlgX	71,6	Sulfolobus solfataricus
23/25	161	AL512975	glycogen operon protein GlgX	75,3	Sulfolobus solfataricus
23/25	162	AL512975	alpha-amylase	52,9	Sulfolobus solfataricus
23/25	163	AL512975	glycogen operon protein GlgX	68,8	Sulfolobus solfataricus
23/25	164	AL512975	alpha-amylase	50	Sulfolobus solfataricus
23/25	165	AL512975	glycogen operon protein GlgX	75,3	Sulfolobus solfataricus

23/25	166	AL512975	glycogen operon protein GlgX	70,4	Sulfolobus solfataricus
23/25	167	AL512975	glycogen operon protein GlgX	70,4	Sulfolobus solfataricus
23/25	168	AL512975	glycogen operon protein GlgX	72,8	Sulfolobus solfataricus
23/25	169	AL512975	alpha-amylase	62,7	Sulfolobus solfataricus
23/25	170	AL512975	alpha-amylase	50	Sulfolobus solfataricus
23/25	171	AL512975	alpha-amylase	50	Sulfolobus solfataricus
23/25	172	AL512975	glycogen operon protein GlgX	69,1	Sulfolobus solfataricus
23/25	173	AL512975	glycogen operon protein GlgX	75,3	Sulfolobus solfataricus
23/25	174	AL512975	glycogen operon protein GlgX	56,8	Sulfolobus solfataricus
23/25	175	AL589708	glycogen debranching enzyme	74,1	Streptomyces coelicolor
23/25	176	AX063741	putative ORF	72	Corynebacterium glutamicum
23/25	177	D63343	maltooligosyl trehalose trehalohydrolase	62,9	Arthrobacter sp.
23/25	178	D63343	maltooligosyl trehalose trehalohydrolase	62,7	Arthrobacter sp.
23/25	179	D78001	maltooligosyl trehalose trehalohydrolase	50,7	Rhizobium sp
23/25	180	D83245	glycogen debranching enzyme	56,8	Sulfolobus acidocaldarius
23/25	181	D83245	glycogen debranching enzyme	74,1	Sulfolobus acidocaldarius
23/25	182	D83245	glycogen debranching enzyme	61,7	Sulfolobus acidocaldarius
23/25	183	D83245	glycogen debranching enzyme	74,1	Sulfolobus acidocaldarius
23/25	184	D83245	glycogen debranching enzyme	80,2	Sulfolobus acidocaldarius
23/25	185	D83245	glycogen debranching enzyme	69,1	Sulfolobus acidocaldarius
23/25	186	D83245	glycogen debranching enzyme	70,4	Sulfolobus acidocaldarius
23/25	187	D83245	glycogen debranching enzyme	71,6	Sulfolobus acidocaldarius
23/25	188	D90900	glycogen operon protein GlgX	64,2	Synechocystis sp
23/25	189	D90900	glycogen operon protein GlgX	64,2	Synechocystis sp
23/25	190	D90908	glycogen operon protein GlgX	55,6	Synechocystis sp

210-211/25	191	D90908	glycogen operon protein GlgX	91,1	Synechocystis sp
210-211/25	192	D90908	glycogen operon protein GlgX .	88,6	Synechocystis sp
210-211/25	193	D90908	glycogen operon protein GlgX	56,8	Synechocystis sp
210-211/25	194	U18997	glgX	69,6	E. coli
210-211/25	195	Y08256	glycogen operon protein GlgX	71,6	Sulfolobus sol-fataricus
210-211/25	196	Y08256	glycogen operon protein GlgX	72,8	Sulfolobus sol-fataricus
210-211/25	197	Y08256	glycogen operon protein GlgX	58	Sulfolobus sol-fataricus
210-211/25	198	Y08256	alpha-amylase precursor	51,5	Sulfolobus sol-fataricus
210-211/25	199	Y08256	alpha-amylase precursor	59,7	Sulfolobus sol-fataricus
210-211/25	200	Y08256	alpha-amylase precursor	52,9	Sulfolobus sol-fataricus
36/38	201	Y08256	alpha-amylase precursor	44,8	Sulfolobus sol-fataricus
36/38	202	Y08256	glycogen operon protein GlgX	70,4	Sulfolobus sol-fataricus
36/38	203	Z74020	glgX	36,4	Mycobacterium tuberculosis
36/38	204	Z74020	glgX	74,1	Mycobacterium tuberculosis
36/38	205	Z74020	glgX	77,8	Mycobacterium tuberculosis
24/26	7	A20154	alpha-amylase	42	Bacillus amyloli-quefaciens
24/26	8	AE006357	alpha-amylase	44,3	Lactococcus lactis
24/26	9	AF153911	beta-agarase	60	Pseudomonas sp.
24/26	10	AF153911	beta-agarase	58	Pseudomonas sp.
24/26	11	AF153911	beta-agarase	59	Pseudomonas sp.
24/26	12	AF153911	beta-agarase	60	Pseudomonas sp.

24/26	13	AF153911	beta-agarase	58	Pseudomonas sp.
24/26	14	AF208003	alpha-amylase	45,4	Diabrotica virgifera
24/26	15	AL352956	secreted alpha-amylase	74	Streptomyces coelicolor
24/26	16	AL352956	secreted alpha-amylase	89	Streptomyces coelicolor
24/26	17	AL352956	secreted alpha-amylase	81	Streptomyces coelicolor
24/26	18	AL352956	secreted alpha-amylase	88	Streptomyces coelicolor
24/26	19	AL352956	secreted alpha-amylase	80	Streptomyces coelicolor
24/26	20	AL352956	secreted alpha-amylase	80	Streptomyces coelicolor
24/26	21	AL352956	secreted alpha-amylase	80	Streptomyces coelicolor
24/26	22	AL352956	secreted alpha-amylase	82	Streptomyces coelicolor
24/26	23	AX036894	unnamed protein product	54,2	Bacillus stearothermo-philus
24/26	24	J01542	alpha-amylase protein precursor	42,9	Bacillus amyloli-quefaciens
24/26	25	L01642	alpha-amylase	56,6	E.coli
24/26	26	L01642	alpha-amylase	52,8	E.coli
24/26	27	M13255	amyS	52,3	B.stearothermophilus
24/26	28	M13255	amyS	57	B.stearothermophilus
24/26	29	M13255	amyS	50,5	B.stearothermophilus
24/26	30	M13255	amyS	52,3	B.stearothermophilus
24/26	31	M13255	amyS	50,5	B.stearothermophilus
24/26	32	M18244	alpha-amylase	55,4	Streptomyces limosus
24/26	33	M25263	alpha-amylase	65	Streptomyces venezuelae
24/26	34	M25263	alpha-amylase	91	Streptomyces venezuelae
24/26	35	U51129	amylase	85	Streptomyces albus
24/26	36	U51129	amylase	85	Streptomyces albus
24/26	37	U75445	alpha-amylase	56,1	Bacillus sp. MK 716
24/26	38	U75445	alpha-amylase	54,2	Bacillus sp. MK 717

24/26	39	U75445	alpha-amylase	55,9	Bacillus sp. MK 718
24/26	40	X15752	cyclomaltodextrin glucanotransferase	33,9	Bacillus licheni-formis
24/26	41	X57568	alpha-amylase	64	Streptomyces griseus
24/26	42	X57568	alpha-amylase	56,4	Streptomyces griseus
24/26	43	X57568	alpha-amylase	75	Streptomyces griseus
24/26	44	X57568	alpha-amylase	45,2	Streptomyces griseus
24/26	45	X57568	alpha-amylase	47,1	Streptomyces griseus
24/26	46	X59279	putative alpha-glucanotransferase /hydrolase	35,1	Anabaena variabilis
24/26	47	X70255	alpha-amylase	43,1	Streptomyces lividans
24/26	48	X70255	alpha-amylase	44	Streptomyces lividans
24/26	49	Y13332	alpha-amylase	58,4	Streptomyces sp.
24/26	50	Y13332	alpha-amylase	94	Streptomyces sp.
24/26	51	Y13332	alpha-amylase	89	Streptomyces sp.
24/26	52	Y13332	alpha-amylase	93	Streptomyces sp.
24/26	53	Y13332	alpha-amylase	86	Streptomyces sp.
24/26	54	Y13332	alpha-amylase	91	Streptomyces sp.
24/26	55	Y13332	alpha-amylase	92	Streptomyces sp.
24/26	56	Y13332	alpha-amylase	92	Streptomyces sp.

Table 3: Temperature profile of the α-amylase from HA11

Temperature [°C]	Activity relative to that at 45°C [%]
45	100
46,4	123
50,4	146

55,8	121
61	38
64,7	28

Table 4: Ha13 amylase

The temperature profile (in analogy to HA11) and the temperature stability after preincubation for 15 min was determined. The remaining activity at 50°C in 100 mM Tris HCl pH 8.6 was then determined.

Substrate conversion in the temperature gradients from 50°C to 69.7°C in the DNSS assay (Tris-HCl pH 8.6).

Temperature	Percent activity remaining
50°C	100
51.4°C	110
55.5°C	139
60.8°C	118
66°C	98
69.7°C	41

Table 5: Temperature stability of the amylase domain from HA13

Substrate conversion in the DNSA assay after preincubation in the temperature gradient from 50°C to 69.7°C for 15 min, then substrate conversion at 50°C in Tris-HCl pH 8.6.

Temperature	Percent activity remaining
50°C	100
51.4°C	97

55.5°C	82
60.8°C	38
66°C	2
69.7°C	2

Table 6: pH stability of the amylases HA11 and HA13

Substrate conversion in the respective buffer system in the DNSA assay at 50°C for 15 min.

pH	Buffer	Percent activity remaining	
		HA 11	HA 13
4,0	Glycine-HCl	60	90
4,5	Glycine-HCl	59	96
5,0	Na-Acetate	110	107
6,5	Na-Acetate	104	106
6,5	Tris-Maleate	100	100
8,6	Tris-HCl	64	99
10	Glycine-NaOH	41	83
12	Glycine-NaOH	6	74

Table 7: Effect of surfactants on the activity of amylase HA 11

Substrate conversion in the DNSA assay with 100 mM with Tris-HCl pH 8.6 at 50°C for 15 min.

Surfactants added	Percent activity remaining
+ 0,05 % SDS	100
+ 0,10 % SDS	86
+ 0,50 % SDS	66
+ 1,00 % SDS	49

Table 8: Effect of surfactants on the activity of amylase HA 13

Substrate conversion in the DNSA assay with 100 mM with Tris-HCl pH 8.6 at 50°C for 15 min.

Surfactants added	Percent activity remaining
without SDS	100
+ 0,05 % SDS	90
+ 0,10 % SDS	74
+ 0,50 % SDS	71
+ 1,00 % SDS	67

Table 9: Effect of bleaches on HA11 amylase

Substrate conversion in the DNSA assay with 100 mM glycine-NaOH pH 9.5. Preincubation at room temperature under appropriate conditions is followed by further incubation at 50°C for 15 min.

Bleach	Percent activity remaining
without H_2O_2	100
+ 0,05 % H_2O_2	99
+ 0,10 % H_2O_2	87
+ 0,20 % H_2O_2	4
+ 0,35 % H_2O_2	1
+ 0,70 % H_2O_2	1

Table 10: Effect of bleaches on HA13 amylase

Substrate conversion in the DNSA assay with 100 mM glycine-NaOH pH 9.5. Preincubation at room temperature under appropriate conditions is followed by further incubation at 50°C for 15 min.

Bleach	Percent activity remaining
without H_2O_2	100
+ 0,05 % H_2O_2	81
+ 0,10 % H_2O_2	85
+ 0,20 % H_2O_2	80
+ 0,35 % H_2O_2	68
+ 0,70 % H_2O_2	60

Table 11: Calcium dependence of the HA11 amylase

Substrate conversion in the DNSA assay with 100 mM Tris-HCl pH 8.6 at 50°C for 15 min.

Addition	Percent activity remaining
2 mM CaCl2	100
1 mM EDTA	44

Table 12: Calcium dependence of the HA13 amylase domain

Substrate conversion in the DNSA assay with 100 mM Tris-HCl pH 8.6 at 50°C for 15 min.

Addition	Percent activity remaining
2 mM CaCl2	100
1 mM EDTA	104

Table 13: Investigation of the washing performance

	with bleach		without bleach	
	CFT PCS 26	CFT PCS 26	CFT PCS 26	CFT PCS 26
without enzyme	3,8	0,4	3,7	0,2
HA11 (S213)	7,7	3	9,4	4,5
HA 13 (S213Pb)	5,7	2	5,9	1,8
BAN	4,2	0,7	7,3	2,9
Termamyl	5,6	1,7	8,1	3,5

CLAIMS:

1. Glycosyl hydrolase the amino acid sequence of which is identical with the amino acid sequence set forth in Seq. 1 to at least 80%.

2. Glycosyl hydrolase the amino acid sequence of which is identical with the amino acid sequence set forth in Seq. 1 to at least 85%, in particular at least 90%, particularly preferred at least 95% and exceptionally preferred to 100%.

3. Glycosyl hydrolase the amino acid sequence of which is identical with the amino acid sequence set forth in Seq. 2 to at least 50%.

4. Glycosyl hydrolase the amino acid sequence of which is identical with the amino acid sequence set forth in Seq. 2 to at least 60%, preferably at least 70%, in particular at least 80%, particularly preferred at least 90% and exceptionally preferred to 100%.

5. Glycosyl hydrolase the amino acid sequence of which is identical with the amino acid sequence set forth in Seq. 3 to at least 60%.

6. Glycosyl hydrolase the amino acid sequence of which is identical with the amino acid sequence set forth in Seq. 3 to at least 70%, preferably at least 80%, in particular at least 90%, particularly preferred at least 95% and exceptionally preferred to 100%.

7. Nucleic acid coding for a glycosyl hydrolase the amino acid sequence of which is identical with one of the amino acid sequences set forth in Seq. 1 to 3 to at least 80%, in particular at least 90%, particularly

preferred at least 95% and exceptionally preferred to 100%.

8. A nucleic acid which codes for a glycosyl hydrolase and whose nucleotide sequence agrees at least 75% with the nucleotide sequence indicated in Seq. 4.

9. A nucleic acid which codes for a glycosyl hydrolase and whose nucleotide sequence agrees at least 80%, preferably at least 85%, in particular at least 90%, particularly preferably at least 95% and very particularly preferably 100% with the nucleotide sequence indicated in Seq. 4.

10. A nucleic acid which codes for a glycosyl hydrolase and whose nucleotide sequence agrees at least 50% with the nucleotide sequence indicated in Seq. 5.

11. A nucleic acid which codes for a glycosyl hydrolase and whose nucleotide sequence agrees at least 60%, preferably at least 70%, in particular at least 80%, particularly preferably at least 90% and very particularly preferably 100% with the nucleotide sequence indicated in Seq. 5.

12. A nucleic acid which codes for a glycosyl hydrolase and whose nucleotide sequence agrees at least 60% with the nucleotide sequence indicated in Seq. 6.

13. A nucleic acid which codes for a glycosyl hydrolase and whose nucleotide sequence agrees at least 70%, preferably at least 80%, in particular at least 90%, particularly preferably at least 95% and very particularly preferably 100% with the nucleotide sequence indicated in Seq. 6.

14. A glycosyl hydrolase obtainable by one or more conservative amino acid exchanges from a glycosyl hydrolase as claimed in any of claims 1 to 6.

15. A glycosyl hydrolase whose amino acid sequence comprises a part which is at least 96%, in particular at least 97%, preferentially at least 98%, preferably at least 99%, particularly preferably 100% identical to the consensus sequence of SEQ ID No. 308, in particular over the partial region corresponding to positions 13 to 71.

16. A glycosyl hydrolase whose amino acid sequence comprises a part which is at least 96%, in particular at least 97%, preferentially at least 98%, preferably at least 99%, particularly preferably 100% identical to the consensus sequence of SEQ ID No. 307, in particular over the partial region corresponding to positions 13 to 101.

17. A glycosyl hydrolase whose amino acid sequence comprises a part which is at least 92%, in particular at least 94%, preferentially at least 96%, preferably at least 99%, particularly preferably 100% identical to the consensus sequence of SEQ ID No. 305, in particular over the partial region corresponding to positions 9 to 115.

18. A glycosyl hydrolase whose amino acid sequence comprises a part which is at least 92%, in particular at least 94%, preferentially at least 96%, preferably at least 99%, particularly preferably 100% identical to the consensus sequence of SEQ ID No. 306, in particular over the partial region corresponding to positions 9 to 113.

19. A glycosyl hydrolase whose amino acid sequence comprises a part which is at least 92%, in particular at least 94%, preferentially at least 96%, preferably at least 99%, particularly preferably 100% identical to one of the amino acid sequences indicated under SEQ ID No. 7 to 56, in particular over the partial region corresponding to positions 9 to 113 according to the consensus sequence of SEQ ID No. 306.

20. A glycosyl hydrolase whose amino acid sequence comprises a part which is at least 92%, in particular at least 94%, preferentially at least 96%, preferably at least 99%, particularly preferably 100% identical to one of the amino acid sequences indicated under SEQ ID No. 7 to 56, in particular over the partial region corresponding to positions 9 to 115 according to the consensus sequence of SEQ ID No. 305.

21. A glycosyl hydrolase whose amino acid sequence comprises a part which is 96%, in particular at least 97%, preferentially at least 98%, preferably at least 99%, particularly preferably 100% identical to one of the amino acid sequences indicated under SEQ ID No. 107 to 205, in particular over the partial region corresponding to positions 13 to 71 according to the consensus sequence of SEQ ID No. 308.

22. A glycosyl hydrolase whose amino acid sequence comprises a part which is 96%, in particular at least 97%, preferentially at least 98%, preferably at least 99%, particularly preferably 100% identical to one of the amino acid sequences indicated under SEQ ID No. 107 to 205, in particular over the partial region corresponding to positions 13 to 101 according to the consensus sequence of SEQ ID No. 307.

23. A glycosyl hydrolase obtainable by one or more conservative amino acid exchanges from a glycosyl hydrolase as claimed in any of claims 15 to 22.

24. A glycosyl hydrolase obtainable by derivatization, fragmentation, deletion mutation or insertion mutation of a glycosyl hydrolase as claimed in any of claims 1 to 6, or 15 to 22.

25. A nucleic acid coding for a glycosyl hydrolase whose amino acid sequence comprises a part which is at least 96%, in particular at least 97%, preferentially at least 98%, preferably at least 99%, particularly preferably 100% identical to the consensus sequence of SEQ ID No. 308, in particular over the partial region corresponding to positions 13 to 71.

26. A nucleic acid coding for a glycosyl hydrolase whose amino acid sequence comprises a part which is at least 96%, in particular at least 97%, preferentially at least 98%, preferably at least 99%, particularly preferably 100% identical to the consensus sequence of SEQ ID No. 307, in particular over the partial region corresponding to positions 13 to 101.

27. A nucleic acid coding for a glycosyl hydrolase whose amino acid sequence comprises a part which is at least 92%, in particular at least 94%, preferentially at least 96%, preferably at least 99%, particularly preferably 100% identical to the consensus sequence of SEQ ID No. 305, in particular over the partial region corresponding to positions 9 to 115.

28. A nucleic acid coding for a glycosyl hydrolase whose amino acid sequence comprises a part which is at least 92%, in particular at least 94%, preferentially at least 96%, preferably at least 99%, particularly

preferably 100% identical to the consensus sequence of SEQ ID No. 306, in particular over the partial region corresponding to positions 9 to 113.

29. A nucleic acid coding for a glycosyl hydrolase whose amino acid sequence comprises a part which is at least 92%, in particular at least 94%, preferentially at least 96%, preferably at least 99%, particularly preferably 100% identical to one of the amino acid sequences indicated under SEQ ID No. 7 to 56, in particular over the partial region corresponding to positions 9 to 113 according to the consensus sequence of SEQ ID No. 306.

30. A nucleic acid coding for a glycosyl hydrolase whose amino acid sequence comprises a part which is at least 92%, in particular at least 94%, preferentially at least 96%, preferably at least 99%, particularly preferably 100% identical to one of the amino acid sequences indicated under SEQ ID No. 7 to 56, in particular over the partial region corresponding to positions 9 to 115 according to the consensus sequence of SEQ ID No. 305.

31. A nucleic acid coding for a glycosyl hydrolase whose amino acid sequence comprises a part which is 96%, in particular at least 97%, preferentially at least 98%, preferably at least 99%, particularly preferably 100% identical to one of the amino acid sequences indicated under SEQ ID No. 107 to 205, in particular over the partial region corresponding to positions 13 to 71 according to the consensus sequence of SEQ ID No. 308.

32. A nucleic acid coding for a glycosyl hydrolase whose amino acid sequence comprises a part which is 96%, in particular at least 97%, preferentially at

least 98%, preferably at least 99%, particularly preferably 100% identical to one of the amino acid sequences indicated under SEQ ID No. 107 to 205, in particular over the partial region corresponding to positions 13 to 101 according to the consensus sequence of SEQ ID No. 307.

33. A nucleic acid coding for a glycosyl hydrolase as claimed in claim 14, 23 or 24.

34. A nucleic acid which codes for a glycosyl hydrolase and whose sequence is at least 80%, preferably at least 90%, particularly preferably 100% identical to one of the nucleotide sequence indicated under SEQ ID No. 57 to 106, or 206 to 304, preferably in the positions which are not specified by the PCR primer sequences.

35. An oligonucleotide, in particular a PCR primer, having one of the sequences indicated in SEQ ID No. 309 to 316.

36. The use of a nucleic acid as claimed in any of claims 7 to 13 or 25 to 34, or of an oligonucleotide as claimed in claim 35 for identifying and/or isolating a novel glycosyl hydrolase.

37. The use of a glycosyl hydrolase as claimed in any of claims 1 to 6 or 15 to 24 for fusion or linkage to another protein, in particular for developing a novel enzyme.

38. The use of a nucleic acid as claimed in any of claims 7 to 13 or 25 to 34 for fusion to another nucleic acid, in particular to develop a novel enzyme.

39. A vector which comprises one of the nucleic acid regions designated in claims 7 to 13 or 25 to 34.

40. A cloning vector as claimed in claim 39.

41. An expression vector as claimed in claim 40.

42. A host cell which expresses one of the proteins or derivatives designated in claims 1 to 6 or 15 to 24 or can be induced to the expression thereof, preferably using an expression vector as claimed in claim 39 or 41.

43. A host cell as claimed in claim 42, characterized in that it is a bacterium, in particular one which secretes the produced protein or derivative into the surrounding medium.

44. A host cell as claimed in claim 43, characterized in that it is a Gram-positive bacterium.

45. A host cell as claimed in claim 44, characterized in that it belongs to the genus Bacillus, preferably the species Bacillus licheniformis, Bacillus amyloliquefaciens, Bacillus subtilis or Bacillus alcalophilus.

46. A host cell as claimed in claim 42, characterized in that it is a eukaryotic cell, in particular one which modifies the produced protein post-translationally.

47. A method for identifying and isolating new glycosyl hydrolases, characterized in that

a) metagenomic DNA is obtained from various habitats,

b) the metagenomic DNA is subcloned into a suitable cloning system, and

c) the gene library produced in this way is subjected to a screening for new glycosyl hydrolases.

48. The method as claimed in claim 47, characterized in that the biodiversity of the metagenomic DNA is determined by PCR typing using glycosyl hydrolase-specific PCR primers and subsequent sequencing.

49. The method as claimed in claim 47 or 48, characterized in that the screening in step c) is carried out by selection for glycosyl hydrolase activity.

50. The method as claimed in claim 48, characterized in that the screening in step c) is carried out by colony hybridization using probes based on the PCR fragments generated in accordance with claim 48.

51. The method as claimed in claim 48, characterized in that glycosyl hydrolase-specific PCR primers selected from those listed in Table 1 are employed, preferably those selected from the primer pairs GEX 24/26 and GEX 23/25.

52. A washing or cleaning composition, characterized in that it comprises a glycosyl hydrolase as claimed in any of claims 1 to 6 or 15 to 24.

53. A composition as claimed in claim 52, characterized in that it comprises from 0.000001 percent by weight to 5% by weight, and increasingly preferably 0.00001 to 4% by weight, 0.0001 to 3% by weight, 0.001 to 2% by weight or 0.01 to 1% by weight, of the glycosyl hydrolase.

54. A method for cleaning textiles or hard surfaces, characterized in that a glycosyl hydrolase as claimed

in any of claims 1 to 6 or 15 to 24 is used in at least one of the method steps, preferably in a composition as claimed in claim 52 or 53, particularly preferably in machines.

55. The method as claimed in claim 54, characterized in that the glycosyl hydrolase is used in an amount of from 0.01 mg to 400 mg, preferably from 0.02 mg to 200 mg, particularly preferably from 0.02 mg to 100 mg, in the method step characterized by this enzyme.

56. The method as claimed in claim 54 or 55, characterized in that the glycosyl hydrolase is used in a concentration of from 0.0005 to 20 mg per l, preferably 0.005 to 10 mg per l, particularly preferably from 0.005 to 8 mg per l of wash liquor.

57. The use of a glycosyl hydrolase as claimed in any of claims 1 to 6 or 15 to 24, preferably in a washing or cleaning composition as claimed in either of claims 52 or 53 for cleaning textiles or hard surfaces.

58. The use as claimed in claim 57, characterized in that the glycosyl hydrolase is used in an amount of from 0.01 mg to 400 mg, preferably from 0.02 mg to 200 mg, particularly preferably from 0.02 mg to 100 mg.

59. The use as claimed in claim 57 or 58, characterized in that the glycosyl hydrolase is used in a concentration of from 0.0005 to 20 mg per l, preferably 0.005 to 10 mg per l, particularly preferably from 0.005 to 8 mg per l of wash liquor.

60. The use of a glycosyl hydrolase as claimed in any of claims 1 to 6 or 15 to 24 for treating raw materials or intermediate products in the manufacture of textiles, in particlar for desizing cotton.

61. A method for liquefaction of starch, in particular for the production of ethanol, characterized in that a glycosyl hydrolase as claimed in any of claims 1 to 6 or 15 to 24 is employed therein.

62. The use of a glycosyl hydrolase as claimed in any of claims 1 to 6 or 15 to 24 for producing linear and/or short-chain oligosaccharides.

63. The use of a glycosyl hydrolase as claimed in any of claims 1 to 6 or 15 to 24 for hydrolyzing cyclodextrins.

64. The use of a glycosyl hydrolase as claimed in any of claims 1 to 6 or 15 to 24 for releasing low molecular weight compounds from polysaccharide carriers or cyclodextrins.

65. The use of a glycosyl hydrolase as claimed in any of claims 1 to 6 or 15 to 24 for the manufacture of food products and/or food ingredients.

66. The use of a glycosyl hydrolase as claimed in any of claims 1 to 6 or 15 to 24 for producing animal feed and/or animal feed ingredients.

67. The use of a glycosyl hydrolase as claimed in any of claims 1 to 6 or 15 to 24 for breaking down starch-containing adhesive bondings.

68. A method for temporary gluing, characterized in that a glycosyl hydrolase as claimed in any of claims 1 to 6 or 15 or 24 is employed therein.