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(54) Title: ALKALINE BACILLUS AMYLASE

(57) Abstract: This invention relates to an amylase derived from Bacillus horikoshii. The amylase has a preference for soluble substrates, shows substantial exo-amylase activity and has a pH optimum of about 9.8.

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ALKALINE BACILLUS AMYLASE

FIELD OF THE INVENTION

The present invention relates to amylases having a pH optimum around 9.8.

BACKGROUND OF THE INVENTION

For a number of years alpha-amylase enzymes have been used for a variety of different purposes, the most important of which are starch liquefaction, textile desizing, starch modification in the paper and pulp industry, and for brewing and baking.

Examples of commercial alpha-amylase products are Termamyl®, BAN® and Fungamyl®, all available from Novozymes A/S, Denmark. These and similar products from other commercial sources have an acidic to a neutral pH optimum, typically in the range of from pH 5 to pH 7.5, and they do not display optimal activity in detergent solutions at alkaline pH.

WO 95/26397 discloses an alpha-amylase from a *Bacillus* strain, which has optimum activity at pH 8. WO 96/23873 describes variants of *Bacillus* amylases with improved performance under washing conditions.

US 5,147,796 describes an alkaline pullulanase having alpha-amylase activity. Fig. 2b of the document shows optimum amylase activity at pH 8-8.5.

M. Takagi et al., J. Ferment. Bioeng, vol 81, No. 6, 557-559 (1996) describe an alkaliphilic alpha-amylase-pullulanase from *Bacillus* sp. The enzyme has optimum amylase activity at pH 9, but the activity drops rapidly at higher pH, and the activity at pH 10 is lower than at pH 7.

WO 01/14532 and WO 99/50399 disclose alpha-amylases from *Bacillus* strains, which have optimum activity at pH 9.5.

The amylase of the present invention has a highly alkaline profile with an optimum activity at pH 9.8

SUMMARY OF THE INVENTION

The invention provides an alpha-amylase having one or more of the following characteristics:

a) An amino acid sequence which has at least 60% identity with amino acids 1 to 1105 of Seq Id No: 2.

b) An amino acid sequence comprising Seq Id No: 2.

c) An amino acid sequence that is identical to Seq Id No: 2.

d) An amino acid sequence comprised by Seq Id No: 2

In a further aspect the invention relates to an alpha-amylase having one or more characteristics selected from the group consisting of:

a) a theoretical pI of about 4.4; or

b) a pH-optimum of about 9.8; or

c) a temperature optimum of about 50°C at pH 9; or

d) at least 40% residual activity after 30 minutes at 50°C and pH 10; or

e) exhibits substantial exo-amylase activity

f) obtainable from *Bacillus* horikoshii.

In a further aspect the invention relates to an alpha-amylase encoded by a polynucleotide which hybridizes under medium stringency conditions, more preferably high stringency conditions, with the nucleic acid sequence of Seq Id No:1 or its complementary strand.

In a still further aspect the invention relates to a DNA sequence encoding the alpha-amylase of the invention. I a particular embodiment said DNA sequence comprises or is comprised by Seq. ID No.: 1.

Further aspects of the invention provide a recombinant expression vector comprising the DNA sequence, and a cell transformed with the DNA sequence or the recombinant expression vector.

The invention also provides a method for producing the alpha-amylase and for the use of said alpha-amylase for production of malto-oligosaccharides with a DP of less than 10, and as a detergent enzyme.

BRIEF DESCRIPTION OF DRAWINGS

Figure 1 shows a pH activity profile of the alpha-amylase of the invention.

Figure 2 shows a temperature activity profile of the alpha-amylase of the invention.

Figure 3 shows the stability of the alpha-amylase of the invention after incubation at various temperatures.

Figure 4 shows the stability of the alpha-amylase of the invention after incubation at various pH.

Figure 5 shows the wash performance of the alpha-amylase in a commercial detergent.

Figure 6 shows the wash performance of the alpha-amylase at pH 9 in Carbonate buffer.

DETAILED DESCRIPTION OF THE INVENTION

Sequence Listing and Deposited Microorganisms

The present application contains information in the form of a sequence listing, which is appended to the application and also submitted on a data carrier accompanying this application. In addition, the present application refers to deposited microorganisms. The contents of the data carrier and the deposited microorganisms are fully incorporated herein by reference.

The alpha-amylase of the invention may be derived from *Bacillus* sp. strain NCIB 12512. This strain was deposited on 29 July 1987 under the terms of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure at the National Collections of Industrial and Marine Bacteria Limited (NCIMB), 23 St. Machar Drive, Aberdeen AB2 1RY, Scotland, United Kingdom.

The deposit was made by Novo Nordisk A/S and was later assigned to Novozymes A/S.

Production of alpha-amylase

The alpha-amylase of the invention can be produced by cultivating a suitable amylase-producing strain of *Bacillus* or the transformed host cell of the invention in a suitable nutrient medium, and recovering the alpha-amylase from the culture medium.

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

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

Properties of alpha-amylase

A preferred alpha-amylase is derived from *Bacillus* horikoshii. It can be produced as described in the examples. The full length amino acid sequence of the amylase and the DNA encoding it are shown in Seq. ID No. 2 and Seq. ID No. 1 respectively.

In the present context, "derived from" is intended not only to indicate an alpha-amylase produced or producible by a strain of the organism in question, but also an alpha-amylase encoded by a DNA sequence isolated from such strain and produced in a host organism transformed with said DNA sequence. Finally, the term is intended to indicate an alpha-amylase, which is encoded by a DNA sequence of synthetic and/or cDNA origin and which has the identifying characteristics of the alpha-amylase in question.

The following characteristics were found for the amylase of the invention (purified alpha-amylase):

A molecular weight of approximately 130 kDa as determined by SDS-PAGE using a Novex, 4-20% gradient gel. The theoretical MW of the polypeptide chain is 123 kDa, using the pc program GPMAW (Lighthouse data).

A theoretical pI of 4.4 was determined by the pc program Vector NTI 8, 1994-2002 Infomax, Inc.

A pH-activity curve is shown in Figure 1, taking the activity at pH 9.8 as 100%. It was determined using the reducing sugar assay (PAHBAH) using 50 mM Britten-Robinson buffer adjusted to various pH-values. Figure 1 shows that the optimum activity is about pH 9.8.

A temperature-activity curve was measured using the PAHBAH assay at various temperatures with 50 mM Britten-Robinson buffer adjusted to pH 9.0. The results are shown in Figure 2. It is seen from Figure 2 that the amylase of the invention has optimum activity at about 50°C.

Temperature stability of the amylase was measured at pH 10. A stability profile of the amylase of the invention (figure 3) shows superior stability of the amylase of the invention compared to a commercial amylase.

pH stability of the amylase was measured at 37°C. A stability profile of the amylase of the invention (figure 4) shows superior stability of the amylase of the invention compared to a commercial amylase.

Sequence homology and alignment

For purposes of the present invention, alignments of sequences and calculation of homology and identity scores may be done using a full Smith-Waterman alignment, useful for both protein and DNA alignments. The default scoring matrices BLOSUM50 and the identity matrix are used for protein and DNA alignments respectively. The penalty for the first residue in a gap is -12 for proteins and -16 for DNA, while the penalty for additional residues in a gap is -2 for proteins and -4 for DNA. Alignment may be made with the FASTA package version v20u6 (W. R. Pearson and D. J. Lipman (1988), "Improved Tools for Biological Sequence Analysis", PNAS 85:2444-2448, and W. R. Pearson (1990) "Rapid and Sensitive Sequence Comparison with FASTP and FASTA", Methods in Enzymology, 183:63-98).

Multiple alignments of protein sequences may be made using "ClustalW" (Thompson, J.D., Higgins, D.G. and Gibson, T.J. (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. Nucleic Acids Research, 22:4673-4680). Multiple alignments of DNA sequences may be done using the protein alignment as a template, replacing the amino acids with the corresponding codon from the DNA sequence.

The amino acid sequence of the present invention exhibits a degree of identity preferably of at least 60%, or at least 65%, or at least 70%, or at least 75%, preferably at least 80%, or at least 85%, more preferably at least 90%, especially at least 95%, even more especially at least 96%, or at least 97%, or at least 98%, or at least 99% with the amino acid sequence shown in positions 1-1105 of SEQ ID NO: 2.

The DNA construct of the present invention comprises a DNA sequence exhibiting a degree of identity preferably of at least 60%, preferably at least 70%, more preferably at least 80%, especially at least 90% or at least 95%, even more especially at least 97% or at least 99% with the nucleic acid sequence shown in positions 109-3423 of SEQ ID NO: 1.

Hybridization

Suitable experimental conditions for determining hybridization between a nucleotide probe and a homologous DNA or RNA sequence involves presoaking of the filter containing the DNA fragments or RNA to hybridize in 5x SSC (sodium chloride/sodium citrate, Sambrook, et al., 1989) for 10 min, and prehybridization of the filter in a solution of 5x SSC, 5x Denhardt's solution (Sambrook, et al., 1989), 0.5% SDS and 100 µg/ml of denatured sonicated salmon sperm DNA (Sambrook, et al., 1989), followed by hybridization in the same solution containing a random-primed (Feinberg, A. P. and Vogelstein, B. (1983) Anal. Biochem. 132:6-13), 32P-dCTP-labeled (specific activity > 1 x 109 cpm/µg) probe for 12 hours at ca. 45°C. The filter is then washed twice for 30 minutes in 2x SSC, 0.5% SDS at least 55°C (low stringency), preferably at least 60°C (medium stringency), more preferably at least 65°C (medium/high stringency), more preferably at least 70°C (high stringency), even more preferably at least 75°C (very high stringency).

Molecules which hybridize to the oligonucleotide probe under these conditions are detected by exposure to x-ray film.

Recombinant expression vector

The present invention also relates to recombinant expression vectors comprising a polynucleotide of the present invention, a promoter, and transcriptional and translational stop signals. The various nucleic acids and control sequences described above may be joined together to produce a recombinant expression vector which may include one or more convenient restriction sites to allow for insertion or substitution of the nucleotide sequence encoding the polypeptide at such sites. Alternatively, a nucleotide sequence of the present invention may be expressed by inserting the nucleotide sequence or a nucleic acid construct comprising the sequence into an appropriate vector for expression. In creating the expression vector, the coding sequence is located in the vector so that the coding sequence is operably linked with the appropriate control sequences for expression.

The recombinant expression vector may be any vector (e.g., a plasmid or virus) which can be conveniently subjected to recombinant DNA procedures and can bring about expression of the nucleotide sequence. The choice of the vector will typically depend on the compatibility of the vector with the host cell into which the vector is to be introduced. The vectors may be linear or closed circular plasmids.

The vector may be an autonomously replicating vector, i.e., a vector which exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication, e.g., a plasmid, an extrachromosomal element, a minichromosome, or an artificial chromosome. The vector may contain any means for assuring self-replication. Alternatively, the vector may be one which, when introduced into the host cell, is integrated into the genome and replicated together with the chromosome(s) into which it has been integrated. Furthermore, a single vector or plasmid or two or more vectors or plasmids which together contain the total DNA to be introduced into the genome of the host cell, or a transposon may be used.

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

Examples of bacterial selectable markers are the dal genes from *Bacillus* subtilis or *Bacillus* licheniformis, or markers which confer antibiotic resistance such as ampicillin, kanamycin, chloramphenicol, or tetracycline resistance.

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

For integration into the host cell genome, the vector may rely on the polynucleotide's sequence encoding the polypeptide or any other element of the vector for integration into the genome by homologous or nonhomologous recombination. Alternatively, the vector may contain additional nucleotide sequences for directing integration by homologous recombination into the genome of the host cell at a precise location(s) in the chromosome(s). To increase the likelihood of integration at a precise location, the integrational elements should preferably contain a sufficient number of nucleic acids, such as 100 to 10,000 base pairs, preferably 400 to 10,000 base pairs, and most preferably 800 to 10,000 base pairs, which have a high degree of identity with the corresponding target sequence to enhance the probability of homologous recombination. The integrational elements may be any sequence that is homologous with the target sequence in the genome of the host cell. Furthermore, the integrational elements may be non-encoding or encoding nucleotide sequences. On the other hand, the vector may be integrated into the genome of the host cell by non-homologous recombination.

For autonomous replication, the vector may further comprise an origin of replication enabling the vector to replicate autonomously in the host cell in question. The origin of replication may be any plasmid replicator mediating autonomous replication which functions in a cell. The term "origin of replication" or "plasmid replicator" is defined herein as a nucleotide sequence that enables a plasmid or vector to replicate in vivo.

Examples of bacterial origins of replication are the origins of replication of plasmids pBR322, pUC19, pACYC177, and pACYC184 permitting replication in E. coli, and pUB110, pE194, pTA1060, and pAMß1 permitting replication in *Bacillus*.

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

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

Host cells

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

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

Examples of bacterial host cells which, on cultivation, are capable of producing the enzyme of the invention are gram positive bacteria such as strains of *Bacillus*, such as strains of B. subtilis, B. licheniformis, B. lentus, B. clausii, B. brevis, B. stearothermophilus, B. alkalophilus, B. amyloliquefaciens, B. coagulans, B. circulans, B. lautus, B. megaterium or B. thuringiensis, or strains of Streptomyces, such as S. lividans or S. murinus, or gram negative bacteria such as Escherichia coli. The transformation of the bacteria may be effected by protoplast transformation, electroporation, conjugation, or by using competent cells in a manner known per se (cf. Sambrook et al., 1989: Molecular Cloning: A Laboratory Manunal, Second Edition (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York).

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

Industrial Applications – please provide information about relevant applications

Owing to their activity at alkaline pH values, the alpha-amylases of the invention are well suited for use in a variety of industrial processes, e.g. laundry and hard surface cleaning detergent compositions, the production of sweeteners and ethanol from starch. Thus, it may be used in conventional starch-converting processes, such as liquefaction and saccharification processes described in US Patent No. 3,912,590 and EP patent publications Nos. 252,730 and 63,909.

Pulp and paper production

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

a) disintegrating the paper to produce a pulp,

b) treating with a starch-degrading enzyme before, during or after step a), and

c) separating ink particles from the pulp after steps a) and b).

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

Desizing

The alpha-amylase of the invention may also be very useful in textile desizing. In the textile processing industry, alpha-amylases are traditionally used as auxiliaries in the desizing process to facilitate the removal of starch-containing size, which has served as a protective coating on weft yarns during weaving. Complete removal of the size coating after weaving is important

to ensure optimum results in the subsequent processes, in which the fabric is scoured, bleached and dyed. Enzymatic starch break-down is preferred because it does not involve any harmful effect on the fibre material. In order to reduce processing cost and increase mill throughput, the desizing processing is sometimes combined with the scouring and bleaching steps. In such cases, non-enzymatic auxiliaries such as alkali or oxidation agents are typically used to break down the starch, because traditional alpha-amylases are not very compatible with high pH levels and bleaching agents. The non-enzymatic breakdown of the starch size does lead to some fibre damage because of the rather aggressive chemicals used. Accordingly, it would be desirable to use the alpha-amylases of the invention as they have an improved performance in alkaline solutions. The alpha-amylases may be used alone or in combination with a cellulase when desizing cellulose-containing fabric or textile.

Beer making

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

Production of malto-oligosaccharides

The alpha-amylase of the invention exhibits substantial exo-alpha-amylase (see break down pattern of amylase and waxy corn starch, Example 4). This allows for the use of the enzyme of the present invention for production of malto-oligosaccharides of a specific degree of polymerisation (DP), a process that otherwise is tedious and expensive. Malto-oligosaccharides with a DP around 10 or less have potential applications in e.g. the food industry. These malto-oligosaccharides have a less sweet taste compared to sucrose's, and a partial replacement of sucrose with e.g. maltotetraose (DP=4) reduces the sweetness of the foods without affecting their inherent taste and flavour. At the same time they have a high moisture retention power, and can thus serve to prevent retrogradation of starch ingredient and makes retain a suitable moisture in foods. A further aspect of application of malto-oligosaccharides is that they show a less Maillard reaction, and thus less coloration. Malto-oligosaccharides show higher viscosity than that of sucrose, and are useful in improving the texture of foods. A further aspect of the application of malto-oligosaccharides is that they may be used to control the freezing points of frozen foods, as they depresses the freezing point of water more moderately than sucrose or high-fructose syrup.

Detergent Compositions

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

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

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

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

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

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

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

The present invention is further described by the following examples, which should not be construed as limiting the scope of the invention.

MATERIALS AND METHODS

50 mM Britton-Robinson buffer

50 mM acetic acid, 50 mM phosphoric acid, 50 mM boric acid, pH adjusted to the value of interest with NaOH.

Detergent:

Commercial European color detergent; alpha-amylases present in the detergent were inactivated by microwaves before adding the alpha-amylase of the present invention.

EXAMPLES

Example 1: Cloning and expression of the alpha-amylase of the invention

Bacterial strains and growth conditions

TH1 is a *Bacillus* subtilis strain (amy-,spo-,apr-,npr-), that has been modified by insertion of a construct, from the strain DN3 (Noone et al.2000, J Bacteriol 182 (6) 1592-1599) by transformation and selection for erytromycin. The changed genotype is: ykdA::pDN3 (PykdA-lacZ Pspac-ykdA) Ermr. TH1 contains the following features: the full ykdA promoter is fused to the LacZ reporter gene. In addition the ykdA gene is placed under control of the IPTG-inducible Pspac promoter, so the ykdA gene no longer has it's naturally regulation. The strain can be used as host for expression clones and libraries and transformants expressing and secreting protein can be selected on plates containing X-gal and IPTG. TH1 can be maintained and propagated in Luria-Bertani (LB) or supplemented with agar (1.5% wt/vol) as appropriate, addition of 6 µg/ml erythromycin and grown at 37°C with aeration.

DNA manipulations

Bacillus subtilis transformations were performed as described previously (Anagnostopolous, C., and J. Spizizen. 1961. Requirements for transformation in *Bacillus* subtilis. J. Bacteriol. 81:741-746). All routine molecular biological procedures were performed according to the protocols described by Sambrook et al. (1989).

Modification of vector

The shuttle vector for *Bacillus* and E.coli pDG268neo (Widner B; Thomas M; Sternberg D; Lammon D; Behr R; Sloma A (2000): Development of marker-free strains of *Bacillus* subtilis capable of secreting high levels of industrial enzymes. Journal of Industrial Microbiology and Biotechnology, Vol. 25 (4) pp. 204-212) was modified to allow for cloning of partial digested Sau3A or Tsp509I genomic DNA. The vector was modified by inserting a BamHI and EcoRI

site between the Sac1 and Not1 sites (fragment between was deleted and a linker was inserted). In this way genomic fragments can be cloned and genes contained in these fragments can be transcribed from either the strong promoter on the vector or by their own promoter.

Construction of a library in E.coli

Chromosomal DNA from *Bacillus* horikoshii was isolated by QIAmp Tissue Kit (Qiagen, Hilden, Germany). The genomic DNA was partial digested by Sau3A by standard methods. DNA fragments from 3-5 kb were gel purified and ligated into BamHI digested and dephosphorylated vector (the modified pDG268neo (= pDG268BE)). 1 micro L of the ligation was transformed by electroporation into competent E.coli cells (Electromax DH10B Cells, Invitrogen) according to standard protocols. The transformed cells were plated on plates containing solid LB media containing ampicillin as selection marker. The plates were incubated for 16 h at 37 C. 20.000 transformants obtained this way were pooled and plasmid DNA was prepared from the pooled cells by using a midiprep Qiagene kit (Qiagene). This plasmid pool represents the library.

Transformation of library into *Bacillus* subtilis TH1

The obtained plasmid pool DNA was used to transform *Bacillus* TH1 competent cells.

Bacillus transformations were plated on Petri dishes with LB-media supplemented with agar (1.5% wt/vol) and X-Gal (5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside) was added to the media at a concentration of 64 micro g/mL, and IPTG (isopropyl-b-D-thiogalactopyranoside) was added to a final concentration of 0,8 mM. Antibiotics were added at the following concentrations: chloramphenicol, 6 µg/ml; and erythromycin, 3 µg/ml. Transformants were incubated at 37° C overnight.

Selection and analysis of transformants secreting recombinant protein

Blue colonies that occurred after 16h of incubation or in the following 24 h were selected and restreaked on new plates to obtain pure single blue colonies. For expression studies transformants were grown in liquid PS-1 or TY media at 30° C and at 250 rpm. Cells were spun down and the supernatant analysed for secreted recombinant protein on SDS-polyacrylamide gels.

Results

A genomic library of *Bacillus* horikoshii was made in the vector pDGneo268BE. A plasmid pool was made from the E.coli library. The plasmid pool DNA was transformed into *Bacillus* strain TH1 (TH1 allows for blue/white selection of secreting recombinant clones).16-20000 *Bacillus* transformants were obtained on agar plates containing X-gal and IPTG, which allows for blue /white selection of secreting *Bacillus* transformants. Nineteen intense blue colonies appeared

among the 16-20.000 colonies. These blue colonies were fermented in liquid media and the supernatants analysed on SDS-gels. 5 of the 19 blue colonies gave an intense protein band on a SDS gel. A recombinant protein band of around 120 kD from one of the clones was purified and characterised by N-terminal amino acid sequencing. The DNA insert in the corresponding clone was sequenced to identify the gene giving rise to the recombinant protein band. The resulting gene sequence is the sequence of the alpha amylase of the invention listed as the Seq. ID No.:1 and the encoded protein is listed as the Seq. ID No: 2.

Example 2: Purification method for the alpha-amylase of the invention

The culture supernatant was centrifuged and cell debris was discarded.

Fermentation supernatant was then sterile filtered using Seitz-EKS filter with pore size 0.22microns which was purchased from Pall Seitz Schenk Filter system GmbH, Bettringer Strasse 42 D-73550 Germany. Filtration was carried out using pressure filter.

The sterile filtered supernatant was then adjusted to 1 M ammonium sulphate by adding solid ammonium sulphate.

Hydrophobic chromatography on Matrix Phenyl Toyopearl 650M Toshohaas was purchased from Bie and Berntsen Roedovre, Denmark.

Fifty millilitre column was packed with the matrix and equilibrated with 1M ammonium sulphate adjusted to pH 8. Sterile filtered fermentation supernatant was then applied on the column and washed with the 1 Molar ammonium sulphate solution till all unbound material was washed out.

Bound Proteins were then eluted by lowering the salt concentration using 50 mM Borate pH 8 solution without any salt. Fractions 10 ml each were collected and analysed by SDS-PAGE.

Fractions containing protein with 120 kDa molecular weight were then pooled and dialyzed against Buffer containing 50 mM Borate, 5mM Dimethyl glutaric acid pH 6.

Anion exchange chromatography on Fast flow Q sepharose matrix was purchased form Amersham Pharmacia and 50 ml column was packed. The column was then equilibrated with the buffer containing 50 mM Boarte, 5mM Dimetrhyl glutaric acid pH6. The dialyzed pool containing the 120 kDa protein was then applied on the column and washed with the buffer till all unbound material was washed out. The bound protein was then eluted with linear salt gradient using Buffer B pH6 which contained 50 mM Borate, 5 mM Dimethyl glutaric acid and 1 M salt.

Fractions were then analyzed by SDS-PAGE and pooled on the basis of Molecular weight which contained the desired protein.

Example 3: Characterisation of the alpha-amylase of the present invention

Reducing sugar assay (PAHBAH)

The enzyme reaction was initiated by adding 50 µl enzyme sample to 100 micro L 0.15% amylose (Merck, 4561), 50 mM Britton-Robinson buffer (50 mM acetic acid, 50 mM phosphoric acid, 50 mM boric acid, pH adjusted to the value of interest with NaOH), 0.01% Triton-X-100 in PCR tubes. This solution was incubated for 15 min at different temp. in a Peltier Thermal Cycler (PCT-200, MJ research) and stopped with 75 micro L 1.5 % (w/v) p-hydroxybenzoic acid hydrazide (Sigma, H-9882), 5 % (w/v) K-Na-tartrate (Merck, 1.08087), 2% (w/v) NaOH and incubated at 95°C for 10 min. The temperature was lowered to 20°C and 150 micro L was transferred to a 96 well micro titter plate. The absorbance was then measured at 410 nm with a microplate reader (Spectra Max 190, Molecular Devices).

pH profile

The pH profile was measured at 37°C, at pH 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12 using the Reducing sugar assay described above.

The pH profile is shown in figure 1, and it is seen that the alpha-amylase of the invention has an optimum activity at about 9.8.

Temperature profile

The temperature profile was measured at pH 9 at 20°C, 30°C, 40°C, 50°C, 60°C, 70°C, 80°C, 90°C using the Reducing sugar assay described above.

The temperature profile is shown in figure 2, and it is seen that the alpha-amylase of the invention has an optimum activity at about 50°C.

Temperature stability

150 micro L enzyme sample (diluted with 50 mM Britton-Robinson buffer: 50 mM acetic acid, 50 mM phosphoric acid, 50 mM boric acid, pH adjusted to 10 with NaOH, with 1 mM EDTA or 1 mM $CaCl_2$) in an Eppendorf tube was incubated for 30 min a in Eppendorf Thermomixer at different temperatures and 300 rpm shaking. The incubation was stopped by transferring the eppendorf tupe to an ice bath and adding 75 micro L solution to 75 micro L 0.5 M HEPES pH 7. 20 micro L of the above stopped solution was transferred into a microtiter plate and 200 micro L 3.7 mM 4,6-ethylidene-G_7PNP, 3.3 kU/L alpha-glucosidase, 52.2 mM HEPES, 73 mM NaCl, 10.5 mM MgCl2, 0.06 mM $CaCl_2$, pH 7 (AMYL kit, Roche Diagnostics GmbH). The absorbance at 405 nm was measured with 11 seconds intervals with a microplate reader (Spectra Max 190, Molecular Devices) and the activity was determined from the slope generated after 5 minutes measurement.

The temperature stability is depicted in figure 3, where the stability of the amylase of the present invention is compared with the stability of the commercially available amylase BAN

®, available from Novozymes A/S. Figure 3 shows a superior stability of the amylase of the present invention as compared to the commercially available amylase.

pH stability

150 micro L enzyme sample (diluted with 50 mM Britton-Robinson buffer (50 mM acetic acid, 50 mM phosphoric acid, 50 mM boric acid, pH adjusted to the value of interest with NaOH, with and without 1 mM $CaCl_2$)) in an Eppendorf tube was incubated for 30 minutes in a Eppendorf Thermomixer at 37°C and 300 rpm shaking. The incubation was stopped by transferring 75 micro L solution to 75 micro L 0.5 M HEPES pH 7. 20 micro L of the above stopped solution was transferred into a microtiter plate and 200 µl 3.7 mM 4,6-ethylidene-G_7PNP, 3.3 kU/L alpha-glucosidase, 52.2 mM HEPES, 73 mM NaCl, 10.5 mM MgCl2, 0.06 mM $CaCl_2$, pH 7 (AMYL kit, Roche Diagnostics GmbH). The absorbance at 405 nm was measured with 11 seconds intervals with a micro plate reader (Spectra Max 190, Molecular Devices) and the activity was determined from the slope generated after 5 minutes measurement.

The pH stability is depicted in figure 4, where the stability of the amylase of the present invention is compared with the stability of the commercially available amylase BAN ®, available from Novozymes A/S. Figure 4 shows a superior stability of the amylase of the present invention as compared to the commercially available amylase.

pI determination

The pI of the polypeptide chain is determined theoretically by the pc program Vector NTI 8, 1994-2002 Infomax, Inc. pI was determined to 4.4.

Example 4: Amylose/starch degradation pattern

20 micro L sample were added to 1 mL 1 % amylose (Merck, 4561) or 1 % waxy corn starch (Cerestar), 10 mM sodium borate buffer, 0.1 mM $CaCl_2$, pH 9 and incubated for various times at 37°C. The reaction was stopped by adding 20 micro L 1 M HCl and incubated at 95°C for 10 minutes. The samples were filtered through a 0.22 micro meter filter and the carbohydrate composition was determined by IMPC HPLC.

The data below is expressed in % area in a top in the HPLC chromatogram, compared to total area of all oligosacharides in the 1035 minutes chromatogram.

1 % amylose degradation pattern					
	0 min	17 min	45 min	120 min	1035 min

	%	%	%	%	%
DP6	< 1	3	5	8	< 1
DP5	< 1	0	1	1	< 1
DP4	< 1	2	6	15	48
DP3	< 1	< 1	< 1	1	5
DP2	< 1	1	2	6	26
DP1	< 1	1	1	3	17

Table 1: Degradation pattern of 1% amylose.

1 % waxy corn starch					
	0 min	17 min	45 min	120 min	1035 min
	%	%	%	%	%
DP6	< 1	8	11	8	2
DP5	< 1	< 1	1	2	1
DP4	< 1	3	7	18	30
DP3	< 1	< 1	< 1	< 1	5
DP2	< 1	1	3	8	19
DP1	< 1	1	1	4	14

Table 2: Degradation pattern of 1% waxy corn starch.

During amylose/amylopectin degradation no DP products larger than DP6 is seen. Initially the domination degradation product is DP6 and after prolonged incubation, this DP6 is hydrolysed to DP4 and DP2. Thus, this amylase exhibits substantially exo alpha amylase activity.

Example 5: Wash performance of the amylase of the invention

Wash in detergent solution.

Washing performance is evaluated by washing starch-soiled CS-28 swatches (obtainable from CFT, Centre for Test Material, Netherlands) in a beaker under constant shaking.

4 g/L of a commercially available detergent (Mega Perls Color from Henkel GmbH) is added to water. Water hardness is adjusted to 15° dH by addition of Ca:Mg in the ratio 4:1 prior to the addition of the detergent. pH is not adjusted upon addition of detergent.

The soiled swatches are washed for 20 minutes at 40°C. Upon drying the reflectance is measured.

As can be seen from Figure 5 the alpha-amylase of the invention clearly is able to remove starch from the soiled swatches.

5

Wash in buffer solution.

Washing performance is evaluated by washing starch-soiled CS-28 swatches (obtainable from CFT, Centre for Test Material, Netherlands) in a beaker under constant shaking.

5 mM Carbonate buffer is used to adjust pH to 9.

10 The soiled swatches are washed for 20 minutes at 40°C. Upon drying the reflectance is measured.

As can be seen from Figure 6 the alpha-amylase of the invention clearly is able to remove starch from the soiled swatches.

CLAIMS

1. An alpha-amylase having one or more of the following characteristics:

a) An amino acid sequence which has at least 60% identity with amino acids 1 to 1105 of Seq Id No: 2.

b) An amino acid sequence comprising Seq Id No: 2.

c) An amino acid sequence that is identical to Seq Id No: 2.

d) An amino acid sequence comprised by Seq Id No: 2.

2. The alpha-amylase of claim 1 having one or more characteristics selected from the group consisting of:

a) a theoretical pI of about 4.4; or

b) a pH-optimum of about 9.8; or

c) a temperature optimum of about 50°C at pH 9; or

d) at least 40% residual activity after 30 minutes at 50°C and pH 10; or

e) exhibits substantial exo-amylase activity

f) obtainable from *Bacillus* horikoshii.

3. The variant of any of the preceding claims, wherein the alpha-amylase of claim 1 is encoded by a polynucleotide which hybridizes under medium stringency conditions, more preferably high stringency conditions, with the nucleic acid sequence of Seq Id No:1 or its complementary strand.

4. A DNA sequence encoding the alpha-amylase of claim 1.

5. A DNA sequence according to claim 4 which comprises:

a) a DNA sequence which has a degree of identity of at least 60% with the DNA sequence shown in positions 109-3423 of Seq. Id No: 1; or

b) the DNA sequence shown in positions 109-3423 of Seq. Id No: 1; or

c) the DNA sequence of Seq. Id No: 1.

6. A recombinant expression vector comprising the DNA sequence according to any of claims 4-5.

7. A cell transformed with the DNA sequence according to any of claims 3-4 or the recombinant expression vector according to claim 6.

8. The cell according to claim 7, which is a prokaryotic cell, in particular a bacterial cell.

9. The cell according to claim 8, wherein the cell belongs to *Bacillus* or *Escherichia*, preferably E. Coli.

10. A method of producing the alpha-amylase of the invention, comprising cultivating a cell according to any of claims 7-9 under conditions permitting the production of the alpha-amylase and recovering the alpha-amylase of the culture.

11. A method for producing the alpha-amylase of any of the preceding claims, comprising cultivating a amylase-producing strain of *Bacillus* in a suitable nutrient medium, and recovering the alpha-amylase from the culture medium.

12. Use of the alpha-amylase of any of claims 1-2 for production of malto-oligosaccharides with a DP of less than 10.

13. A detergent composition comprising the alpha-amylase of any of claims 1-2.

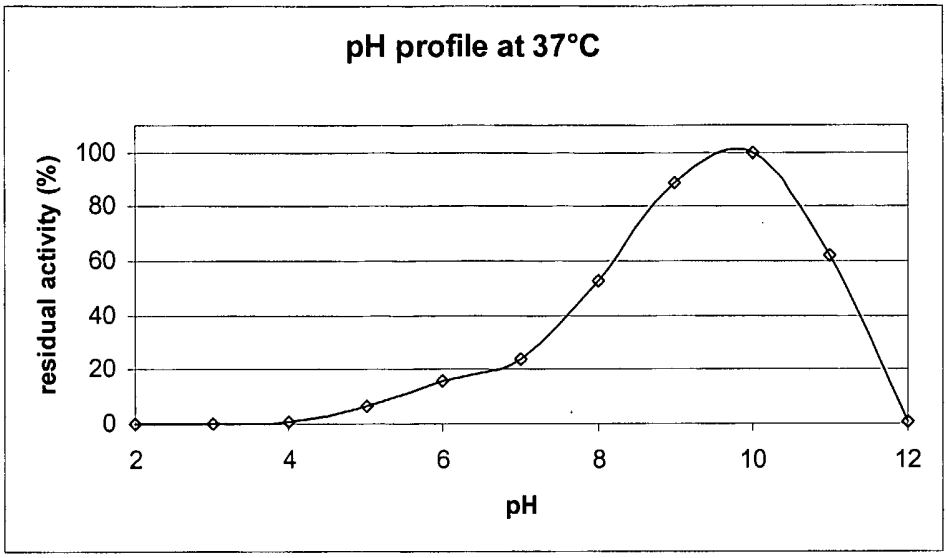

pH profile at 37°C
residual activity (%)
100
80
60
40
20
0
2
4
6
8
10
12
pH

Fig. 1

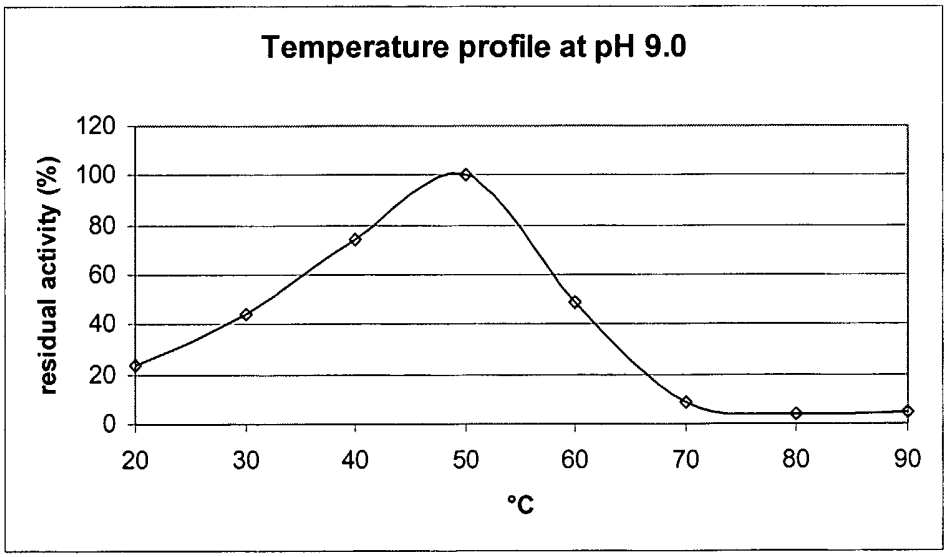

Temperature profile at pH 9.0
residual activity (%)
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100
80
60
40
20
0
20
30
40
50
60
70
80
90
°C

Fig. 2

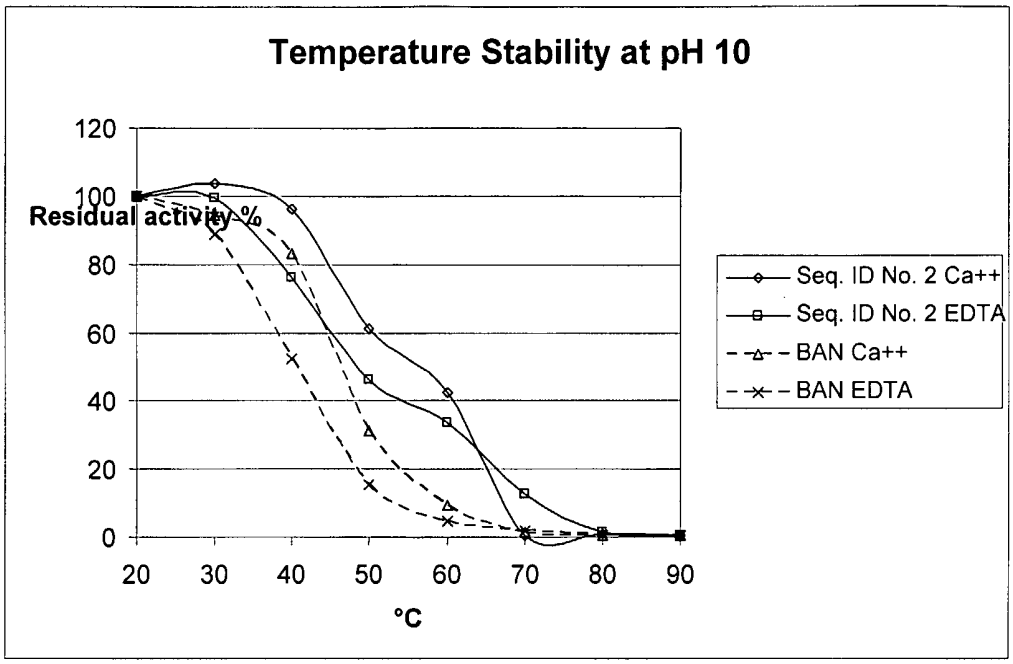

Temperature Stability at pH 10
Residual activity %
120
100
80
60
40
20
0
20
30
40
50
60
70
80
90
°C
Seq. ID No. 2 Ca++
Seq. ID No. 2 EDTA
BAN Ca++
BAN EDTA

Fig. 3

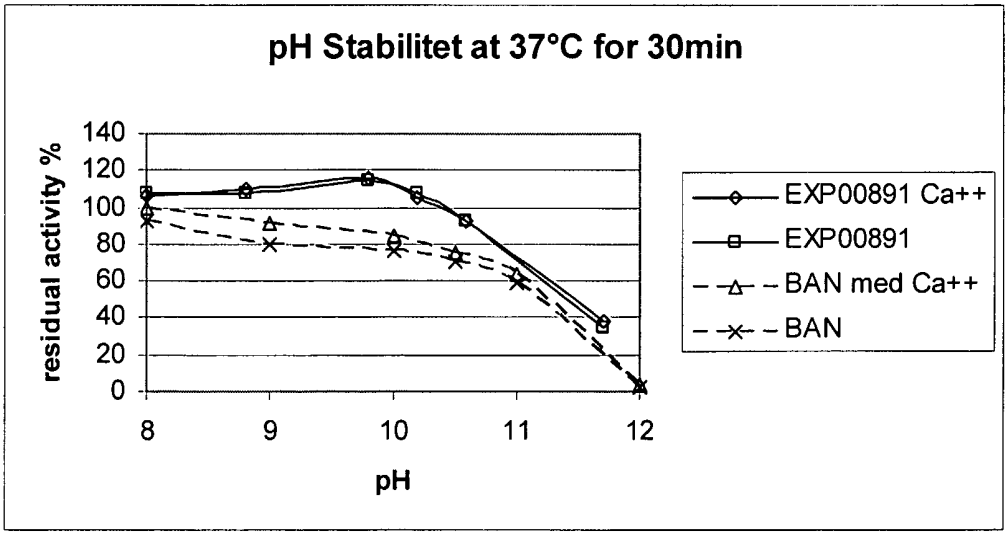

pH Stabilitet at 37°C for 30min
residual activity %
140
120
100
80
60
40
20
0
8
9
10
11
12
pH
EXP00891 Ca++
EXP00891
BAN med Ca++
BAN

Fig. 4

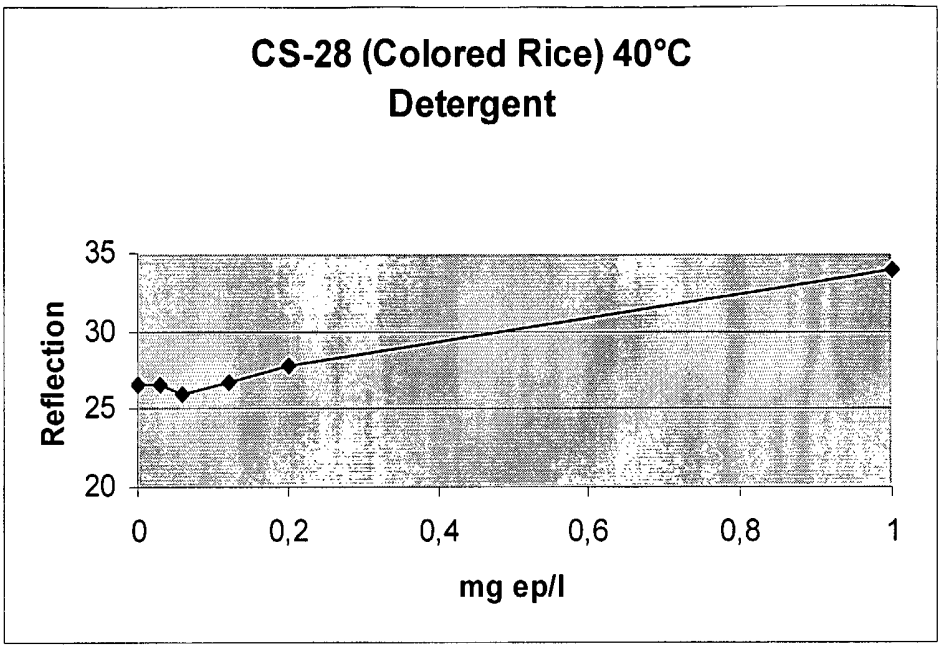

CS-28 (Colored Rice) 40°C
Detergent
Reflection
35
30
25
20
0
0,2
0,4
0,6
0,8
1
mg ep/l

Fig. 5

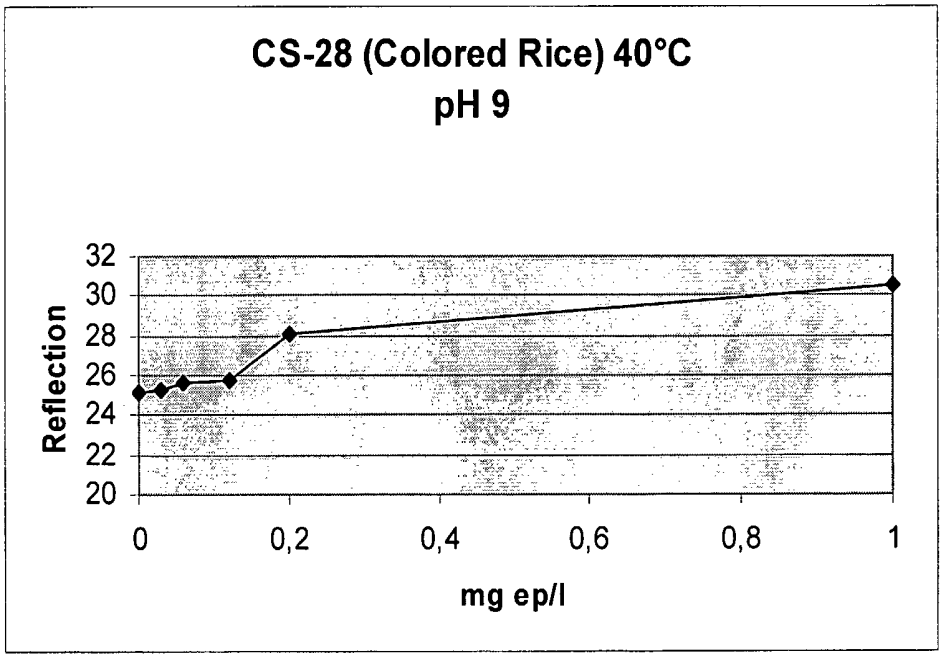

CS-28 (Colored Rice) 40°C
pH 9
Reflection
32
30
28
26
24
22
20
0
0,2
0,4
0,6
0,8
1
mg ep/l

Fig. 6

SEQUENCE LISTING

<110> Novozymes A/S

<120> Alkaline amylase with two starch binding domains

<130> 10699

<160> 2

<170> PatentIn version 3.2

<210> 1
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<213> Bacillus horikoshii

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<220>
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<222> (109)..(3423)
<223> mature peptide

2881-3131 and 3160-3423 encodes starch binding domains

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Ala Glu Gln
1

aca gac aat ttc act tgg gat aat gca aca gtc tat ttt gcg atg acg 165
Thr Asp Asn Phe Thr Trp Asp Asn Ala Thr Val Tyr Phe Ala Met Thr
5 10 15

gat cga ttt tac gat ggc aat cct tca aat aac aat tca tat ggc agg 213
Asp Arg Phe Tyr Asp Gly Asn Pro Ser Asn Asn Asn Ser Tyr Gly Arg
20 25 30 35

ccg caa caa gat gca tgg ggt caa aat ata ggg act ttc cat ggt ggg 261
Pro Gln Gln Asp Ala Trp Gly Gln Asn Ile Gly Thr Phe His Gly Gly
40 45 50

gac ctt caa ggg tta acc gac aaa tta aat gaa gga tac ttt act gac 309
Asp Leu Gln Gly Leu Thr Asp Lys Leu Asn Glu Gly Tyr Phe Thr Asp
55 60 65

tta gga att aat gcg att tgg att acc gca cca tat gag caa ata cat 357
Leu Gly Ile Asn Ala Ile Trp Ile Thr Ala Pro Tyr Glu Gln Ile His
70 75 80

ggt tgg gtt ggc ggt ggg tca gat ggt gat ttt gcc cat tat gct ttt 405
Gly Trp Val Gly Gly Gly Ser Asp Gly Asp Phe Ala His Tyr Ala Phe
85 90 95

cac ggg tat tac gct ctt gac tac acg aca gtc gac caa aat atg gga 453

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His Gly Tyr Tyr Ala Leu Asp Tyr Thr Thr Val Asp Gln Asn Met Gly
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Thr Val Glu Glu Met Arg Glu Phe Val Asp Thr Ala His Glu Asn Gly
                120                 125                 130

atc cgg gtt gtc ctt gat gtg gta atg aat cat cct ggc tac aac aca      549
Ile Arg Val Val Leu Asp Val Val Met Asn His Pro Gly Tyr Asn Thr
            135                 140                 145

tta aaa gat atg gag cag tat ggt ttt ggt acg caa caa tta aat tca      597
Leu Lys Asp Met Glu Gln Tyr Gly Phe Gly Thr Gln Gln Leu Asn Ser
        150                 155                 160

tct tgg act cct gga cct ggc caa aca tgg cat agc cac cac gaa caa      645
Ser Trp Thr Pro Gly Pro Gly Gln Thr Trp His Ser His His Glu Gln
    165                 170                 175

att gat tat aat aat gcg aat gca tgg gca aat tgg tgg gga gat tgg      693
Ile Asp Tyr Asn Asn Ala Asn Ala Trp Ala Asn Trp Trp Gly Asp Trp
180                 185                 190                 195

att aga gct gat att ggt ggg tac gag aaa tgt ggt aat agt gaa att      741
Ile Arg Ala Asp Ile Gly Gly Tyr Glu Lys Cys Gly Asn Ser Glu Ile
                200                 205                 210

gaa atg tgt tta gct ggt tta cct gac ttt cgt acc gat tta act cat      789
Glu Met Cys Leu Ala Gly Leu Pro Asp Phe Arg Thr Asp Leu Thr His
            215                 220                 225

agt gtc ggg ttg ccg cca tta tta caa aca aaa tgg caa cag gaa tca      837
Ser Val Gly Leu Pro Pro Leu Leu Gln Thr Lys Trp Gln Gln Glu Ser
        230                 235                 240

agt ggt tat gaa aat tgg atc gtt cct gct gct agt aat tta cgt caa      885
Ser Gly Tyr Glu Asn Trp Ile Val Pro Ala Ala Ser Asn Leu Arg Gln
    245                 250                 255

gat tta gga att gct ccg gct gat tat att gtg aaa tgg ctt tca gct      933
Asp Leu Gly Ile Ala Pro Ala Asp Tyr Ile Val Lys Trp Leu Ser Ala
260                 265                 270                 275

tgg gtt gag gaa ttt ggt att gat ggt ttt cgt gtc gat acg gca aaa      981
Trp Val Glu Glu Phe Gly Ile Asp Gly Phe Arg Val Asp Thr Ala Lys
                280                 285                 290

cat gtg gaa atg cac cgt tgg gac caa tta aag caa gcc gca aat gaa     1029
His Val Glu Met His Arg Trp Asp Gln Leu Lys Gln Ala Ala Asn Glu
            295                 300                 305

gca ctt tgg aca tgg cga gaa aat aat cca gat aag cct ggt gca aat     1077
Ala Leu Trp Thr Trp Arg Glu Asn Asn Pro Asp Lys Pro Gly Ala Asn
        310                 315                 320

tgg gat gat gat ttt tgg atg gtt gca gaa gta tgg ggc cat ggt gta     1125
Trp Asp Asp Asp Phe Trp Met Val Ala Glu Val Trp Gly His Gly Val
    325                 330                 335

gga agg agc tca tat ttt gat cat ggt ttt gat tct gtc atc aat ttt     1173
Gly Arg Ser Ser Tyr Phe Asp His Gly Phe Asp Ser Val Ile Asn Phe
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340                 345                 350                 355

acg ttc caa gga gaa cat ggc aat ggt cca gcg tat aat tta gcg aca     1221
Thr Phe Gln Gly Glu His Gly Asn Gly Pro Ala Tyr Asn Leu Ala Thr
                360                 365                 370

atg gaa tca acg ttt tct cgt tat gcg cgt gac att aat tct gac cca     1269
Met Glu Ser Thr Phe Ser Arg Tyr Ala Arg Asp Ile Asn Ser Asp Pro
            375                 380                 385

tcg ttt aat gtg tta agt tat ata tca caa cat gat acg atg tta tac     1317
Ser Phe Asn Val Leu Ser Tyr Ile Ser Gln His Asp Thr Met Leu Tyr
        390                 395                 400

ccg aga aat cgt ctt ctc gat ggt gga aca tat tta atg ttg tta cct     1365
Pro Arg Asn Arg Leu Leu Asp Gly Gly Thr Tyr Leu Met Leu Leu Pro
    405                 410                 415

ggg gca gtt caa gtg ttt tat gga gat gaa acg gcc cgt ccg ttc ggt     1413
Gly Ala Val Gln Val Phe Tyr Gly Asp Glu Thr Ala Arg Pro Phe Gly
420                 425                 430                 435

cca aca ggc tct gat tct cac caa gga aca cgt tct tcg atg aat tgg     1461
Pro Thr Gly Ser Asp Ser His Gln Gly Thr Arg Ser Ser Met Asn Trp
                440                 445                 450

ggt tca gtt aat gaa caa gtt ctt gaa cac tgg caa aag atg ggg caa     1509
Gly Ser Val Asn Glu Gln Val Leu Glu His Trp Gln Lys Met Gly Gln
            455                 460                 465

ttc cgt aat aat cat gtt tca ata gga gca ggg aaa cac aaa caa att     1557
Phe Arg Asn Asn His Val Ser Ile Gly Ala Gly Lys His Lys Gln Ile
        470                 475                 480

gga agc agt cca tat aca ttc agt cgt gta cat gat gac gat cga gtt     1605
Gly Ser Ser Pro Tyr Thr Phe Ser Arg Val His Asp Asp Asp Arg Val
    485                 490                 495

gtt gtt tct atc ggt gcg agt ggt tct act aca att gat gtt tct tcc     1653
Val Val Ser Ile Gly Ala Ser Gly Ser Thr Thr Ile Asp Val Ser Ser
500                 505                 510                 515

gta ttt gat aac gga atg gaa tta cgg gac ttt tat aca ggg caa aca     1701
Val Phe Asp Asn Gly Met Glu Leu Arg Asp Phe Tyr Thr Gly Gln Thr
                520                 525                 530

gca aca gtt tca aat ggc cat gtg aca ttt gaa gct cat tct aat ggg     1749
Ala Thr Val Ser Asn Gly His Val Thr Phe Glu Ala His Ser Asn Gly
            535                 540                 545

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Val Ile Leu Ile Glu Gln Val Lys Ser Glu Leu Pro Thr Leu Ser Ala
        550                 555                 560

aat cca ggt ggt gag gaa ttt gat aca gag gaa att gaa gtg gaa tta     1845
Asn Pro Gly Gly Glu Glu Phe Asp Thr Glu Glu Ile Glu Val Glu Leu
    565                 570                 575

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cct agc aaa gaa ggc att gcc ttt gct aac gga gat gtc att tca att     1941
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ggt gct aat tta gcg gtt gat gaa tcg ctc acc ttg aag tta ttt gct     1989
Gly Ala Asn Leu Ala Val Asp Glu Ser Leu Thr Leu Lys Leu Phe Ala
            615                 620                 625

gaa aat aaa atg gga agt gca acg gaa gaa tat aca ttt aca aaa aca     2037
Glu Asn Lys Met Gly Ser Ala Thr Glu Glu Tyr Thr Phe Thr Lys Thr
        630                 635                 640

cca cca tat gta agc gtt tct gca acc cct cct ggt gga aca ttt aca     2085
Pro Pro Tyr Val Ser Val Ser Ala Thr Pro Pro Gly Gly Thr Phe Thr
    645                 650                 655

aca gag ttt gtt gaa atc aca tta aag tct aaa aat aca acg gat gct     2133
Thr Glu Phe Val Glu Ile Thr Leu Lys Ser Lys Asn Thr Thr Asp Ala
660                 665                 670                 675

ttt tac agt atc gat gga acc aat cca aag gat aat ggc att tct ttt     2181
Phe Tyr Ser Ile Asp Gly Thr Asn Pro Lys Asp Asn Gly Ile Ser Phe
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act gat ggg gag act att gtt atc ggt gag gaa gct act cat gga gat     2229
Thr Asp Gly Glu Thr Ile Val Ile Gly Glu Glu Ala Thr His Gly Asp
            695                 700                 705

gag ata aca tta cgg ctt tgg ggt caa aat gaa tac ggt gaa gtg gaa     2277
Glu Ile Thr Leu Arg Leu Trp Gly Gln Asn Glu Tyr Gly Glu Val Glu
        710                 715                 720

gaa cag tat acg tat aca aaa att gaa ggg ttg acc att cat ttc aaa     2325
Glu Gln Tyr Thr Tyr Thr Lys Ile Glu Gly Leu Thr Ile His Phe Lys
    725                 730                 735

aaa cca tct tct tgg gga caa cca caa cta tat tac tat ggt aca aat     2373
Lys Pro Ser Ser Trp Gly Gln Pro Gln Leu Tyr Tyr Tyr Gly Thr Asn
740                 745                 750                 755

cca act gta tct gaa ccg aca tgg gca aac gca ccg aac atg gaa cat     2421
Pro Thr Val Ser Glu Pro Thr Trp Ala Asn Ala Pro Asn Met Glu His
                760                 765                 770

act ggt ggt gac tgg tat aaa tac aca att agc ggt gtt aaa gaa gcc     2469
Thr Gly Gly Asp Trp Tyr Lys Tyr Thr Ile Ser Gly Val Lys Glu Ala
            775                 780                 785

ttt gtt att ttt aaa gat aat caa gga aat caa aca cca gga caa agt     2517
Phe Val Ile Phe Lys Asp Asn Gln Gly Asn Gln Thr Pro Gly Gln Ser
        790                 795                 800

caa tca gga tat ttc cgt gat aaa gaa gga tgg tat gac ggt tct tgg     2565
Gln Ser Gly Tyr Phe Arg Asp Lys Glu Gly Trp Tyr Asp Gly Ser Trp
    805                 810                 815

cat aat caa aac cca gaa gag cca aca aaa cct gct tcc cct aaa aat     2613
His Asn Gln Asn Pro Glu Glu Pro Thr Lys Pro Ala Ser Pro Lys Asn
820                 825                 830                 835
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ata gcc gtt cat gat gtc act gac aca aca gcg gtt att tca tgg gat     2661
Ile Ala Val His Asp Val Thr Asp Thr Thr Ala Val Ile Ser Trp Asp
                840                 845                 850

gtt gtc act ggt gct gtc gaa ggg tac cga tta tat gat gaa gat aat     2709
Val Val Thr Gly Ala Val Glu Gly Tyr Arg Leu Tyr Asp Glu Asp Asn
            855                 860                 865

aat gtt gta caa gaa aca aca aac gaa acg att aca tta gaa aac ctt     2757
Asn Val Val Gln Glu Thr Thr Asn Glu Thr Ile Thr Leu Glu Asn Leu
        870                 875                 880

acg tct gaa acg gaa tat tct ttt tac gtc acc gct ttt aat tcg gtt     2805
Thr Ser Glu Thr Glu Tyr Ser Phe Tyr Val Thr Ala Phe Asn Ser Val
    885                 890                 895

ggg gaa tcc gaa cga agt gaa caa att gca ttc act aca aaa aga aca     2853
Gly Glu Ser Glu Arg Ser Glu Gln Ile Ala Phe Thr Thr Lys Arg Thr
900                 905                 910                 915

aac ata gga aaa aac gaa gtg act att tat tat aat gga ttt gaa gct     2901
Asn Ile Gly Lys Asn Glu Val Thr Ile Tyr Tyr Asn Gly Phe Glu Ala
                920                 925                 930

cct tat att cat tat aaa ccg gtt gga gga atg tgg aca aca gta ccc     2949
Pro Tyr Ile His Tyr Lys Pro Val Gly Gly Met Trp Thr Thr Val Pro
            935                 940                 945

ggg gaa gcg atg tca cct tct gat aaa cct ggg ttt tcg aaa att aca     2997
Gly Glu Ala Met Ser Pro Ser Asp Lys Pro Gly Phe Ser Lys Ile Thr
        950                 955                 960

att aat gtt ggg gat gct gac gga att gtc gct gca ttt aat aac ggt     3045
Ile Asn Val Gly Asp Ala Asp Gly Ile Val Ala Ala Phe Asn Asn Gly
    965                 970                 975

cat ggt caa tgg gat aat aat caa gga cag gat tat acg ttt ggt tta     3093
His Gly Gln Trp Asp Asn Asn Gln Gly Gln Asp Tyr Thr Phe Gly Leu
980                 985                 990                 995

ggt acg ttt acc gta  atg aac ggt aaa att  agt gaa ggc gaa cct       3138
Gly Thr Phe Thr Val  Met Asn Gly Lys Ile  Ser Glu Gly Glu Pro
                1000                1005                1010

acg aat cag ttc act  caa att tat tac caa  aca ggc tgg tcg aat       3183
Thr Asn Gln Phe Thr  Gln Ile Tyr Tyr Gln  Thr Gly Trp Ser Asn
                1015                1020                1025

ccg cat att cat tat  gct atc gat ggt ggt  caa tgg aca cag tca       3228
Pro His Ile His Tyr  Ala Ile Asp Gly Gly  Gln Trp Thr Gln Ser
                1030                1035                1040

cct ggt gtc aga atg  caa cgt tct tct gaa  tat aca gga tat tct       3273
Pro Gly Val Arg Met  Gln Arg Ser Ser Glu  Tyr Thr Gly Tyr Ser
                1045                1050                1055

gtt gta acg att gat  gta ggc tca cga agt  ggt ctc acc gct gtc       3318
Val Val Thr Ile Asp  Val Gly Ser Arg Ser  Gly Leu Thr Ala Val
                1060                1065                1070

ttt aat aac gga agt  ggt caa tgg gat aat  aac caa ggc cga aac       3363
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Phe Asn Asn Gly Ser  Gly Gln Trp Asp Asn  Asn Gln Gly Arg Asn
                1075                1080                1085

tat caa ttt ggg aca  ggg att tac aca ctt  tca tca gga aga atc      3408
Tyr Gln Phe Gly Thr  Gly Ile Tyr Thr Leu  Ser Ser Gly Arg Ile
                1090                1095                1100

ata gaa ggg aca cct                                             3423
Ile Glu Gly Thr Pro
                1105
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<212> PRT
<213> Bacillus horikoshii

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Ala Met Thr Asp Arg Phe Tyr Asp Gly Asn Pro Ser Asn Asn Asn Ser
            20                  25                  30

Tyr Gly Arg Pro Gln Gln Asp Ala Trp Gly Gln Asn Ile Gly Thr Phe
        35                  40                  45

His Gly Gly Asp Leu Gln Gly Leu Thr Asp Lys Leu Asn Glu Gly Tyr
    50                  55                  60

Phe Thr Asp Leu Gly Ile Asn Ala Ile Trp Ile Thr Ala Pro Tyr Glu
65                  70                  75                  80

Gln Ile His Gly Trp Val Gly Gly Gly Ser Asp Gly Asp Phe Ala His
                85                  90                  95

Tyr Ala Phe His Gly Tyr Tyr Ala Leu Asp Tyr Thr Thr Val Asp Gln
            100                 105                 110

Asn Met Gly Thr Val Glu Glu Met Arg Glu Phe Val Asp Thr Ala His
        115                 120                 125

Glu Asn Gly Ile Arg Val Val Leu Asp Val Val Met Asn His Pro Gly
    130                 135                 140

Tyr Asn Thr Leu Lys Asp Met Glu Gln Tyr Gly Phe Gly Thr Gln Gln
145                 150                 155                 160

Leu Asn Ser Ser Trp Thr Pro Gly Pro Gly Gln Thr Trp His Ser His
                165                 170                 175
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His Glu Gln Ile Asp Tyr Asn Asn Ala Asn Ala Trp Ala Asn Trp Trp
            180                 185                 190

Gly Asp Trp Ile Arg Ala Asp Ile Gly Gly Tyr Glu Lys Cys Gly Asn
        195                 200                 205

Ser Glu Ile Glu Met Cys Leu Ala Gly Leu Pro Asp Phe Arg Thr Asp
    210                 215                 220

Leu Thr His Ser Val Gly Leu Pro Pro Leu Leu Gln Thr Lys Trp Gln
225                 230                 235                 240

Gln Glu Ser Ser Gly Tyr Glu Asn Trp Ile Val Pro Ala Ala Ser Asn
                245                 250                 255

Leu Arg Gln Asp Leu Gly Ile Ala Pro Ala Asp Tyr Ile Val Lys Trp
            260                 265                 270

Leu Ser Ala Trp Val Glu Glu Phe Gly Ile Asp Gly Phe Arg Val Asp
        275                 280                 285

Thr Ala Lys His Val Glu Met His Arg Trp Asp Gln Leu Lys Gln Ala
    290                 295                 300

Ala Asn Glu Ala Leu Trp Thr Trp Arg Glu Asn Asn Pro Asp Lys Pro
305                 310                 315                 320

Gly Ala Asn Trp Asp Asp Asp Phe Trp Met Val Ala Glu Val Trp Gly
                325                 330                 335

His Gly Val Gly Arg Ser Ser Tyr Phe Asp His Gly Phe Asp Ser Val
            340                 345                 350

Ile Asn Phe Thr Phe Gln Gly Glu His Gly Asn Gly Pro Ala Tyr Asn
        355                 360                 365

Leu Ala Thr Met Glu Ser Thr Phe Ser Arg Tyr Ala Arg Asp Ile Asn
    370                 375                 380

Ser Asp Pro Ser Phe Asn Val Leu Ser Tyr Ile Ser Gln His Asp Thr
385                 390                 395                 400

Met Leu Tyr Pro Arg Asn Arg Leu Leu Asp Gly Gly Thr Tyr Leu Met
                405                 410                 415
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Leu Leu Pro Gly Ala Val Gln Val Phe Tyr Gly Asp Glu Thr Ala Arg
            420                 425                 430

Pro Phe Gly Pro Thr Gly Ser Asp Ser His Gln Gly Thr Arg Ser Ser
        435                 440                 445

Met Asn Trp Gly Ser Val Asn Glu Gln Val Leu Glu His Trp Gln Lys
    450                 455                 460

Met Gly Gln Phe Arg Asn Asn His Val Ser Ile Gly Ala Gly Lys His
465                 470                 475                 480

Lys Gln Ile Gly Ser Ser Pro Tyr Thr Phe Ser Arg Val His Asp Asp
                485                 490                 495

Asp Arg Val Val Val Ser Ile Gly Ala Ser Gly Ser Thr Thr Ile Asp
            500                 505                 510

Val Ser Ser Val Phe Asp Asn Gly Met Glu Leu Arg Asp Phe Tyr Thr
        515                 520                 525

Gly Gln Thr Ala Thr Val Ser Asn Gly His Val Thr Phe Glu Ala His
    530                 535                 540

Ser Asn Gly Val Ile Leu Ile Glu Gln Val Lys Ser Glu Leu Pro Thr
545                 550                 555                 560

Leu Ser Ala Asn Pro Gly Gly Glu Glu Phe Asp Thr Glu Glu Ile Glu
                565                 570                 575

Val Glu Leu Met Val Ser Gly Ala Glu Gln Gly Tyr Tyr Thr Ile Asp
            580                 585                 590

Gly Ser Asp Pro Ser Lys Glu Gly Ile Ala Phe Ala Asn Gly Asp Val
        595                 600                 605

Ile Ser Ile Gly Ala Asn Leu Ala Val Asp Glu Ser Leu Thr Leu Lys
    610                 615                 620

Leu Phe Ala Glu Asn Lys Met Gly Ser Ala Thr Glu Glu Tyr Thr Phe
625                 630                 635                 640

Thr Lys Thr Pro Pro Tyr Val Ser Val Ser Ala Thr Pro Pro Gly Gly
                645                 650                 655
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Thr Phe Thr Thr Glu Phe Val Glu Ile Thr Leu Lys Ser Lys Asn Thr
            660                 665                 670

Thr Asp Ala Phe Tyr Ser Ile Asp Gly Thr Asn Pro Lys Asp Asn Gly
        675                 680                 685

Ile Ser Phe Thr Asp Gly Glu Thr Ile Val Ile Gly Glu Glu Ala Thr
    690                 695                 700

His Gly Asp Glu Ile Thr Leu Arg Leu Trp Gly Gln Asn Glu Tyr Gly
705                 710                 715                 720

Glu Val Glu Glu Gln Tyr Thr Tyr Thr Lys Ile Glu Gly Leu Thr Ile
                725                 730                 735

His Phe Lys Lys Pro Ser Ser Trp Gly Gln Pro Gln Leu Tyr Tyr Tyr
            740                 745                 750

Gly Thr Asn Pro Thr Val Ser Glu Pro Thr Trp Ala Asn Ala Pro Asn
        755                 760                 765

Met Glu His Thr Gly Gly Asp Trp Tyr Lys Tyr Thr Ile Ser Gly Val
    770                 775                 780

Lys Glu Ala Phe Val Ile Phe Lys Asp Asn Gln Gly Asn Gln Thr Pro
785                 790                 795                 800

Gly Gln Ser Gln Ser Gly Tyr Phe Arg Asp Lys Glu Gly Trp Tyr Asp
                805                 810                 815

Gly Ser Trp His Asn Gln Asn Pro Glu Glu Pro Thr Lys Pro Ala Ser
            820                 825                 830

Pro Lys Asn Ile Ala Val His Asp Val Thr Asp Thr Thr Ala Val Ile
        835                 840                 845

Ser Trp Asp Val Val Thr Gly Ala Val Glu Gly Tyr Arg Leu Tyr Asp
    850                 855                 860

Glu Asp Asn Asn Val Val Gln Glu Thr Thr Asn Glu Thr Ile Thr Leu
865                 870                 875                 880

Glu Asn Leu Thr Ser Glu Thr Glu Tyr Ser Phe Tyr Val Thr Ala Phe
                885                 890                 895

Asn Ser Val Gly Glu Ser Glu Arg Ser Glu Gln Ile Ala Phe Thr Thr
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           900                 905                 910

Lys Arg Thr Asn Ile Gly Lys Asn Glu Val Thr Ile Tyr Tyr Asn Gly
        915                 920                 925

Phe Glu Ala Pro Tyr Ile His Tyr Lys Pro Val Gly Gly Met Trp Thr
    930                 935                 940

Thr Val Pro Gly Glu Ala Met Ser Pro Ser Asp Lys Pro Gly Phe Ser
945                 950                 955                 960

Lys Ile Thr Ile Asn Val Gly Asp Ala Asp Gly Ile Val Ala Ala Phe
                965                 970                 975

Asn Asn Gly His Gly Gln Trp Asp Asn Asn Gln Gly Gln Asp Tyr Thr
           980                 985                 990

Phe Gly Leu Gly Thr Phe Thr Val  Met Asn Gly Lys Ile  Ser Glu Gly
        995                 1000                1005

Glu Pro  Thr Asn Gln Phe Thr  Gln Ile Tyr Tyr Gln  Thr Gly Trp
    1010                1015                1020

Ser Asn  Pro His Ile His Tyr  Ala Ile Asp Gly Gly  Gln Trp Thr
    1025                1030                1035

Gln Ser  Pro Gly Val Arg Met  Gln Arg Ser Ser Glu  Tyr Thr Gly
    1040                1045                1050

Tyr Ser  Val Val Thr Ile Asp  Val Gly Ser Arg Ser  Gly Leu Thr
    1055                1060                1065

Ala Val  Phe Asn Asn Gly Ser  Gly Gln Trp Asp Asn  Asn Gln Gly
    1070                1075                1080

Arg Asn  Tyr Gln Phe Gly Thr  Gly Ile Tyr Thr Leu  Ser Ser Gly
    1085                1090                1095

Arg Ile  Ile Glu Gly Thr Pro
    1100                1105
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PCT/DK2005/000794

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34, Chemin des Colombettes
1211 Geneva 20
SWITZERLAND

24-MAR-2006

Our ref: 10699.204-WO

International Patent Application No PCT/DK2005/000794

Dear Madam/Sir

Subsequent to the filing of the above-identified international patent application it has come to our attention that we inadvertently omitted to indicate that the invention included a deposited microorganism in the Request Form, see page 2, line 30 to page 3, line 6:

Deposit no.	Date of Deposit	Deposit institute
12512	29-JUL-1987	National Collections of Industrial and Marine Bacteria Ltd. (NCIMB)

A statement regarding expert option under the Budapest Treaty is enclosed.

We herewith respectfully submit that the above-identified be recorded relative to said international application.

Please confirm the change by return mail.

Yours sincerely,
Novozymes A/S

Bo Kalum, Patent counsel

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Internet:
www.novozymes.com

CVR number:
10 00 71 27

Our ref: 10699.204-WO

Additional Indications (Form PCT/RO/134)

Statement regarding the "expert option"

Deposited microorganisms:

With respect to the deposited microorganism set out on Form PCT/RO/134 we request the so-called expert option:

Deposit no.	Date of Deposit	Deposit institute
12512	29-JUL-1987	NCIMB

Until the publication of the mention of grant of a European patent or, where applicable, for twenty years from the date of filing if the application has been refused, withdrawn or deemed withdrawn, a sample of the deposited micro-organism is only to be provided to an independent expert nominated by the person requesting the sample (cf. Rule 28(4) EPC). And as far as Australia is concerned, the expert option is likewise requested, reference being had to Regulation 3.25 of Australia Statutory Rules 1991 No 71. Also, for Canada we request that only an independent expert nominated by the Commissioner is authorized to have access to a sample of the microorganism deposited.

24-MAR-2006

Novozymes A/S

Bo Kalum

Bo Kalum, Patent Counsel

INTERNATIONAL SEARCH REPORT

International application No
PCT/DK2005/000794

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N9/28 C12N15/56 C12N1/21 C12P19/14 C11D3/386

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N C11D C12P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 01/14532 A (NOVOZYMES A/S) 1 March 2001 (2001-03-01) cited in the application the whole document	1-13
Y	WO 95/26397 A (NOVO NORDISK A/S; OUTTRUP, HELLE; BISGAARD-FRANTZEN, HENRIK; OESTERGAA) 5 October 1995 (1995-10-05) cited in the application the whole document	1-13
X	WO 00/58447 A (DANISCO A/S; KRAGH, KARSTEN, MATHIAS; LARSEN, BJARNE; DUEDAHL-OLESEN,) 5 October 2000 (2000-10-05)	1-12
Y		1-13

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[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
21 April 2006	18/05/2006

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Van der Schaal, C

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Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE SWISS-PROT [Online] ebi; 1 October 2000 (2000-10-01), XP002377812 accession no. Q9KFR4_BACHD Database accession no. Q9KFR4	1-12
Y	abstract -& DATABASE EMBL [Online] 2 December 2004 (2004-12-02), TAKAMI H AND TAKAKI Y: "Bacillus halodurans C-123 DNA" XP002377813 accession no. BA000004 Database accession no. BA000004 abstract & TAKAMI H ET AL: "COMPLETE GENOME SEQUENCE OF THE ALKALIPHILIC BACTERIUM BACILLUS HALODURANS AND GENOMIC SEQUENCE COMPARISON WITH BACILLUS SUBTILIS" NUCLEIC ACIDS RESEARCH, OXFORD UNIVERSITY PRESS, SURREY, GB, vol. 28, no. 21, 2000, pages 4317-4331, XP008044903 ISSN: 0305-1048	1-13

X	DATABASE UniProt ebi; 23 November 2004 (2004-11-23), TAKAKI Y ET AL: "Bacillus clausii" XP002377814 retrieved from EBI accession no. Q5WHK9_BACSK Database accession no. Q5WHK9	1-12
Y	the whole document	1-12

Y	SHIROKIZAWA O ET AL: "NUCLEOTIDE SEQUENCE OF THE G-6 AMYLASE GENE FROM ALKALOPHILIC BACILLUS-SP H-167" FEMS MICROBIOLOGY LETTERS, AMSTERDAM, NL, vol. 70, no. 2, 1990, pages 131-136, XP002145563 ISSN: 0378-1097 the whole document	1-12

Y	SHIROKIZAWA O ET AL: "CLONING AND EXPRESSION OF THE MALTOHEXAOSE-FORMING AMYLASE GENE FROM ALKALOPHILIC BACILLUS SP. H-167 IN ESCHERICHIA COLI" AGRICULTURAL AND BIOLOGICAL CHEMISTRY, JAPAN SOC. FOR BIOSCIENCE, BIOTECHNOLOGY AND AGROCHEM, TOKYO, JP, vol. 53, no. 2, 1 February 1989 (1989-02-01), pages 491-495, XP000026624 ISSN: 0002-1369 page 491, right-hand column, paragraph 2	1-12

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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	NIELSEN P ET AL: "PHENETIC DIVERSITY OF ALKALIPHILIC BACILLUS STRAINS: PROPOSAL FOR NINE NEW SPECIES" MICROBIOLOGY, SOCIETY FOR GENERAL MICROBIOLOGY, READING, GB, vol. 141, no. 7, 1995, pages 1745-1761, XP001203463 ISSN: 1350-0872 page 1745, left-hand column page 1760, left-hand column, line 2 -----	1-12
Y	HORIKOSHI KOKI: "Alkaliphiles: From an industrial point of view" FEMS MICROBIOLOGY REVIEWS, vol. 18, no. 2-3, 1996, pages 259-270, XP002377542 ISSN: 0168-6445 page 259, paragraph 1 page 262, right-hand column, paragraph 2 page 262, right-hand column, paragraph 5 - page 263, left-hand column -----	1-12

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/DK2005/000794

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 0114532 A	01-03-2001	AU 6686200 A	19-03-2001
		CN 1399676 A	26-02-2003
		EP 1212409 A2	12-06-2002
		JP 2003507059 T	25-02-2003
WO 9526397 A	05-10-1995	AT 305031 T	15-10-2005
		AU 2067795 A	17-10-1995
		BR 9507229 A	16-09-1997
		CA 2186592 A1	05-10-1995
		DK 0753057 T3	30-01-2006
		EP 0753057 A1	15-01-1997
		FI 963861 A	27-09-1996
		JP 9510617 T	28-10-1997
		US 5824531 A	20-10-1998
WO 0058447 A	05-10-2000	AU 3450200 A	16-10-2000