



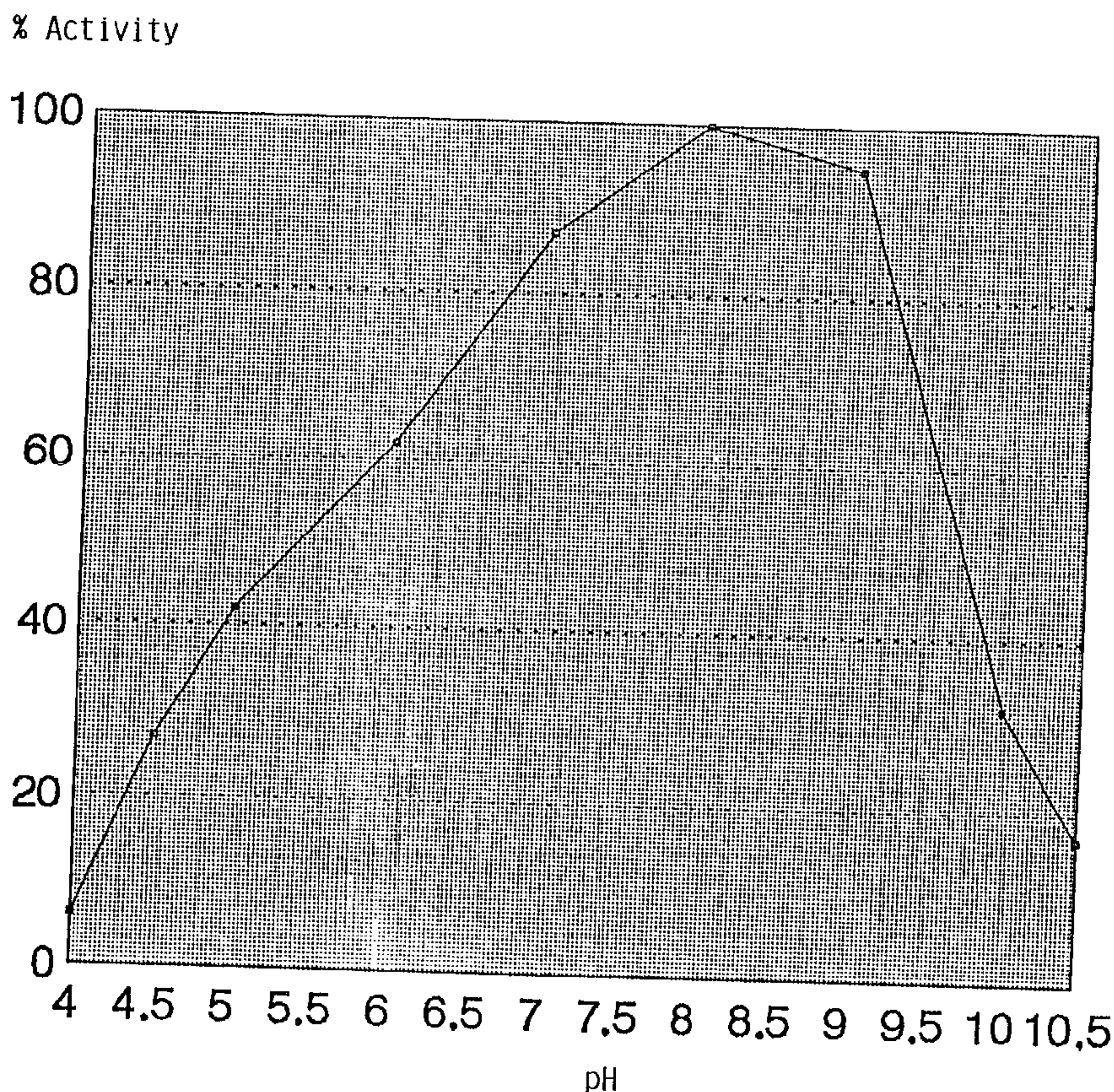
(86) Date de dépôt PCT/PCT Filing Date: 1995/03/29  
 (87) Date publication PCT/PCT Publication Date: 1995/10/05  
 (45) Date de délivrance/Issue Date: 2008/02/19  
 (85) Entrée phase nationale/National Entry: 1996/09/26  
 (86) N° demande PCT/PCT Application No.: DK 1995/000142  
 (87) N° publication PCT/PCT Publication No.: 1995/026397  
 (30) Priorités/Priorities: 1994/03/29 (DK353/94);  
 1994/11/03 (DK1271/94); 1995/02/03 (DK123/95)

(51) Cl.Int./Int.Cl. *C12N 15/56* (2006.01),  
*C11D 3/386* (2006.01), *C12C 7/04* (2006.01),  
*C12N 1/21* (2006.01), *C12N 9/28* (2006.01),  
*C12P 19/14* (2006.01), *C12P 21/02* (2006.01),  
*C12S 11/00* (2006.01), *C12S 3/08* (2006.01),  
*D06L 1/14* (2006.01), *D06M 16/00* (2006.01),  
*D21C 5/00* (2006.01), *D21C 5/02* (2006.01)

(72) Inventeurs/Inventors:  
 OUTTRUP, HELLE, DK;  
 BISGARD-FRANTZEN, HENRIK, DK;  
 ISTERGAARD, PETER RAHBEK, DK;  
 RASMUSSEN, MICHAEL DOLBERG, DK;  
 VAN DER ZEE, PIA, DK

(73) Propriétaire/Owner:

(54) Titre : AMYLASE ALCALINE ISSUE D'UN BACILLE  
 (54) Title: ALKALINE BACILLUS AMYLASE



(57) Abrégé/Abstract:

An  $\alpha$ -amylase characterized by having a specific activity at least 25 % higher than the specific activity of Termamyl® at a temperature in the range of 25 °C to 55 °C and at a pH value in the range of pH 8 to pH 10.

(73) Propriétaires(suite)/Owners(continued):NOVOZYMES A/S, DK

(74) Agent: MCCARTHY TETRAULT LLP





INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<p>(51) International Patent Classification <sup>6</sup> : <b>C12N 9/28, C11D 3/386</b></p>	<p><b>A1</b></p>	<p>(11) International Publication Number: <b>WO 95/26397</b></p>
		<p>(43) International Publication Date: 5 October 1995 (05.10.95)</p>

<p>(21) International Application Number: <b>PCT/DK95/00142</b></p>	<p>(74) Common Representative: <b>NOVO NORDISK A/S; Corporate Patents, Novo Allé, DK-2880 Bagsværd (DK).</b></p>									
<p>(22) International Filing Date: <b>29 March 1995 (29.03.95)</b></p>										
<p>(30) Priority Data:</p> <table border="0"> <tr> <td>353/94</td> <td>29 March 1994 (29.03.94)</td> <td>DK</td> </tr> <tr> <td>1271/94</td> <td>3 November 1994 (03.11.94)</td> <td>DK</td> </tr> <tr> <td>123/95</td> <td>3 February 1995 (03.02.95)</td> <td>DK</td> </tr> </table>	353/94	29 March 1994 (29.03.94)	DK	1271/94	3 November 1994 (03.11.94)	DK	123/95	3 February 1995 (03.02.95)	DK	<p>(81) Designated States: <b>AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LU, LV, MD, MG, MN, MW, MX, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TT, UA, UG, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ, UG).</b></p>
353/94	29 March 1994 (29.03.94)	DK								
1271/94	3 November 1994 (03.11.94)	DK								
123/95	3 February 1995 (03.02.95)	DK								
<p>(71) Applicant (for all designated States except US): <b>NOVO NORDISK A/S [DK/DK]; Novo Allé, DK-2880 Bagsværd (DK).</b></p>										
<p>(72) Inventors; and</p>										
<p>(75) Inventors/Applicants (for US only): <b>OUTTRUP, Helle [DK/DK]; Novo Nordisk a/s, Novo Allé, DK-2880 Bagsværd (DK). BISGÅRD-FRANTZEN, Henrik [DK/DK]; Novo Nordisk a/s, Novo Allé, DK-2880 Bagsværd (DK). ØSTERGAARD, Peter, Rahbek [DK/DK]; Novo Nordisk a/s, Novo Allé, DK-2880 Bagsværd (DK). RASMUSSEN, Michael, Dolberg [DK/DK]; Novo Nordisk a/s, Novo Allé, DK-2880 Bagsværd (DK). VAN DER ZEE, Pia [DK/DK]; Novo Nordisk a/s, Novo Allé, DK-2880 Bagsværd (DK).</b></p>										

**2186592**

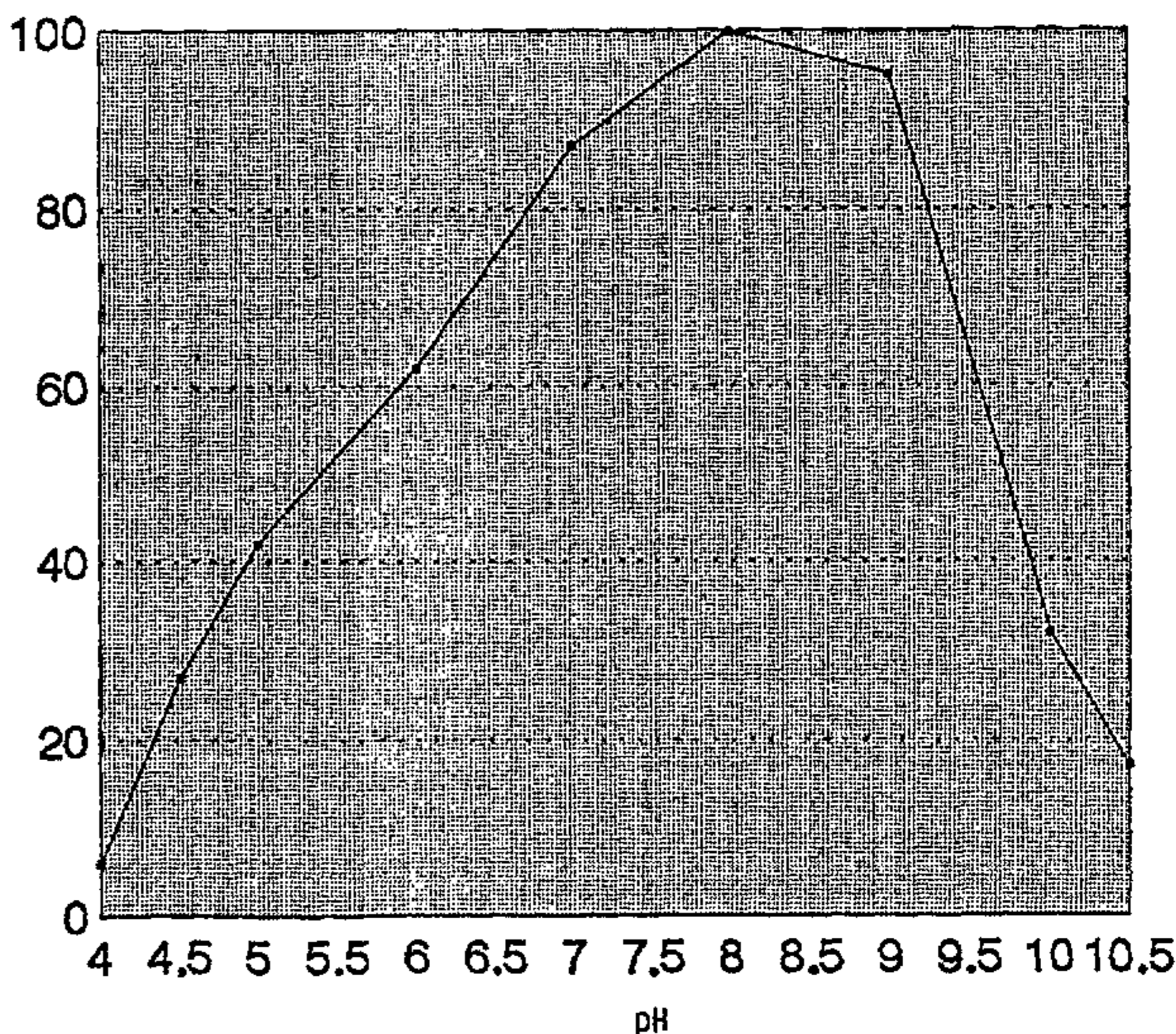
**Published**  
With international search report.

(54) Title: **ALKALINE BACILLUS AMYLASE**

(57) Abstract

An  $\alpha$ -amylase characterized by having a specific activity at least 25 % higher than the specific activity of Termamyl® at a temperature in the range of 25 °C to 55 °C and at a pH value in the range of pH 8 to pH 10.

% Activity



**ALKALINE BACILLUS AMYLASE****FIELD OF INVENTION**

The present invention relates to amylases having improved dishwashing and/or washing performance.

**5 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  
10 baking. A further use of  $\alpha$ -amylases, which is becoming increasingly important is the removal of starchy stains during washing and dishwashing.

Examples of commercial  $\alpha$ -amylase products are Termamyl<sup>®</sup>, BAN<sup>®</sup> and Fungamyl<sup>®</sup>, all available from Novo Nordisk  
15 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, which means that they do not display optimal activity in detergent solutions owing to the alkaline character of the detergents.

20 It is an object of the present invention to provide novel  $\alpha$ -amylases with improved performance in alkaline solutions, especially in alkaline detergent solutions.

**SUMMARY OF THE INVENTION**

The present invention provides  $\alpha$ -amylases with a very  
25 high specific activity at pH 8-10 and at temperatures of from 30°C to around 60°C, conditions normal in detergent solutions.

Accordingly, the present invention relates to an  $\alpha$ -amylase having a specific activity at least 25% higher than the specific activity of Termamyl<sup>®</sup> at a temperature in the range of  
30 25°C to 55°C and at a pH value in the range of pH 8 to pH 10, measured by the  $\alpha$ -amylase activity assay as described herein.



2186592

2

## BRIEF DESCRIPTION OF DRAWINGS

The present invention is further illustrated with reference to the accompanying drawings, in which

Fig. 1 shows the relation between pH and the  $\alpha$ -amylase activity of a novel amylase (obtained from Bacillus strain NCIB 12289), determined as described in Example 2.

Fig. 2 shows the pH profile of an  $\alpha$ -amylase obtained from Bacillus strain NCIB 12512 (I), of an  $\alpha$ -amylase obtained from Bacillus strain NCIB 12513 (II) and of Termamyl® (III) determined at 55°C in the pH interval of from 4 to 10.5, the test being performed as described in Example 3.

Fig. 3 shows the temperature profile of an  $\alpha$ -amylase obtained from Bacillus strain NCIB 12512 (I), of an  $\alpha$ -amylase obtained from Bacillus strain NCIB 12513 (II) and of Termamyl® (III) determined at pH 10.0 in the temperature interval of from 25°C to 95°C, the test being performed as described in Example 3.

Fig. 4 shows the RSF-rating - removal of starch film from dish- and glassware, as a function of the dosage of a novel  $\alpha$ -amylase (obtained from Bacillus strain NCIB 12289) at 55°C, the test being performed as described in Example 4.

Fig. 5 shows the RSF-rating - removal of starch film from dish- and glassware, as a function of the dosage of a novel  $\alpha$ -amylase (obtained from Bacillus strain NCIB 12512) at 45°C (\*), at 55°C (\*) and at 65°C (x), the test being performed as described in Example 4.

## DETAILED DESCRIPTION OF THE INVENTION

### The $\alpha$ -Amylases of the Invention

One embodiment of the present invention provides an  $\alpha$ -amylase having a specific activity at least 25% higher or at least 35% higher or at least 45% higher or at least 55% higher or at least 65% higher or at least 75% or at least 25-75% higher than the specific activity of Termamyl® at a temperature in the range of 25°C to 55°C or at a temperature in the range

of 25°C to 35°C or at a temperature in the range of 35°C to 45°C or at a temperature in the range of 45°C to 55°C and at a pH value in the range of pH 8 to pH 10 or at a pH value in the range of pH 8 to 8.5 or at a pH value in the range of pH 8.5 to 9.0 or at a pH value in the range of pH 9.0 to 9.5 or at a pH value in the range of pH 9.5 to 10.0, measured by the  $\alpha$ -amylase activity assay as described herein.

It has surprisingly been found that preferred novel  $\alpha$ -amylases of the invention may be characterized by having a specific activity at least 25% higher than the specific activity of Termamyl® at any temperature in the range of 25°C to 55°C and at any pH value in the range of from pH 8 to pH 10, measured by the  $\alpha$ -amylase activity assay as described herein.

Compared with known  $\alpha$ -amylases it is very remarkable how well the  $\alpha$ -amylases of the invention perform at pH 10; accordingly in a preferred embodiment the  $\alpha$ -amylase is characterized by having a specific activity at least 25% higher than the specific activity of Termamyl® at any temperature in the range of 25°C to 55°C and at pH 10, using the  $\alpha$ -amylase activity assay as described herein.

In another aspect the invention relates to an  $\alpha$ -amylase comprising the amino acid sequence shown in SEQ ID No. 1 or an  $\alpha$ -amylase being at least 80% homologous with the amino acid sequence (SEQ ID No.1), preferably being at least 85% homologous with SEQ ID No. 1, more preferably being at least 90% homologous with SEQ ID No.1.

A polypeptide is considered to be X% homologous to the parent  $\alpha$ -amylase if a comparison of the respective amino acid sequences, performed via known algorithms, such as the one described by Lipman and Pearson in Science 227, 1985, p. 1435, reveals an identity of X%.

In a further aspect the invention relates to an  $\alpha$ -amylase comprising the amino acid sequence shown in SEQ ID No. 2 or an  $\alpha$ -amylase being at least 80% homologous with the amino acid sequence (SEQ ID No.2), preferably being at least 85% homologous with SEQ ID No. 2, more preferably being at least 90% homologous with SEQ ID No.2.

In another embodiment the invention relates to an  $\alpha$ -amylase comprising an N-terminal amino acid sequence identical to that shown in SEQ ID No. 3 or an  $\alpha$ -amylase being at least 80% homologous with SEQ ID No.3 in the N-terminal, preferably being at least 90% homologous with SEQ ID No.3 in the N-terminal.

Preferred  $\alpha$ -amylases of the invention are obtainable from an alkaliphilic Bacillus species, particularly from one of the Bacillus strains NCIB 12289, NCIB 12512, NCIB 12513 and DSM 10 9375. In the context of the present invention, the term "obtainable from" is intended not only to indicate an  $\alpha$ -amylase produced by a Bacillus strain but also an  $\alpha$ -amylase encoded by a DNA sequence isolated from such a Bacillus strain and produced in a host organism transformed with said DNA sequence.

15 The strain NCIB 12289 is described in detail in EP 0 277 216. The strain NCIB 12289 has been deposited according to the Budapest Treaty on the International Recognition of the Deposits of Microorganisms for the Purpose of Patent Procedures, on 8 July 1986 at The National Collection of Industrial Bacteria (NCIB) under accession no. NCIB 12289.

The strain NCIB 12512 is described in detail in EP 0 277 216. The strain NCIB 12512 has been deposited according to the Budapest Treaty on the International Recognition of the Deposits of Microorganisms for the Purpose of Patent Procedures, on 5 August 1987 at The National Collection of Industrial Bacteria (NCIB) under accession no. NCIB 12512.

The strain NCIB 12513 is described in detail in EP 0 277 216. The strain NCIB 12513 has been deposited according to the Budapest Treaty on the International Recognition of the Deposits of Microorganisms for the Purpose of Patent Procedures, on 5 August 1987 at The National Collection of Industrial Bacteria (NCIB) under accession no. NCIB 12513.

35 The strain DSM 9375 has been deposited according to the Budapest Treaty on the International Recognition of the Deposits of Microorganisms for the Purpose of Patent Procedures, on 16 August 1994 at Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSM) under Accession No. DSM



9375.

Cloning a DNA sequence encoding an  $\alpha$ -amylase

The DNA sequence encoding an  $\alpha$ -amylase of the invention may be isolated from any cell or microorganism producing the  $\alpha$ -amylase in question, using various methods well known in the art. First, a genomic DNA and/or cDNA library should be constructed using chromosomal DNA or messenger RNA from the organism that produces the  $\alpha$ -amylase to be studied. Then, if the amino acid sequence of the  $\alpha$ -amylase is known, homologous, labelled oligonucleotide probes may be synthesized and used to identify  $\alpha$ -amylase-encoding clones from a genomic library prepared from the organism in question. Alternatively, a labelled oligonucleotide probe containing sequences homologous to a known  $\alpha$ -amylase gene could be used as a probe to identify  $\alpha$ -amylase-encoding clones, using hybridization and washing conditions of lower stringency. According to the present invention preferred probes may be constructed on the basis of SEQ ID No. 1 or on the basis of SEQ ID No. 2 or on the basis of SEQ ID No. 4 or on the basis of SEQ ID No 5.

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

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

Finally, the DNA sequence may be of mixed genomic and synthetic origin, mixed synthetic and cDNA origin or mixed



2186592

6

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

### Expression of $\alpha$ -amylase

10 According to the invention, an  $\alpha$ -amylase-encoding DNA sequence produced by methods described above, or by any alternative methods known in the art, can be expressed, in enzyme form, using an expression vector which typically includes control sequences encoding a promoter, operator, 15 ribosome binding site, translation initiation signal, and, optionally, a repressor gene or various activator genes.

The recombinant expression vector carrying the DNA sequence encoding an  $\alpha$ -amylase of the invention may be any vector which may conveniently be subjected to recombinant DNA 20 procedures, and the choice of vector will often depend on the host cell into which it is to be introduced. Thus, the vector may be an autonomously replicating vector, i.e. a vector which exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication, e.g., a plasmid, a 25 bacteriophage or an extrachromosomal element, minichromosome or an artificial chromosome. Alternatively, the vector may be one which, when introduced into a host cell, is integrated into the host cell genome and replicated together with the chromosome(s) into which it has been integrated.

30 In the vector, the DNA sequence should be operably connected to a suitable promoter sequence. The promoter may be any DNA sequence which shows transcriptional activity in the host cell of choice and may be derived from genes encoding proteins either homologous or heterologous to the host cell. 35 Examples of suitable promoters for directing the transcription of the DNA sequence encoding an  $\alpha$ -amylase of the invention,

especially in a bacterial host, are the promoter of the lac operon of E.coli, the Streptomyces coelicolor agarase gene dagA promoters, the promoters of the Bacillus licheniformis  $\alpha$ -amylase gene (amyL), the promoters of the Bacillus stearothermophilus maltogenic amylase gene (amyM), the promoters of the Bacillus Amyloliquefaciens  $\alpha$ -amylase (amyQ), the promoters of the Bacillus subtilis xylA and xylB genes etc. For transcription in a fungal host, examples of useful promoters are those derived from the gene encoding A. oryzae TAKA amylase, Rhizomucor miehei aspartic proteinase, A. niger neutral  $\alpha$ -amylase, A. niger acid stable  $\alpha$ -amylase, A. niger glucoamylase, Rhizomucor miehei lipase, A. oryzae alkaline protease, A. oryzae triose phosphate isomerase or A. nidulans acetamidase.

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

20 The vector may further comprise a DNA sequence enabling the vector to replicate in the host cell in question. Examples of such sequences are the origins of replication of plasmids pUC19, pACYC177, pUB110, pE194, pAMB1 and pIJ702.

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

While intracellular expression may be advantageous  
35 in some respects, e.g., when using certain bacteria as host cells, it is generally preferred that the expression is extracellular.



2186592

8

Procedures suitable for constructing vectors of the invention encoding an  $\alpha$ -amylase and containing the promoter, terminator and other elements, respectively, are well known to persons skilled in the art (cf., for instance, Sambrook et al. in Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor, 1989).

The cell of the invention, either comprising a DNA construct or an expression vector of the invention as defined above, is advantageously used as a host cell in the recombinant production of an  $\alpha$ -amylase of the invention. The cell may be transformed with the DNA construct of the invention encoding the  $\alpha$ -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 suitable bacteria are grampositive bacteria such as Bacillus subtilis, Bacillus licheniformis, Bacillus lentus, Bacillus brevis, Bacillus stearothermophilus, Bacillus alkalophilus, Bacillus amyloliquefaciens, Bacillus coagulans, Bacillus circulans, Bacillus lautus, Bacillus megaterium, Bacillus thuringiensis, or Streptomyces lividans or Streptomyces murinus, or gramnegative bacteria such as E.coli.

The transformation of the bacteria may, for instance, be effected by protoplast transformation or by using competent cells in a manner known per se.

The yeast organism may favourably be selected from a species of Saccharomyces or Schizosaccharomyces, e.g., Saccharomyces cerevisiae. The filamentous fungus may advan-

tageously belong to a species of Aspergillus, e.g., Aspergillus oryzae or Aspergillus niger. Fungal cells may be transformed by a process involving protoplast formation and transformation of the protoplasts followed by regeneration of the cell wall in a manner known per se. A suitable procedure for transformation of Aspergillus host cells is described in EP 238 023.

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

The medium used to cultivate the cells may be any conventional medium suitable for growing the host cell in  
15 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).

20 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  
25 sulphate, followed by the use of chromatographic procedures such as ion exchange chromatography, affinity chromatography, or the like.

#### Assay for $\alpha$ -Amylase Activity

$\alpha$ -Amylase activity was determined by a method  
30 employing Phadebas<sup>®</sup> tablets as substrate. Phadebas tablets (Phadebas<sup>®</sup> Amylase Test, supplied by Pharmacia Diagnostic) contain a cross-linked insoluble blue-coloured starch polymer which has been mixed with bovine serum albumin and a buffer substance and tabletted.

35 For every single measurement one tablet is suspended in a tube containing 5 ml 50 mM Britton-Robinson buffer (50 mM



acetic acid, 50 mM phosphoric acid, 50 mM boric acid, 0.1 mM  $\text{CaCl}_2$ , pH adjusted to the value of interest with NaOH). The test is performed in a water bath at the temperature of interest. The  $\alpha$ -amylase to be tested is diluted in x ml of 50 mM Britton-Robinson buffer. 1 ml of this  $\alpha$ -amylase solution is added to the 5 ml 50 mM Britton-Robinson buffer. The starch is hydrolysed by the  $\alpha$ -amylase giving soluble blue fragments. The absorbance of the resulting blue solution, measured spectrophotometrically at 620 nm, is a function of the  $\alpha$ -amylase activity.

It is important that the measured 620 nm absorbance after 10 or 15 minutes of incubation (testing time) is in the range of 0.2 to 2.0 absorbance units at 620 nm. In this absorbance range there is linearity between activity and absorbance (Lambert-Beer law). The dilution of the enzyme must therefore be adjusted to fit this criterion.

Under a specified set of conditions (temp., pH, reaction time, buffer conditions) 1 mg of a given  $\alpha$ -amylase will hydrolyse a certain amount of substrate and a blue colour will be produced. The colour intensity is measured at 620 nm. The measured absorbance is directly proportional to the specific activity (activity/mg of pure  $\alpha$ -amylase protein) of the  $\alpha$ -amylase in question under the given set of conditions. Thus, by testing different  $\alpha$ -amylases of interest (including Termamyl<sup>®</sup>, the  $\alpha$ -amylase used for reference) under identical conditions, the specific activity of each of the  $\alpha$ -amylases at a given temperature and at a given pH can be compared directly, and the ratio of the specific activity of each of the  $\alpha$ -amylases of interest relative to the specific activity of Termamyl<sup>®</sup> can be determined.

### Industrial 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, in particular the enzyme finds potential applications as a component in washing, dishwashing and hard surface cleaning detergent compositions, but it may

also be useful in the production of sweeteners and ethanol from starch. Conditions for conventional starch-converting processes and liquefaction and/or saccharification processes are described in, for instance, US Patent No. 3,912,590 and EP patent publications Nos. 252,730 and 63,909.

Being alkaline the  $\alpha$ -amylases of the invention also possess valuable properties in the production of lignocellulosic materials, such as pulp, paper and cardboard, from starch reinforced waste paper and cardboard, especially where  
10 repulping occurs at pH above 7 and where amylases can facilitate the disintegration of the waste material through degradation of the reinforcing starch. The  $\alpha$ -amylases of the invention are especially useful in the deinking/recycling processes of making paper out of old starch-coated or starch-containing  
15 printed paper. It is usually desirable to remove the printing ink in order to produce new paper of high brightness; examples of how the  $\alpha$ -amylases of the invention may be used in this way are described in PCT/DK 94/00437.

The  $\alpha$ -amylases of the invention may also be very  
20 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  
25 simpler integrated process.

The  $\alpha$ -amylases 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  
30 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.  
35 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.

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.

#### Detergent Compositions

According to the invention, the  $\alpha$ -amylases may typically be a component of a detergent composition, e.g., a laundry detergent composition or a dishwashing detergent composition. As such, it may be included in the detergent composition in the form of a non-dusting granulate, a stabilized liquid, or a protected enzyme. Non-dusting granulates may be produced, e.g., as disclosed in US 4,106,991 and 4,661,452 (both to Novo Industri A/S) 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 molecular weights of 1000 to 20000; ethoxylated nonylphenols 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 patent GB 1483591. Liquid enzyme preparations may, for instance, be stabilized by adding a polyol such as propylene

glycol, a sugar or sugar alcohol, lactic acid or boric acid according to established methods. Other enzyme stabilizers are well known in the art. Protected enzymes may be prepared according to the method disclosed in EP 238,216.

5 The detergent composition of the invention may be in any convenient form, e.g. as powder, granules, paste or liquid. A liquid detergent may be aqueous, typically containing up to 70% water and 0-30% organic solvent, or nonaqueous.

The detergent composition comprises one or more surf-  
10 actants, each of which may be anionic, nonionic, cationic, or amphoteric (zwitterionic). The detergent will usually contain 0-50% of anionic surfactant such as linear alkylbenzene-sulfonate (LAS), alpha-olefinsulfonate (AOS), alkyl sulfate (fatty alcohol sulfate) (AS), alcohol ethoxysulfate (AEOS or  
15 AES), secondary alkanesulfonates (SAS), alpha-sulfo fatty acid methyl esters, alkyl- or alkenylsuccinic acid, or soap. It may also contain 0-40% of nonionic surfactant such as alcohol ethoxylate (AEO or AE), alcohol propoxylate, carboxylated alcohol ethoxylates, nonylphenol ethoxylate, alkylpolygly-  
20 coside, alkyldimethylamine oxide, ethoxylated fatty acid monoethanolamide, fatty acid monoethanolamide, or polyhydroxy alkyl fatty acid amide (e.g. as described in WO 92/06154).

The detergent composition may additionally comprise one or more other enzymes, such as pullulanase, esterase,  
25 lipase, cutinase, protease, cellulase, peroxidase, or oxidase, e.g., laccase.

Normally the detergent contains 1-65% of a detergent builder, but some dishwashing detergents may contain even up to 90% of a detergent builder, or complexing agent such as  
30 zeolite, diphosphate, triphosphate, phosphonate, citrate, nitrilotriacetic acid (NTA), ethylenediaminetetraacetic acid (EDTA), diethylenetriaminepentaacetic acid (DTMPA), alkyl- or alkenylsuccinic acid, soluble silicates or layered silicates (e.g. SKS-6 from Hoechst).

35 The detergent builders may be subdivided into phosphorus-containing and non-phosphorous-containing types. Examples of phosphorus-containing inorganic alkaline detergent

builders include the water-soluble salts, especially alkali metal pyrophosphates, orthophosphates, polyphosphates and phosphonates. Examples of non-phosphorus-containing inorganic builders include water-soluble alkali metal carbonates, borates and silicates as well as layered disilicates and the various types of water-insoluble crystalline or amorphous aluminosilicates of which zeolites is the best known representative.

Examples of suitable organic builders include alkali metal, ammonium or substituted ammonium salts of succinates, malonates, fatty acid malonates, fatty acid sulphonates, carboxymethoxy succinates, polyacetates, carboxylates, polycarboxylates, aminopolycarboxylates and polyacetyl carboxylates.

The detergent may also be unbuil, i.e. essentially free of detergent builder.

The detergent may comprise one or more polymers. Examples are carboxymethylcellulose (CMC), poly(vinylpyrrolidone) (PVP), polyethyleneglycol (PEG), poly(vinyl alcohol) (PVA), polycarboxylates such as polyacrylates, polymaleates, maleic/acrylic acid copolymers and lauryl methacrylate/acrylic acid copolymers.

The detergent composition may contain bleaching agents of the chlorine/bromine-type or the oxygen-type. The bleaching agents may be coated or encapsulated. Examples of inorganic chlorine/bromine-type bleaches are lithium, sodium or calcium hypochlorite or hypobromite as well as chlorinated trisodium phosphate. The bleaching system may also comprise a  $H_2O_2$  source such as perborate or percarbonate which may be combined with a peracid-forming bleach activator such as tetraacetylenediamine (TAED) or nonanoyloxybenzene-sulfonate (NOBS).

Examples of organic chlorine/bromine-type bleaches are heterocyclic N-bromo and N-chloro imides such as trichloroisocyanuric, tribromoisocyanuric, dibromoisocyanuric and dichloroisocyanuric acids, and salts thereof with water solubilizing cations such as potassium and sodium. Hydantoin compounds are also suitable. The bleaching system may also comprise peroxyacids of, e.g., the amide, imide, or sulfone



type.

In dishwashing detergents the oxygen bleaches are preferred, for example in the form of an inorganic persalt, preferably with a bleach precursor or as a peroxy acid compound. Typical examples of suitable peroxy bleach compounds are alkali metal perborates, both tetrahydrates and monohydrates, alkali metal percarbonates, persilicates and perphosphates. Preferred activator materials are TAED or NOBS.

The enzymes of the detergent composition of the invention may be stabilized using conventional stabilizing agents, e.g. a polyol such as propylene glycol or glycerol, a sugar or sugar alcohol, lactic acid, boric acid, or a boric acid derivative such as, e.g., an aromatic borate ester, and the composition may be formulated as described in, e.g., WO 92/19709 and WO 92/19708. The enzymes of the invention may also be stabilized by adding reversible enzyme inhibitors, e.g., of the protein type as described in EP 0 544 777 B1.

The detergent may also contain other conventional detergent ingredients such as, e.g., fabric conditioners including clays, deflocculant material, foam boosters/foam depressors (in dishwashing detergents foam depressors), suds suppressors, anti-corrosion agents, soil-suspending agents, anti-soil-redeposition agents, dyes, dehydrating agents, bactericides, optical brighteners, or perfume.

The pH (measured in aqueous solution at use concentration) will usually be neutral or alkaline, e.g. in the range of 7-11.

Particular forms of laundry detergent compositions within the scope of the invention include:

1) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

Linear alkylbenzenesulfonate (calculated as acid)	7 - 12%
Alcohol ethoxysulfate (e.g. C <sub>12-18</sub> alcohol, 1-2 EO) or alkyl sulfate (e.g. C <sub>16-18</sub> )	1 - 4%

	Alcohol ethoxylate (e.g. C <sub>14-15</sub> alcohol, 7 EO)	5	-	9%
	Sodium carbonate (as Na <sub>2</sub> CO <sub>3</sub> )	14	-	20%
	Soluble silicate (as Na <sub>2</sub> O, 2SiO <sub>2</sub> )	2	-	6%
5	Zeolite (as NaAlSiO <sub>4</sub> )	15	-	22%
	Sodium sulfate (as Na <sub>2</sub> SO <sub>4</sub> )	0	-	6%
	Sodium citrate/citric acid (as C <sub>6</sub> H <sub>5</sub> Na <sub>3</sub> O <sub>7</sub> /C <sub>6</sub> H <sub>8</sub> O <sub>7</sub> )	0	-	15%
	Sodium perborate (as NaBO <sub>3</sub> ·H <sub>2</sub> O)	11	-	18%
10	TAED	2	-	6%
	Carboxymethylcellulose	0	-	2%
	Polymers (e.g. maleic/acrylic acid copolymer, PVP, PEG)	0	-	3%
15	Enzymes (calculated as pure enzyme protein)	0.0001	-	0.1%
	Minor ingredients (e.g. suds suppressors, perfume, optical brightener, photobleach)	0	-	5%

2) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

	Linear alkylbenzenesulfonate (calculated as acid)	6	-	11%
25	Alcohol ethoxysulfate (e.g. C <sub>12-18</sub> alcohol, 1-2 EO or alkyl sulfate (e.g. C <sub>16-18</sub> ))	1	-	3%
	Alcohol ethoxylate (e.g. C <sub>14-15</sub> alcohol, 7 EO)	5	-	9%
	Sodium carbonate (as Na <sub>2</sub> CO <sub>3</sub> )	15	-	21%
30	Soluble silicate (as Na <sub>2</sub> O, 2SiO <sub>2</sub> )	1	-	4%
	Zeolite (as NaAlSiO <sub>4</sub> )	24	-	34%
	Sodium sulfate (as Na <sub>2</sub> SO <sub>4</sub> )	4	-	10%
	Sodium citrate/citric acid (as C <sub>6</sub> H <sub>5</sub> Na <sub>3</sub> O <sub>7</sub> /C <sub>6</sub> H <sub>8</sub> O <sub>7</sub> )	0	-	15%
35	Carboxymethylcellulose	0	-	2%
	Polymers (e.g. maleic/acrylic acid copolymer, PVP, PEG)	1	-	6%

Enzymes (calculated as pure enzyme protein)	0.0001 - 0.1%
Minor ingredients (e.g. suds suppressors, perfume)	0 - 5%

5 3) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

	Linear alkylbenzenesulfonate (calculated as acid)	5 - 9%
10	Alcohol ethoxylate (e.g. C <sub>12-15</sub> alcohol, 7 EO)	7 - 14%
	Soap as fatty acid (e.g. C <sub>16-22</sub> fatty acid)	1 - 3%
	Sodium carbonate (as Na <sub>2</sub> CO <sub>3</sub> )	10 - 17%
	Soluble silicate (as Na <sub>2</sub> O, 2SiO <sub>2</sub> )	3 - 9%
15	Zeolite (as NaAlSiO <sub>4</sub> )	23 - 33%
	Sodium sulfate (as Na <sub>2</sub> SO <sub>4</sub> )	0 - 4%
	Sodium perborate (as NaBO <sub>3</sub> ·H <sub>2</sub> O)	8 - 16%
	TAED	2 - 8%
	Phosphonate (e.g. EDTMPA)	0 - 1%
20	Carboxymethylcellulose	0 - 2%
	Polymers (e.g. maleic/acrylic acid copolymer, PVP, PEG)	0 - 3%
	Enzymes (calculated as pure enzyme protein)	0.0001 - 0.1%
25	Minor ingredients (e.g. suds suppressors, perfume, optical brightener)	0 - 5%

4) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising



	Linear alkylbenzenesulfonate (calculated as acid)	8	- 12%
	Alcohol ethoxylate (e.g. C <sub>12-15</sub> alcohol, 7 EO)	10	- 25%
5	Sodium carbonate (as Na <sub>2</sub> CO <sub>3</sub> )	14	- 22%
	Soluble silicate (as Na <sub>2</sub> O, 2SiO <sub>2</sub> )	1	- 5%
	Zeolite (as NaAlSiO <sub>4</sub> )	25	- 35%
	Sodium sulfate (as Na <sub>2</sub> SO <sub>4</sub> )	0	- 10%
	Carboxymethylcellulose	0	- 2%
10	Polymers (e.g. maleic/acrylic acid copolymer, PVP, PEG)	1	- 3%
	Enzymes (calculated as pure enzyme protein)	0.0001	- 0.1%
15	Minor ingredients (e.g. suds suppressors, perfume)	0	- 5%

5) An aqueous liquid detergent composition comprising

	Linear alkylbenzenesulfonate (calculated as acid)	15	- 21%
20	Alcohol ethoxylate (e.g. C <sub>12-15</sub> alcohol, 7 EO or C <sub>12-15</sub> alcohol, 5 EO)	12	- 18%
	Soap as fatty acid (e.g. oleic acid)	3	- 13%
	Alkenylsuccinic acid (C <sub>12-14</sub> )	0	- 13%
	Aminoethanol	8	- 18%
25	Citric acid	2	- 8%
	Phosphonate	0	- 3%
	Polymers (e.g. PVP, PEG)	0	- 3%
	Borate (as B <sub>4</sub> O <sub>7</sub> )	0	- 2%
	Ethanol	0	- 3%
30	Propylene glycol	8	- 14%
	Enzymes (calculated as pure enzyme protein)	0.0001	- 0.1%
35	Minor ingredients (e.g. dispersants, suds suppressors, perfume, optical brightener)	0	- 5%

6) An aqueous structured liquid detergent composition comprising

	Linear alkylbenzenesulfonate (calculated as acid)	15	- 21%
5	Alcohol ethoxylate (e.g. C <sub>12-15</sub> alcohol, 7 EO, or C <sub>12-15</sub> alcohol, 5 EO)	3	- 9%
	Soap as fatty acid (e.g. oleic acid)	3	- 10%
10	Zeolite (as NaAlSiO <sub>4</sub> )	14	- 22%
	Potassium citrate	9	- 18%
	Borate (as B <sub>4</sub> O <sub>7</sub> )	0	- 2%
	Carboxymethylcellulose	0	- 2%
	Polymers (e.g. PEG, PVP)	0	- 3%
15	Anchoring polymers such as, e.g., lauryl methacrylate/acrylic acid copolymer; molar ratio 25:1; MW 3800	0	- 3%
	Glycerol	0	- 5%
20	Enzymes (calculated as pure enzyme protein)	0.0001	- 0.1%
	Minor ingredients (e.g. dispersants, suds suppressors, perfume, optical brighteners)	0	- 5%

25 7) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

	Fatty alcohol sulfate	5	- 10%
	Ethoxylated fatty acid monoethanol- amide	3	- 9%
30	Soap as fatty acid	0	- 3%
	Sodium carbonate (as Na <sub>2</sub> CO <sub>3</sub> )	5	- 10%
	Soluble silicate (as Na <sub>2</sub> O, 2SiO <sub>2</sub> )	1	- 4%
	Zeolite (as NaAlSiO <sub>4</sub> )	20	- 40%
	Sodium sulfate (as Na <sub>2</sub> SO <sub>4</sub> )	2	- 8%
35	Sodium perborate (as NaBO <sub>3</sub> ·H <sub>2</sub> O)	12	- 18%
	TAED	2	- 7%

	Polymers (e.g. maleic/acrylic acid copolymer, PEG)	1	-	5%
	Enzymes (calculated as pure enzyme protein)	0.0001	-	0.1%
5	Minor ingredients (e.g. optical brightener, suds suppressors, perfume)	0	-	5%

8) A detergent composition formulated as a granulate comprising

10	Linear alkylbenzenesulfonate (calculated as acid)	8	-	14%
	Ethoxylated fatty acid monoethanolamide	5	-	11%
	Soap as fatty acid	0	-	3%
	Sodium carbonate (as $\text{Na}_2\text{CO}_3$ )	4	-	10%
15	Soluble silicate (as $\text{Na}_2\text{O}, 2\text{SiO}_2$ )	1	-	4%
	Zeolite (as $\text{NaAlSiO}_4$ )	30	-	50%
	Sodium sulfate (as $\text{Na}_2\text{SO}_4$ )	3	-	11%
	Sodium citrate (as $\text{C}_6\text{H}_5\text{Na}_3\text{O}_7$ )	5	-	12%
20	Polymers (e.g. PVP, maleic/acrylic acid copolymer, PEG)	1	-	5%
	Enzymes (calculated as pure enzyme protein)	0.0001	-	0.1%
	Minor ingredients (e.g. suds suppressors, perfume)	0	-	5%

25 9) A detergent composition formulated as a granulate comprising

	Linear alkylbenzenesulfonate (calculated as acid)	6	-	12%
	Nonionic surfactant	1	-	4%
	Soap as fatty acid	2	-	6%
30	Sodium carbonate (as $\text{Na}_2\text{CO}_3$ )	14	-	22%
	Zeolite (as $\text{NaAlSiO}_4$ )	18	-	32%
	Sodium sulfate (as $\text{Na}_2\text{SO}_4$ )	5	-	20%
	Sodium citrate (as $\text{C}_6\text{H}_5\text{Na}_3\text{O}_7$ )	3	-	8%
	Sodium perborate (as $\text{NaBO}_3 \cdot \text{H}_2\text{O}$ )	4	-	9%



2186592

21

	Bleach activator (e.g. NOBS or TAED)	1	-	5%
	Carboxymethylcellulose	0	-	2%
5	Polymers (e.g. polycarboxylate or PEG)	1	-	5%
	Enzymes (calculated as pure enzyme protein)	0.0001	-	0.1%
	Minor ingredients (e.g. optical brightener, perfume)	0	-	5%

10 10) An aqueous liquid detergent composition comprising

	Linear alkylbenzenesulfonate (calculated as acid)	15	-	23%
	Alcohol ethoxysulfate (e.g. C <sub>12-15</sub> alcohol, 2-3 EO)	8	-	15%
15	Alcohol ethoxylate (e.g. C <sub>12-15</sub> alcohol, 7 EO, or C <sub>12-15</sub> alcohol, 5 EO)	3	-	9%
	Soap as fatty acid (e.g. lauric acid)	0	-	3%
20	Aminoethanol	1	-	5%
	Sodium citrate	5	-	10%
	Hydrotrope (e.g. sodium toluenesulfonate)	2	-	6%
	Borate (as B <sub>4</sub> O <sub>7</sub> )	0	-	2%
25	Carboxymethylcellulose	0	-	1%
	Ethanol	1	-	3%
	Propylene glycol	2	-	5%
	Enzymes (calculated as pure enzyme protein)	0.0001	-	0.1%
30	Minor ingredients (e.g. polymers, dispersants, perfume, optical brighteners)	0	-	5%

11) An aqueous liquid detergent composition comprising

35	Linear alkylbenzenesulfonate (calculated as acid)	20	-	32%
----	---	----	---	-----

	Alcohol ethoxylate (e.g. C <sub>12-15</sub> alcohol, 7 EO, or C <sub>12-15</sub> alcohol, 5 EO)	6	- 12%
	Aminoethanol	2	- 6%
5	Citric acid	8	- 14%
	Borate (as B <sub>4</sub> O <sub>7</sub> )	1	- 3%
10	Polymer (e.g. maleic/acrylic acid copolymer, anchoring polymer such as, e.g., lauryl methacrylate/acrylic acid copolymer)	0	- 3%
	Glycerol	3	- 8%
	Enzymes (calculated as pure enzyme protein)	0.0001	- 0.1%
15	Minor ingredients (e.g. hydro-tropes, dispersants, perfume, optical brighteners)	0	- 5%

12) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

20	Anionic surfactant (linear alkylbenzenesulfonate, alkyl sulfate, alpha-olefinsulfonate, alpha-sulfo fatty acid methyl esters, alkanesulfonates, soap)	25	- 40%
25	Nonionic surfactant (e.g. alcohol ethoxylate)	1	- 10%
	Sodium carbonate (as Na <sub>2</sub> CO <sub>3</sub> )	8	- 25%
	Soluble silicates (as Na <sub>2</sub> O, 2SiO <sub>2</sub> )	5	- 15%
	Sodium sulfate (as Na <sub>2</sub> SO <sub>4</sub> )	0	- 5%
30	Zeolite (as NaAlSiO <sub>4</sub> )	15	- 28%
	Sodium perborate (as NaBO <sub>3</sub> ·4H <sub>2</sub> O)	0	- 20%
	Bleach activator (TAED or NOBS)	0	- 5%
	Enzymes (calculated as pure enzyme protein)	0.0001	- 0.1%
35	Minor ingredients (e.g. perfume, optical brighteners)	0	- 3%

13) Detergent formulations as described in 1) - 12) wherein all or part of the linear alkylbenzenesulfonate is replaced by (C<sub>12</sub>-C<sub>18</sub>) alkyl sulfate.

14) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

	(C <sub>12</sub> -C <sub>18</sub> ) alkyl sulfate	9	- 15%
	Alcohol ethoxylate	3	- 6%
	Polyhydroxy alkyl fatty acid amide	1	- 5%
	Zeolite (as NaAlSiO <sub>4</sub> )	10	- 20%
10	Layered disilicate (e.g. SK56 from Hoechst)	10	- 20%
	Sodium carbonate (as Na <sub>2</sub> CO <sub>3</sub> )	3	- 12%
	Soluble silicate (as Na <sub>2</sub> O, 2SiO <sub>2</sub> )	0	- 6%
	Sodium citrate	4	- 8%
15	Sodium percarbonate	13	- 22%
	TAED	3	- 8%
	Polymers (e.g. polycarboxylates and PVP)	0	- 5%
20	Enzymes (calculated as pure enzyme protein)	0.0001	- 0.1%
	Minor ingredients (e.g. optical brightener, photo bleach, perfume, suds suppressors)	0	- 5%

15) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

	(C <sub>12</sub> -C <sub>18</sub> ) alkyl sulfate	4	- 8%
	Alcohol ethoxylate	11	- 15%
	Soap	1	- 4%
	Zeolite MAP or zeolite A	35	- 45%
30	Sodium carbonate (as Na <sub>2</sub> CO <sub>3</sub> )	2	- 8%
	Soluble silicate (as Na <sub>2</sub> O, 2SiO <sub>2</sub> )	0	- 4%
	Sodium percarbonate	13	- 22%



	TAED	1	-	8%
	Carboxymethyl cellulose	0	-	3%
	Polymers (e.g. polycarboxylates and PVP)	0	-	3%
5	Enzymes (calculated as pure enzyme protein)	0.0001	-	0.1%
	Minor ingredients (e.g. optical brightener, phosphonate, perfume)	0	-	3%

16) Detergent formulations as described in 1) - 15) which contain a stabilized or encapsulated peracid, either as an additional component or as a substitute for already specified bleach systems.

17) Detergent compositions as described in 1), 3), 7), 9) and 12) wherein perborate is replaced by percarbonate.

18) Detergent compositions as described in 1), 3), 7), 9), 12), 14) and 15) which additionally contain a manganese catalyst. The manganese catalyst may, e.g., be one of the compounds described in "Efficient manganese catalysts for low-temperature bleaching", Nature 369, 1994, pp. 637-639.

19) Detergent composition formulated as a nonaqueous detergent liquid comprising a liquid nonionic surfactant such as, e.g., linear alkoxyated primary alcohol, a builder system (e.g. phosphate), enzyme and alkali. The detergent may also comprise anionic surfactant and/or a bleach system.

Particular forms of dishwashing detergent compositions within the scope of the invention include:

#### 1) POWDER AUTOMATIC DISHWASHING COMPOSITION

Nonionic surfactant	0.4	-	2.5%
Sodium metasilicate	0	-	20%

	Sodium disilicate	3	- 20%
	Sodium triphosphate	20	- 40%
	Sodium carbonate	0	- 20%
	Sodium perborate	2	- 9%
5	Tetraacetythylenediamine (TAED)	1	- 4%
	Sodium sulphate	5	- 33%
	Enzymes	0.0001	- 0.1%

## 2) POWDER AUTOMATIC DISHWASHING COMPOSITION

10	Nonionic surfactant (e.g. alcohol ethoxylate)	1	- 2%
	Sodium disilicate	2	- 30%
	Sodium carbonate	10	- 50%
	Sodium phosphonate	0	- 5%
	Trisodium citrate dihydrate	9	- 30%
15	Nitrilotrisodium acetate (NTA)	0	- 20%
	Sodium perborate monohydrate	5	- 10%
	Tetraacetythylenediamine (TAED)	1	- 2%
20	Polyacrylate polymer (e.g. maleic acid/acrylic acid co- polymer)	6	- 25%
	Enzymes	0.0001	- 0.1%
	Perfume	0.1	- 0.5%
	Water	5	- 10

## 3) POWDER AUTOMATIC DISHWASHING COMPOSITION

25	Nonionic surfactant	0.5	- 2.0%
	Sodium disilicate	25	- 40%
	Sodium citrate	30	- 55%
	Sodium carbonate	0	- 29%
	Sodium bicarbonate	0	- 20%
30	Sodium perborate monohydrate	0	- 15%
	Tetraacetythylenediamine (TAED)	0	- 6%

	Maleic acid/acrylic acid copolymer	0	-	5%
	Clay	1	-	3%
	Poly(amino acids)	0	-	20%
5	Sodium polyacrylate	0	-	8%
	Enzymes	0.0001	-	0.1%

## 4) POWDER AUTOMATIC DISHWASHING COMPOSITION

	Nonionic surfactant	1	-	2%
	Zeolite MAP	15	-	42%
10	Sodium disilicate	30	-	34%
	Sodium citrate	0	-	12%
	Sodium carbonate	0	-	20%
	Sodium perborate monohydrate	7	-	15%
	Tetraacetylenediamine (TAED)	0	-	3%
15	Polymer	0	-	4%
	Maleic acid/acrylic acid copolymer	0	-	5%
	Organic phosphonate	0	-	4%
	Clay	1	-	2%
	Enzymes	0.0001	-	0.1%
20	Sodium sulphate	Balance		

## 5) POWDER AUTOMATIC DISHWASHING COMPOSITION

	Nonionic surfactant	1	-	7%
	Sodium disilicate	18	-	30%
	Trisodium citrate	10	-	24%
25	Sodium carbonate	12	-	20%
	Monopersulphate (2 KHSO <sub>5</sub> .KHSO <sub>4</sub> .K <sub>2</sub> SO <sub>4</sub> )	15	-	21%
	Bleach stabilizer	0.1	-	2%
	Maleic acid/acrylic acid copolymer	0	-	6%



Diethylenetriaminepentaacetate, pentasodium salt	0	-	2.5%
Enzymes	0.0001	-	0.1%
Sodium sulphate, water	Balance		

5 6) POWDER AND LIQUID DISHWASHING COMPOSITION WITH CLEANING SURFACTANT SYSTEM

	Nonionic surfactant	0	-	1.5%
	Octadecyl dimethylamine N-oxide dihydrate	0	-	5%
10	80:20 wt.C18/C16 blend of octadecyl dimethylamine N-oxide dihydrate and hexadecyldimethyl amine N-oxide dihydrate	0	-	4%
15	70:30 wt.C18/C16 blend of octadecyl bis (hydroxyethyl)amine N-oxide anhydrous and hexadecyl bis (hydroxyethyl)amine N-oxide anhydrous	0	-	5%
20	C <sub>13</sub> -C <sub>15</sub> alkyl ethoxysulfate with an average degree of ethoxylation of 3	0	-	10%
	C <sub>12</sub> -C <sub>15</sub> alkyl ethoxysulfate with an average degree of ethoxylation of 3	0	-	5%
25	C <sub>13</sub> -C <sub>15</sub> ethoxylated alcohol with an average degree of ethoxylation of 12	0	-	5%
	A blend of C <sub>12</sub> -C <sub>15</sub> ethoxylated alcohols with an average degree of ethoxylation of 9	0	-	6.5%
30	A blend of C <sub>13</sub> -C <sub>15</sub> ethoxylated alcohols with an average degree of ethoxylation of 30	0	-	4%
	Sodium disilicate	0	-	33%
	Sodium tripolyphosphate	0	-	46%
	Sodium citrate	0	-	28%
35	Citric acid	0	-	29%
	Sodium carbonate	0	-	20%
	Sodium perborate monohydrate	0	-	11.5%
	Tetraacetylenediamine (TAED)	0	-	4%
	Maleic acid/acrylic acid copolymer	0	-	7.5%

2186592

28

Sodium sulphate	0	- 12.5%
Enzymes	0.0001	- 0.1%

## 7) NON-AQUEOUS LIQUID AUTOMATIC DISHWASHING COMPOSITION

5	Liquid nonionic surfactant (e.g. alcohol ethoxylates)	2.0	- 10.0%
	Alkali metal silicate	3.0	- 15.0%
	Alkali metal phosphate	20.0	- 40.0%
10	Liquid carrier selected from higher glycols, polyglycols, polyoxides, glycolethers	25.0	- 45.0%
	Stabilizer (e.g. a partial ester of phosphoric acid and a C <sub>16</sub> -C <sub>18</sub> alkanol)	0.5	- 7.0%
	Foam suppressor (e.g. silicone)	0	- 1.5%
15	Enzymes	0.0001	- 0.1%

## 8) NON-AQUEOUS LIQUID DISHWASHING COMPOSITION

	Liquid nonionic surfactant (e.g. alcohol ethoxylates)	2.0	- 10.0%
	Sodium silicate	3.0	- 15.0%
20	Alkali metal carbonate	7.0	- 20.0%
	Sodium citrate	0.0	- 1.5%
25	Stabilizing system (e.g. mixtures of finely divided silicone and low molecular weight dialkyl polyglycol ethers)	0.5	- 7.0%
	Low molecule weight polyacrylate polymer	5.0	- 15.0%
	Clay gel thickener (e.g. bentonite)	0.0	- 10.0%
	Hydroxypropyl cellulose polymer	0.0	- 0.6%
30	Enzymes	0.0001	- 0.1%
	Liquid carrier selected from higher glycols, polyglycols, polyoxides and glycol ethers	Balance	

## 9) THIXOTROPIC LIQUID AUTOMATIC DISHWASHING COMPOSITION

35	C <sub>12</sub> -C <sub>14</sub> fatty acid	0	- 0.5%
----	---	---	--------

	Block co-polymer surfactant	1.5	- 15.0%
	Sodium citrate	0	- 12%
	Sodium tripolyphosphate	0	- 15%
	Sodium carbonate	0	- 8%
5	Aluminium tristearate	0	- 0.1%
	Sodium cumene sulphonate	0	- 1.7%
	Polyacrylate thickener	1.32	- 2.5%
	Sodium polyacrylate	2.4	- 6.0%
	Boric acid	0	- 4.0%
10	Sodium formate	0	- 0.45%
	Calcium formate	0	- 0.2%
	Sodium n-decyldiphenyl oxide disulphonate	0	- 4.0%
	Monoethanol amine (MEA)	0	- 1.86%
15	Sodium hydroxide (50%)	1.9	- 9.3%
	1,2-Propanediol	0	- 9.4%
	Enzymes	0.0001	- 0.1%
	Suds suppressor, dye, perfumes, water	Balance	

## 20 10) LIQUID AUTOMATIC DISHWASHING COMPOSITION

	Alcohol ethoxylate	0	- 20%
	Fatty acid ester sulphonate	0	- 30%
	Sodium dodecyl sulphate	0	- 20%
	Alkyl polyglycoside	0	- 21%
25	Oleic acid	0	- 10%
	Sodium disilicate monohydrate	18	- 33%
	Sodium citrate dihydrate	18	- 33%
	Sodium stearate	0	- 2.5%
	Sodium perborate monohydrate	0	- 13%
30	Tetraacetylenediamine (TAED)	0	- 8%
	Maleic acid/acrylic acid copolymer	4	- 8%
	Enzymes	0.0001	- 0.1%



11) LIQUID AUTOMATIC DISHWASHING COMPOSITION CONTAINING PROTECTED BLEACH PARTICLES

	Sodium silicate	5	- 10%
	Tetrapotassium pyrophosphate	15	- 25%
5	Sodium triphosphate	0	- 2%
	Potassium carbonate	4	- 8%
	Protected bleach particles, e.g. chlorine	5	- 10%
	Polymeric thickener	0.7	- 1.5%
10	Potassium hydroxide	0	- 2%
	Enzymes	0.0001	- 0.1%
	Water	Balance	

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

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

The  $\alpha$ -amylases of the invention may be incorporated in concentrations conventionally employed in detergents. It is at present contemplated that, in the detergent composition of the invention, the  $\alpha$ -amylase may be added in an amount corresponding to 0.00001-1 mg (calculated as pure enzyme protein) of  
25  $\alpha$ -amylase per liter of wash/dishwash liquor.

The present invention is further illustrated in the following examples which are not intended to be in any way limiting to the scope of the invention as claimed.

**EXAMPLE 1**

$\alpha$ -amylase Preparations from *Bacillus* strains NCIB 12289, NCIB 12513, DSM 9375 and NCIB 12512.

Fermentation:

5 Each of the above mentioned *Bacillus* strains was incubated at 26°C on a rotary shaking table (300 r.p.m.) in 500 ml baffled Erlenmeyer flasks containing 100 ml of BP-X medium + 0.1 M Carbonate buffer pH 9.0.

BP-X medium:

10	Potato starch	100 g
	Ground barley	50 g
	Soybean flour	20 g
	Sodium caseinate	10 g
	Na <sub>2</sub> HPO <sub>4</sub> X 12 H <sub>2</sub> O	9 g
15	Termamyl® 60L*	0.1 g
	Pluronic®	0.1 g

\*) available from Novo Nordisk A/S.

The starch in the medium was liquified by slowly heating the medium from 60°C to 85°C for 30 minutes. After this  
20 the temperature of the medium was quickly raised to 95°C for 10 minutes and then cooled. Lastly the medium was sterilized by heating at 121°C for 40 minutes.

Purification of  $\alpha$ -amylase from NCIB 12289, DSM 9375 and NCIB 12512.

25 After 5 days of incubation the culture broth was filtrated and concentrated using a Filtron™ ultrafiltration module with 3KD membranes and washed with deionized water until the conductivity was 1 mS/cm. The pH was adjusted to pH 5.9 with 10% (v/v) acetic acid. A S-sepharose FF column was  
30 equilibrated in EKV-buffer, pH 5.9. If not otherwise stated, the purification buffer was 100 mM boric acid, 10 mM succinic acid, 2 mM CaCl<sub>2</sub>, (EKV-buffer) adjusted to the indicated pH with NaOH.

The enzyme solution was applied to the column, the  
35 column was washed with EKV-buffer, pH 5.9, and the amylase was

2186592

32

eluted with a linear NaCl gradient (0-> 500 mM NaCl). Amylase containing fractions were pooled and the pH adjusted to pH 7 with 3% (w/v) NaOH.

A chelate agarose column was loaded with Cu<sup>++</sup> and 5 equilibrated in the following manner: 50 mM CuSO<sub>4</sub>, pH 5 was pumped on to the column until the whole column was blue, then excess of Cu<sup>++</sup>-ions were removed by washing the column with 500 mM imidazol, pH 7, and finally the column was equilibrated with EKV-buffer, pH 7. The amylase pool from the S-sepharose column 10 was applied to the Cu<sup>++</sup>-loaded Chelate agarose column, the column was washed with EKV-buffer, pH 7, and the enzyme was eluted with a linear gradient of imidazol (0-> 500 mM imidazol). Amylase containing fractions were pooled and a solution of saturated ammonium sulphate was added to give a 15 final concentration of 1M (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> in the pool.

A phenyl sepharose column was equilibrated in EKV-buffer + 1M (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, pH 7. The amylase pool from the Cu<sup>++</sup>-column was applied to the hydrophobic interaction column. Binding experiments had shown that the amylase is a rather 20 hydrophobic enzyme, and hence binds tightly to the phenyl column. Protein which did not bind as tightly to the column was washed off the column with EKV-buffer, pH 7. The amylase was step-eluted from the column with EKV-buffer + 25% (v/v) isopropanol. The amylase containing pool was adjusted to pH 9.5 25 with 3% (w/v) NaOH and diluted 5 times with deionized water.

A Q-sepharose HP column was equilibrated in 20 mM Tris-HCl, pH 9.5. The amylase pool from the phenyl sepharose column was applied to the column and the column was washed with 20 mM Tris-HCl, pH 9.5. The amylase was eluted with a linear 30 gradient of NaCl (0 -> 250 mM NaCl).

The amylase peak was adjusted to pH 7 with 10% (v/v) acetic acid.

A Cu<sup>++</sup>-loaded chelating sepharose FF column (loaded with Cu<sup>++</sup> as described for the chelate agarose column) was 35 equilibrated with EKV-buffer, pH 7. The amylase peak from the Q-sepharose column was applied to the column, and the column was washed thoroughly with EKV-buffer, pH 7. The amylase was



eluted with a steep linear gradient of imidazol (0 -> 500 mM imidazol).

The purified amylase was purity checked by SDS-PAGE electrophoresis. The coomassie stained gel had only one band.

#### 5 Purification of $\alpha$ -amylase from NCIB 12513

After 5 days of incubation the culture broth was filtrated and concentrated using a Filtron™ ultrafiltration module with 3KD membranes. The concentrated solution was filtrated and saturated to 20% w/w with ammoniumsulfate. The  
10 solution was then batch absorbed using a AFFI-T™ matrix from Kem-En-Tec A/S. The amylase was eluted using 25% isopropanol in 20 mM Tris pH 7.5 after wash of the matrix with deionized water. The eluted enzyme was subjected to dialysis (20 mM Tris pH 8.5) and a stepwise batch adsorption on Q-sepharose FF for  
15 colour removal was made.

A chelate agarose column was loaded with  $\text{Cu}^{++}$  and equilibrated in the following manner: 50 mM  $\text{CuSO}_4$ , pH 5 was pumped on to the column until the whole column was blue, then excess of  $\text{Cu}^{++}$ -ions was removed by washing the column with 500  
20 mM imidazol, pH 7, and finally the column was equilibrated with 50 mM borate buffer, pH 7.

In spite of the low pI (5.8) the amylase was not bound to the Q-sepharose FF at pH 8.5.

The run through from the Q-sepharose FF column was  
25 applied on the Cu-chelating agarose and eluted using 250 mM imidazol, 20 mM Tris pH 7.0 and the eluted column was dialysed against 50 mM borate buffer pH 7.0. The pH was adjusted to pH 9.5 and the dialysed solution was bound on a Q-sepharose HP and eluted over 10 columns using a linear gradient from 0-250 mM  
30 NaCl. Amylase containing fractions were pooled and a solution of saturated ammonium sulphate was added to give a final concentration of 20% w/w, and the fractions were applied on a phenyl sepharose column. The column was washed using deionized water and eluted using 25% isopropanol in 50 mM borate buffer  
35 pH 7.0.

The purified amylase was purity checked by SDS-PAGE

2186592

34

electrophoresis. The coomassie stained gel had only one band.

## EXAMPLE 2

### Physical-Chemical Properties of the $\alpha$ -Amylases

The  $\alpha$ -amylase obtained from Bacillus strain NCIB  
5 12289, fermented and purified as described in Example 1, was  
found to possess the following properties:

A pI of about 8.8-9.0 as determined by isoelectric  
focusing on LKB Ampholine® PAG plates (3.5-9.5) - meaning that  
said plates are useful in the pI range of 3.5 to 9.5.

10 A molecular weight of approximately 55 kD as deter-  
mined by SDS-PAGE.

A pH profile as shown in Fig. 1, which was determined  
at 37°C in the pH range of from 4 to 10.5. The assay for  $\alpha$ -  
amylase activity described previously was used, using Britton-  
15 Robinson buffer adjusted to predetermined pH values. It appears  
from Fig. 1 that the enzyme possesses  $\alpha$ -amylase activity at all  
pH values of from 4 to 10.5, having optimum at pH 7.5-8.5, and  
at least 60% of the maximum activity at pH 9.5.

Amino acid sequence of the  $\alpha$ -amylase was determined  
20 using standard methods for obtaining and sequencing peptides,  
for reference see Findlay & Geisow (Eds.), Protein Sequencing -  
a Practical Approach, 1989, IRL Press.

The N-terminal amino acid sequence was found to be :  
His-His-Asn-Gly-Thr-Asn-Gly-Thr-Met-Met-Gln-Tyr-Phe-Glu-Trp-  
25 Tyr-Leu-Pro-Asn-Asp (SEQ ID No. 3).

The  $\alpha$ -amylases obtained from Bacillus strains NCIB  
12512 and DSM 9375, fermented and purified as described in  
Example 1, were found to possess the same pI (8.8-9.0), the  
same molecular weight (55 kD), and the same N-terminal sequence  
30 (SEQ ID No. 3) as the  $\alpha$ -amylase obtained from NCIB 12289; so it  
can be concluded that the  $\alpha$ -amylases obtained from NCIB 12289,  
NCIB 12512 and DSM 9375 have the following common features:

(a) A pI of about 8.6-9.3 determined by isoelectric  
focusing on LKB Ampholine® PAG plates;

b) A molecular weight of approximately 55 kD as determined by SDS-PAGE;

c) An N-terminal amino acid with the amino acid sequence as shown in ID No. 3.

5 The full amino acid sequence of the Bacillus strain NCIB 12512  $\alpha$ -amylase is disclosed in SEQ ID No. 1 of the present invention. The full DNA sequence of the Bacillus strain NCIB 12512  $\alpha$ -amylase is disclosed in SEQ ID No. 4 of the present invention.

10 The  $\alpha$ -amylase obtained from Bacillus strain NCIB 12513, fermented and purified as described in Example 1, was found to possess a pI of about 5.8 and a molecular weight of approximately 55 kD.

The full amino acid sequence of the Bacillus strain  
15 NCIB 12513  $\alpha$ -amylase is disclosed in SEQ ID No. 2 of the present invention. The full DNA sequence of the Bacillus strain NCIB 12513  $\alpha$ -amylase is disclosed in SEQ ID No. 5 of the present invention.

### EXAMPLE 3

#### 20 pH and Temperatures Profiles of the $\alpha$ -Amylases according to the Invention Compared to Termamyl®.

A pH profile of an  $\alpha$ -amylase obtained from Bacillus strain NCIB 12512 (I), of an  $\alpha$ -amylase obtained from Bacillus strain NCIB 12513 (II) and of Termamyl® (III) were determined  
25 at 55°C in the pH interval of from 4 to 10.5. The  $\alpha$ -amylases of the invention were fermented and purified as described in Example 1 and Termamyl® was obtained from Novo Nordisk A/S. The assay for  $\alpha$ -amylase activity described previously was used, using 50 mM Britton-Robinson buffer adjusted to predetermined  
30 pH values and a reaction time of 15 minutes. The results are presented in Fig. 2. It appears from Fig. 2 that the  $\alpha$ -amylases of the invention possess  $\alpha$ -amylase activity at all pH values of from pH 4 to pH 10.5, having optimum at pH 7.5-8.5.

A temperature profile of an  $\alpha$ -amylase obtained from



2186592

36

Bacillus strain NCIB 12512 (I), of an  $\alpha$ -amylase obtained from Bacillus strain NCIB 12513 (II) and of Termamyl® (III) were determined at pH 10.0 in the temperature interval of from 25°C to 95°C. The  $\alpha$ -amylases of the invention were fermented and purified as described in Example 1 and Termamyl® was obtained from Novo Nordisk A/S. The assay for  $\alpha$ -amylase activity described previously was used, using 50 mM Britton-Robinson buffer adjusted to pH 10.0 and a reaction time of 10 minutes. The results are presented in Fig. 3. It appears from Fig. 3 that the  $\alpha$ -amylases of the invention possess  $\alpha$ -amylase activity at all temperature values of from 25°C to 85°C, having optimum at 45°C-55°C, and that the specific activity of the  $\alpha$ -amylase of the invention is 25% higher than the specific activity of Termamyl® at any temperature in the temperature interval of from 25°C to 55°C.

#### EXAMPLE 4

##### Dishwashing Performance of novel $\alpha$ -amylases

$\alpha$ -amylases of the invention obtained from Bacillus strain NCIB 12289 and from Bacillus strain 12512 as described in Example 1, were tested using the following test for detergent amylases for automatic dishwashing:

Plates were dipped in hot corn starch and glasses were soiled by pouring corn starch from one glass to another. The plates and glasses were left to dry overnight and then washed in a dishwasher under the following conditions:

Amylase dosage:	0-0.50 mg of enzyme protein per litre of washing liquor
Detergent:	Commercial European
Detergent dosage:	4.0 g per litre of washing liquor
Dishwashing:	45°C, 55°C or 65°C program, Cylinda
pH:	10.1 during dishwashing.

##### Evaluation/Rating System:

Removal of starch film (RSF) from the plates and

glasses was evaluated after colouring the items with iodine (iodine turns starch blue). The following rating scale was used:

<u>Rating</u>	<u>Dishware</u>	<u>Glassware</u>
5 6	clean	clean
5	spots	thin
4	thin	moderate
3	moderate	heavy
2	heavy	very heavy
10 1	very heavy	extreme heavy
0	blind*	blind.

\*) unwashed

After each item had been evaluated according to the above mentioned rating system, the total value of the scores 15 obtained was divided by the total number of items. The resulting RSF-value was then plotted against the mg  $\alpha$ -amylase protein used per litre of washing liquor.

#### Results:

Bacillus strain NCIB 12289  $\alpha$ -amylase: This  $\alpha$ -amylase 20 was tested at 55°C and the results are shown in Fig. 4. It can be seen from Fig. 4 that an RSF value of between 3 and 4 is obtained at an enzyme dosage of 0.1 mg of  $\alpha$ -amylase protein per litre of washing liquor.

Bacillus strain NCIB 12512  $\alpha$ -amylase: This  $\alpha$ -amylase 25 was tested at 45°C (•), at 55°C (\*) and at 65°C (x), and the results are shown in Fig. 5. It can be seen from Fig. 5 that an RSF value of between 3 and 4.5 is obtained at an enzyme dosage of 0.1 mg of  $\alpha$ -amylase protein per litre of washing liquor (the RSF-value increasing with increasing temperature).

**EXAMPLE 5**Mini Dishwashing Performance of Novel  $\alpha$ -Amylases

The following mini dishwashing assay was used: A suspension of starchy material was boiled and cooled to 20°C. The cooled starch suspension was applied on small, individually identified glass plates (approx. 2 x 2 cm) and dried at a temperature in the range of 60-140°C in a drying cabinet. The individual plates were then weighed. For assay purposes, a solution of standard European-type automatic dishwashing detergent (5 g/l) having a temperature of 55°C was prepared. The detergent was allowed a dissolution time of 1 minute, after which the amylase in question was added to the detergent solution (contained in a beaker equipped with magnetic stirring) so as to give an enzyme concentration of 0.5 mg/l. At the same time, the weighed glass plates, held in small supporting clamps, were immersed in a substantially vertical position in the amylase/detergent solution, which was then stirred for 15 minutes at 55°C. The glass plates were then removed from the amylase/detergent solution, rinsed with distilled water, dried at 60°C in a drying cabinet and re-weighed. The performance of the amylase in question [expressed as an index relative to Termamyl® (index 100)] was then determined from the difference in weight of the glass plates before and after treatment, as follows:

$$\text{Index} = \frac{\text{weight loss for plate treated with } \alpha\text{-amylase}}{\text{weight loss for plate treated with Termamyl}} \cdot 100$$

Results

The above described mini dishwashing test was performed at pH 10.0 with Termamyl®, the novel  $\alpha$ -amylase from NCIB 12513 and the novel  $\alpha$ -amylase from NCIB 12512 (the novel  $\alpha$ -amylases obtained as described in Example 1). The tests gave the following results:

Termamyl®	Index: 100
$\alpha$ -amylase (NCIB 12512)	Index: 163



$\alpha$ -amylase (NCIB 12513)

Index: 175

Surprisingly, the performance in the mini dishwashing test is proportional with the specific activity at pH 10.0, 55°C as can be seen from Fig. 3:

5 Termamyl®	Spec. activity: 2200 U/mg
$\alpha$ -amylase (NCIB 12512)	Spec. activity: 4400 U/mg
$\alpha$ -amylase (NCIB 12513)	Spec. activity: 5200 U/mg.

**EXAMPLE 6**Laundry washing

10 Detergent:	Commercial US heavy duty granulate detergent (HDG)
Detergent dosage:	2 g/l
$\alpha$ -amylase dosage:	0.2 mg enzyme protein/l
Soil:	Potato starch colored with Cibacron Blue
15	3GA on cotton
Water hardness:	9°dH
Time:	15 minutes
Temperature:	40°C

Evaluation:

20 Reflectance at 660 nm. The delta reflectance was calculated from the reflectance obtained for a swatch having been washed with the relevant enzyme and the reflectance obtained for a swatch washed without enzyme. More specifically, the delta reflectance is the reflectance obtained with enzyme  
25 minus the reflectance obtained without enzyme.

Results

The above described laundry washing test was performed with Termamyl®, the novel  $\alpha$ -amylase from NCIB 12513 and the novel  $\alpha$ -amylase from NCIB 12512 (the novel  $\alpha$ -amylases

obtained as described in Example 1). The tests gave the following results:

Termamyl®	Index: 100
α-amylase (NCIB 12512)	Index: 145
5 α-amylase (NCIB 12513)	Index: 133

From the results presented above it is evident that the α-amylases of the invention exert a considerably improved starch removal capacity relative to Termamyl, in other words that the α-amylases of the invention have an improved laundry  
10 washing performance compared to that of Termamyl.

#### EXAMPLE 7

##### Catalytic Efficiency of the Bacillus Strain NCIB 12512 α-Amylase and the Bacillus Strain NCIB 12513 α-Amylase Compared with Termamyl®.

15 The kinetics of hydrolysis catalyzed by the α-amylases of the invention and by Termamyl® at various substrate concentrations were determined using the Somogyi-Nelson method (described below) with amylose (Merck 4561) and amylopectin (Sigma A7780) as substrates.

20 The hydrolysis velocities were measured under different substrate concentrations (1%, 0.5%, 0.3%, 0.25% and 0.2%).

The number of reducing sugars were measured using the Somogyi-Nelson method, and determined as glucose eqv. made/mg  
25 of amylase x h giving the hydrolysis velocity. The data were plotted according to the Michaelis-Menten and Lineweaver-Burk equations. From these equations  $V_{max}/K_m$  can easily be calculated by using the following approximation:

$$30 \quad * \quad V = V_{max} \times \frac{[S]}{[S] + K_m}$$

$$\text{When } [S] \ll K_m : V = V_{max} \times \frac{[S]}{K_m} = \frac{V_{max}}{K_m} \times [S]$$

\* At a given substrate concentration, that substrate concentration being less than  $K_m$ , the expression  $V_{max}/K_m$  is equivalent to the catalytic efficiency of a given  $\alpha$ -amylase. In Table 1 below  $V_{max}/K_m$  is calculated for three  
5 different  $\alpha$ -amylases.

Table 1.

Catalytic efficiency [ $V_{max}/K_m$ ] determined at 55°C, pH 7.3 in 50 mM Britton-Robinson buffer

	$\alpha$ -amylase (NCIB 12513)	$\alpha$ -amylase (NCIB 12512)	Termamyl®
Amylopectin	11.9 $\text{sec}^{-1}\text{x}[\text{g/l}]^{-1}$	11.2 $\text{sec}^{-1}\text{x}[\text{g/l}]^{-1}$	3.2 $\text{sec}^{-1}\text{x}[\text{g/l}]^{-1}$
10 Amylose	31.3 $\text{sec}^{-1}\text{x}[\text{g/l}]^{-1}$	30.2 $\text{sec}^{-1}\text{x}[\text{g/l}]^{-1}$	5.4 $\text{sec}^{-1}\text{x}[\text{g/l}]^{-1}$

The catalytic efficiency of  $\alpha$ -amylase (NCIB 12513) and  $\alpha$ -amylase (NCIB 12512) have shown to be surprisingly high towards both Amylopectin and Amylose compared to Termamyl. Especially the high catalytic efficiency towards amylose is  
15 considered to be of significant importance for the improved specific activities and dishwasher/laundry performance compared to Termamyl.

Linear amylose molecules can align themselves next to each other and form interchain hydrogenbonds through the  
20 hydroxyl groups. This network of amylose molecules has crystalline characteristics and are difficult to solubilize and hydrolyze by any known amylase.

#### Somogyi Method for the Determination of Reducing Sugars

The method is based on the principle that the sugar  
25 reduces cupric ions to cuprous oxide which reacts with arsenate molybdate reagent to produce a blue colour which is measured spectrophotometrically. The solution which is to be examined must contain between 50 and 600 mg of glucose per litre.

1 ml of sugar solution is mixed with 1 ml of copper



reagent and placed in a boiling water bath for 20 minutes. The resulting mixture is cooled and admixed with 1 ml of Nelson's colour reagent and 10 ml of deionized water. The absorbancy at 520 nm is measured.

5 In the region 0-2 the absorbance is proportional to the amount of sugar, which may thus be calculated as follows:

$$\text{mg glucose/l} = \frac{100 (\text{sample} - \text{blank})}{(\text{standard} - \text{blank})}$$

$$10 \quad \% \text{ glucose} = \frac{(\text{sample} - \text{blank})}{100 (\text{standard} - \text{blank})}$$

#### REAGENTS

##### 1. Somogyi's copper reagent

35.1 g of  $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ , and

40.0 g of potassium sodium tartrate ( $\text{KNaC}_4\text{H}_4\text{O}_2 \cdot 4\text{H}_2\text{O}$ )

15 are dissolved in

700 ml of deionized water.

100 ml of 1 N sodium hydroxide and

80 ml of 10% cupric sulphate ( $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ ) are added,

180 g of anhydrous sodium sulphate are dissolved in the mixture, and the volume is brought to 1 litre with deionized water.

##### 2. Nelson's colour reagent

50 g of ammonium molybdate are dissolved in

900 ml of deionized water. Then

25 42 ml of concentrated sulphuric acid (Merck) are added, followed by

6 g of disodium hydrogen arsenate heptahydrate dissolved in 50 ml of deionized water, and the volume is brought to 1 litre with deionized water.

30 The solution must stand for 24-48 hours at 37°C before use. It must be stored in the dark in a brown glass bottle with a glass stopper.

3. Standard

100 mg of glucose (May & Baker, anhydrous) are dissolved in 1 litre of deionized water.

Reference: J. Biol. Chem. 153, 375 (1944)

2186592

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

- (A) NAME: NOVO NORDISK A/S
- 5 (B) STREET: Novo Alle
- (C) CITY: Bagsvaerd
- (E) COUNTRY: Denmark
- (F) POSTAL CODE (ZIP): DK-2880
- (G) TELEPHONE: +45 44 44 88 88
- 10 (H) TELEFAX: +45 44 49 05 55
- (I) TELEX: 37173

(ii) TITLE OF INVENTION: ALKALINE BACILLUS AMYLASE

(iii) NUMBER OF SEQUENCES: 5

(iv) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Floppy disk
- (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- (D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

20 (2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 485 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- 25 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

	His	His	Asn	Gly	Thr	Asn	Gly	Thr	Met	Met	Gln	Tyr	Phe	Glu	Trp	Tyr
	1				5					10					15	
30	Leu	Pro	Asn	Asp	Gly	Asn	His	Trp	Asn	Arg	Leu	Arg	Asp	Asp	Ala	Ala
				20					25					30		
	Asn	Leu	Lys	Ser	Lys	Gly	Ile	Thr	Ala	Val	Trp	Ile	Pro	Pro	Ala	Trp
			35					40					45			
35	Lys	Gly	Thr	Ser	Gln	Asn	Asp	Val	Gly	Tyr	Gly	Ala	Tyr	Asp	Leu	Tyr
		50					55					60				
	Asp	Leu	Gly	Glu	Phe	Asn	Gln	Lys	Gly	Thr	Val	Arg	Thr	Lys	Tyr	Gly
	65					70					75					80



Thr Arg Asn Gln Leu Gln Ala Ala Val Thr Ser Leu Lys Asn Asn Gly  
 85 90 95  
 Ile Gln Val Tyr Gly Asp Val Val Met Asn His Lys Gly Gly Ala Asp  
 100 105 110  
 5 Gly Thr Glu Ile Val Asn Ala Val Glu Val Asn Arg Ser Asn Arg Asn  
 115 120 125  
 Gln Glu Thr Ser Gly Glu Tyr Ala Ile Glu Ala Trp Thr Lys Phe Asp  
 130 135 140  
 10 Phe Pro Gly Arg Gly Asn Asn His Ser Ser Phe Lys Trp Arg Trp Tyr  
 145 150 155 160  
 His Phe Asp Gly Thr Asp Trp Asp Gln Ser Arg Gln Leu Gln Asn Lys  
 165 170 175  
 Ile Tyr Lys Phe Arg Gly Thr Gly Lys Ala Trp Asp Trp Glu Val Asp  
 180 185 190  
 15 Thr Glu Asn Gly Asn Tyr Asp Tyr Leu Met Tyr Ala Asp Val Asp Met  
 195 200 205  
 Asp His Pro Glu Val Ile His Glu Leu Arg Asn Trp Gly Val Trp Tyr  
 210 215 220  
 20 Thr Asn Thr Leu Asn Leu Asp Gly Phe Arg Ile Asp Ala Val Lys His  
 225 230 235 240  
 Ile Lys Tyr Ser Phe Thr Arg Asp Trp Leu Thr His Val Arg Asn Thr  
 245 250 255  
 Thr Gly Lys Pro Met Phe Ala Val Ala Glu Phe Trp Lys Asn Asp Leu  
 260 265 270  
 25 Gly Ala Ile Glu Asn Tyr Leu Asn Lys Thr Ser Trp Asn His Ser Val  
 275 280 285  
 Phe Asp Val Pro Leu His Tyr Asn Leu Tyr Asn Ala Ser Asn Ser Gly  
 290 295 300  
 30 Gly Tyr Tyr Asp Met Arg Asn Ile Leu Asn Gly Ser Val Val Gln Lys  
 305 310 315 320  
 His Pro Thr His Ala Val Thr Phe Val Asp Asn His Asp Ser Gln Pro  
 325 330 335  
 Gly Glu Ala Leu Glu Ser Phe Val Gln Gln Trp Phe Lys Pro Leu Ala  
 340 345 350  
 35 Tyr Ala Leu Val Leu Thr Arg Glu Gln Gly Tyr Pro Ser Val Phe Tyr  
 355 360 365  
 Gly Asp Tyr Tyr Gly Ile Pro Thr His Gly Val Pro Ala Met Lys Ser

	370		375		380														
	Lys	Ile	Asp	Pro	Leu	Leu	Gln	Ala	Arg	Gln	Thr	Phe	Ala	Tyr	Gly	Thr			
	385					390					395								400
5	Gln	His	Asp	Tyr	Phe	Asp	His	His	Asp	Ile	Ile	Gly	Trp	Thr	Arg	Glu			
					405					410						415			
	Gly	Asn	Ser	Ser	His	Pro	Asn	Ser	Gly	Leu	Ala	Thr	Ile	Met	Ser	Asp			
					420				425					430					
	Gly	Pro	Gly	Gly	Asn	Lys	Trp	Met	Tyr	Val	Gly	Lys	Asn	Lys	Ala	Gly			
			435					440					445						
10	Gln	Val	Trp	Arg	Asp	Ile	Thr	Gly	Asn	Arg	Thr	Gly	Thr	Val	Thr	Ile			
		450					455					460							
	Asn	Ala	Asp	Gly	Trp	Gly	Asn	Phe	Ser	Val	Asn	Gly	Gly	Ser	Val	Ser			
	465					470					475					480			
15	Val	Trp	Val	Lys	Gln														
					485														

(2) INFORMATION FOR SEQ ID NO: 2:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 485 amino acids
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

25	His	His	Asn	Gly	Thr	Asn	Gly	Thr	Met	Met	Gln	Tyr	Phe	Glu	Trp	His			
	1				5					10					15				
	Leu	Pro	Asn	Asp	Gly	Asn	His	Trp	Asn	Arg	Leu	Arg	Asp	Asp	Ala	Ser			
				20					25					30					
	Asn	Leu	Arg	Asn	Arg	Gly	Ile	Thr	Ala	Ile	Trp	Ile	Pro	Pro	Ala	Trp			
			35					40					45						
30	Lys	Gly	Thr	Ser	Gln	Asn	Asp	Val	Gly	Tyr	Gly	Ala	Tyr	Asp	Leu	Tyr			
		50					55					60							
	Asp	Leu	Gly	Glu	Phe	Asn	Gln	Lys	Gly	Thr	Val	Arg	Thr	Lys	Tyr	Gly			
	65					70					75					80			
35	Thr	Arg	Ser	Gln	Leu	Glu	Ser	Ala	Ile	His	Ala	Leu	Lys	Asn	Asn	Gly			
					85					90					95				

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

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

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

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

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

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

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

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

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

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

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

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

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

Gly Asn Tyr Asp Met Ala Lys Leu Leu Asn Gly Thr Val Val Gln Lys  
 305 310 315 320

30 His Pro Met His Ala Val Thr Phe Val Asp Asn His Asp Ser Gln Pro  
 325 330 335

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

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

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

Lys Ile Asp Pro Ile Leu Glu Ala Arg Gln Asn Phe Ala Tyr Gly Thr





GGGAATCATT GGAACAGGTT GAGGGATGAC GCAGCTAACT TAAAGAGTAA AGGGATAACA 120  
GCIGIATGGA TCCCACCTGC ATGGAAGGGG ACTTCCCAGA ATGATGIAGG TTATGGAGCC 180  
TATGATTTAT ATGATCTTGG AGAGTTTAAAC CAGAAGGGGA CGGTTGGTAC AAAATATGGA 240  
ACACGCAACC AGCTACAGGC TGGGGIGACC TCTTTAAAAA ATAACGGCAT TCAGGTATAT 300  
5 GGIGATGTCG TCATGAATCA TAAAGGIGGA GCAGATGGTA CGGAAATTGT AAATGCGGTA 360  
GAAGTGAATC GGACCAACCG AAACCAGGAA ACCTCAGGAG AGTATGCAAT AGAAGCGTGG 420  
ACAAAGTTTG ATTTTCCCTGG AAGAGGAAAT AACCATTTCCA GCITTAAGTG GCGCTGGTAT 480  
CATTTTGATG GGACAGATTG GGATCAGTCA CGCCAGCTTC AAAACAAAAT ATATAAATTC 540  
AGGGGAACAG GCAAGGCCCTG GGACTGGGAA GTCGATACAG AGAATGGCAA CTATGACTAT 600  
10 CTTATGTAIG CAGACGTGGA TATGGATCAC CCAGAAGTAA TACATGAACT TAGAACTGG 660  
GGAGTGTGGT ATACGAATAC ACTGAACCTT GATGGATTTA GAATAGATGC AGTGAACAT 720  
ATAAAATATA GCITTAAGAG AGATTTGGCTT ACACATGTGC GTAACACCAC AGGTAAACCA 780  
AIGTTTGCAG TGGCTGAGTT TTGGAAAAAT GACCTTGGTG CAATTGAAA CTATTIGAAT 840  
AAAACAAGTT GGAATCACTC GGIGTTTIGAT GTTCTCTCC ACTATAATTT GTACAATGCA 900  
15 TCIAATAGCG GIGGTTATTA TGATATGAGA AATATTTTAA ATGGTTCIGT GGIGCAAAAA 960  
CATCCAACAC ATGCGGTAC TTTTGTGAT AACCATGATT CTCAGCCCGG GGAAGCAITG 1020  
GAATCCITTG TTCAACAATG GTTAAACCA CTGCAATAG CATTTGGTCT GACAAGGGAA 1080  
CAAGGTTATC CTTCGGTATT TTATGGGGAT TACTACGGTA TCCCAACCCA TGGTGTCCG 1140  
GCTATGAAAT CTAATAATAGA CCTCTTCTG CAGGCACGTC AAACITTTGC CTATGGTACG 1200  
20 CAGCATGATT ACTTTGATCA TCATGATATT ATCGGTTGGA CAAGAGAGCG AAATAGCTCC 1260  
CATCCAAATT CAGGCTTGC CACCATATG TCAGATGGTC CAGGIGGTA CAAATGGATG 1320  
TATGIGGGGA AAAATAAAGC GGGACAAGTT TGGAGAGATA TTAACGGAAA TAGGACAGGC 1380  
ACCGTCACAA TTAATGCAGA CGGATGGGGT AATTTCTCTG TTAATGGAGG GTCCGTTTCG 1440  
GTTTGGGIGA AGCAA 1455

25 (2) INFORMATION FOR SEQ ID NO: 5:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 1455 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

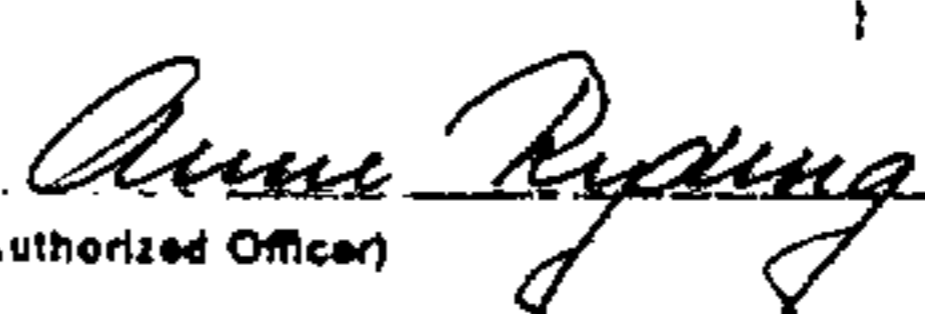
2186592

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

CATCATAATG GGACAAATGG GACGATGATG CAATACTTTG AATGGCAGTT GCCTAATGAT 60  
 GGAATCACT GGAATAGATT AAGAGATGAT GCTAGTAATC TAAGAAATAG AGGTATAACC 120  
 5 GCTATTGGGA TTCGGCCIGC CIGGAAAGGG ACTTCGCAA AATGATGIGGG GTATGGAGCC 180  
 TATGATCFTT ATGATTTAGG GGAATTTAAT CAAAAGGGGA CGGTCGTAC TAAGTATGGG 240  
 ACAOGTAGTC AATTTGGAGTC TGCCATCCAT GCCTTAAAGA ATAATGGCGT TCAAGTTTAT 300  
 GGGGATGTAG TGATGAACCA TAAAGGAGGA GCTGATGCTA CAGAAAACGT TCTTGCIGTC 360  
 GAGGIGAATC CAAATAACCG GAATCAAGAA ATATCIGGGG ACTACACAAT TGAGGCCTGG 420  
 10 ACTAAGTTTG ATTTTCCAGG GAGGGGTAAAT ACATACTCAG ACCTTAAATG GCGTTGGTAT 480  
 CATTTCGATG GGTAGATTTG GGATCAATCA CGACAATTCC AAAATCGTAT CTACAAATTC 540  
 CGAGGTGATG GTAAGGCATG GGATTTGGAA GTAGATTCGG AAAATGGAAA TTATGATTTAT 600  
 TTAATGTATG CAGATGTAGA TAIGGATCAT CCGGAGGTAG TAAATGAGCT TAGAAGATGG 660  
 GGAGAATGGT ATACAAATAC ATTAAATCCT GATGGATTTA GGATCGATGC GGTGAAGCAT 720  
 15 ATTAAATATA GCCTTACACG TGATTTGGTTG ACCCATGTAA GAAACGCAAC GGGAAAAGAA 780  
 ATGTTTGCTG TTGCTGAATF TIGGAAAAAT GATTTAGGTG CCTTGGAGAA CTATTTAAAT 840  
 AAAACAAACT GGAATCATTG TGCTTTTIGAT GTCCCCCTTC ATTATAATCT TTATAACGGG 900  
 TCAANTAGTG GAGGCAACTA TGACATGGCA AACTTCTTA ATGGAACGGT TGTTCAAAAG 960  
 CATCCAATGC ATGCCGTIAC TTTTGIGGAT AATCAGGATT CTCAACCTGG GGAATCATT 1020  
 20 GAATCATTTG TACAAGAATG GTTTAAGCCA CTGTCTTATG CGCTTATTTT AACAGAGAA 1080  
 CAAGGCTATC CCTCTGTCTT CTATGGTGAC TACTATGGAA TTCACACA TAGTGTCCCA 1140  
 GCAATGAAAG CCAAGATTGA TCCAATCTTA GAGGCGCGTC AAAATTTTGC ATATGGAAAC 1200  
 CAACATGATT ATTTTGACCA TCATAATATA ATCGGATGGA CACGTGAAGG AAATACCACG 1260  
 CATCCCAATT CAGGACTTGC GACTATCATG TGGGATGGC CAGGGGGAGA GAAATGGATG 1320  
 25 TACGTAGGGC AAAATAAAGC AGGTCAGTT TGGCATGACA TAACTGGAAA TAAACCAGGA 1380  
 ACAGTTACGA TCAATGCAGA TGGATGGGCT AATTTTTCAG TAAATGGAGG ATCTGTTTCC 1440  
 ATTTGGGTGA AACGA 1455

International Application No: PCT/

<b>MICROORGANISMS</b>	
Optional Sheet in connection with the microorganism referred to on page <u>4</u> , line <u>9-10</u> of the description <sup>1</sup>	
<b>A. IDENTIFICATION OF DEPOSIT <sup>1</sup></b>	
Further deposits are identified on an additional sheet <input type="checkbox"/> <sup>2</sup>	
Name of depositary institution <sup>4</sup>	
DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELL-KULTUREN GmbH	
Address of depositary institution (including postal code and country) <sup>4</sup>	
Mascheroder Weg 1b, D-38124 Braunschweig, Federal Republic of Germany	
Date of deposit <sup>4</sup>	Accession Number <sup>4</sup>
16 August 1994	DSM 9375
<b>B. ADDITIONAL INDICATIONS <sup>7</sup></b> (leave blank if not applicable). This information is continued on a separate attached sheet <input type="checkbox"/>	
<p>In respect of those designations in which a European and/or Australian patent is sought, during the pendency of the patent application a sample of the deposited microorganism is only to be provided to an independent expert nominated by the person requesting the sample (Rule 28(4) EPC / Regulation 3.25 of Australia Statutory Rules 1991 No 71).</p>	
<b>C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE <sup>7</sup></b> (if the indications are not for all designated States)	
<b>D. SEPARATE FURNISHING OF INDICATIONS <sup>8</sup></b> (leave blank if not applicable)	
The indications listed below will be submitted to the International Bureau later <sup>9</sup> (Specify the general nature of the indications e.g., "Accession Number of Deposit")	
<b>E. <input checked="" type="checkbox"/></b> This sheet was received with the international application when filed (to be checked by the receiving Office)	
 (Authorized Officer)	
<input type="checkbox"/> The date of receipt (from the applicant) by the International Bureau <sup>10</sup>	
was	_____
	(Authorized Officer)



1. An  $\alpha$ -amylase characterized by having:
  - (a) a specific activity at least 25% higher than the specific activity of Termamyl® at any temperature in the range of 25°C to 55°C and at pH 10, determined by diluting said  $\alpha$ -amylase in 50 mM Britton-Robinson buffer, adding 1 ml of this  $\alpha$ -amylase solution to 5 ml of 50 mM Britton-Robinson buffer containing one Phadebas® tablet suspended therein, and measuring the absorbance at 620 nm; and
  - (b) the amino acid sequence shown in SEQ ID NO:1 or at least 90% identity with the amino acid sequence shown in SEQ ID NO:1, or
  - (c) the amino acid sequence shown in SEQ ID NO:2 or at least 90% identity with the amino acid sequence shown in SEQ ID NO:2.
2. An  $\alpha$ -amylase according to claim 1, wherein the  $\alpha$ -amylase is obtained from an alkaliphilic *Bacillus* species.
3. An  $\alpha$ -amylase according to claim 2, obtained from any of strains NCIB 12289, NCIB 12512, NCIB 12513, and DSM 9375.
4. A detergent composition comprising an  $\alpha$ -amylase according to any one of claims 1-3 and a surfactant.
5. A laundry detergent composition comprising an  $\alpha$ -amylase according to any one of claims 1-3 and a surfactant.
6. A dishwashing detergent composition comprising an  $\alpha$ -amylase according to any one of claims 1-3 and a surfactant.
7. A detergent composition according to any one of claims 4-6, which further comprises one or more other enzymes selected from the group consisting of a protease, a lipase, a cellulase, a peroxidase, and an oxidase.

8. A detergent additive comprising an  $\alpha$ -amylase according to any one of claims 1-3, provided in the form of a non-dusting granulate, a stabilized liquid, a slurry, or a protected enzyme.
9. Use of a detergent according to any one of claims 5-7 or a detergent comprising an additive according to claim 8, for laundry washing, dishwashing, or hard-surface cleaning.
10. Use of an  $\alpha$ -amylase according to any one of claims 1-3 in a process of starch liquefaction.
11. Use of an  $\alpha$ -amylase according to any one of claims 1-3 in the production of lignocellulosic materials from waste paper containing starch or waste board containing starch.
12. The use according to claim 11, wherein the lignocellulosic materials are selected from the group consisting of pulp, paper, and cardboard.
13. The use according to claim 11 for deinking recycled starch-coated or starch-containing printed paper.
14. Use of an  $\alpha$ -amylase according to any one of claims 1-3, to modify starch for paper-making in a suspension of alkaline mineral filler.
15. The use according to claim 14, wherein the alkaline mineral filler is calcium carbonate.
16. Use of an  $\alpha$ -amylase according to any one of claims 1-3 for textile desizing.
17. The use according to claim 16, wherein said  $\alpha$ -amylase is used in combination with a cellulase.

18. Use of an  $\alpha$ -amylase according to any one of claims 1-3 for a beer-making process.
19. A DNA construct comprising a DNA sequence encoding an  $\alpha$ -amylase according to any one of claims 1-3.
20. A recombinant expression vector which carries a DNA construct according to claim 19.
21. A cell which is transformed with a DNA construct according to claim 19 or a vector according to claim 20.
22. A cell according to claim 21, which is a microorganism.
23. A cell according to claim 22, which is a bacterium or a fungus.
24. A cell according to claim 23, which is a Gram-positive bacterium or a Gram-negative bacterium.
25. A cell according to claim 24, which is a Gram-positive bacterium selected from the group consisting of *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus lentus*, *Bacillus brevis*, *Bacillus stearothermophilus*, *Bacillus alkalophilus*, *Bacillus amyloliquefaciens*, *Bacillus coagulans*, *Bacillus circulans*, *Bacillus lautus*, *Bacillus thuringiensis*, *Streptomyces lividans*, and *Streptomyces murinus*.
26. A cell according to claim 24, which is *E. coli*.
27. A method of producing an  $\alpha$ -amylase characterized by having:
  - (a) a specific activity at least 25% higher than the specific activity of Termamyl® at any temperature in the range of 25°C to 55°C and at pH 10, determined by diluting said  $\alpha$ -amylase in 50 mM Britton-Robinson buffer, adding 1 ml of this  $\alpha$ -amylase solution to 5 ml of 50



mM Britton-Robinson buffer containing one Phadebas® tablet suspended therein, and measuring the absorbance at 620 nm; and

(b) the amino acid sequence shown in SEQ ID NO:1 or at least 90% identity with the amino acid sequence shown in SEQ ID NO:1, or

(c) the amino acid sequence shown in SEQ ID NO:2 or at least 90% identity with the amino acid sequence shown in SEQ ID NO:2;

wherein a cell according to any one of claims 21-26 is cultured under conditions conducive to the production of the  $\alpha$ -amylase, and the  $\alpha$ -amylase is subsequently recovered from the culture.

28. A method of producing an  $\alpha$ -amylase obtained from an alkaliphilic *Bacillus* species and characterized by having:

(a) a specific activity at least 25% higher than the specific activity of Termamyl® at any temperature in the range of 25°C to 55°C and at pH 10, determined by diluting said  $\alpha$ -amylase in 50 mM Britton-Robinson buffer, adding 1 ml of this  $\alpha$ -amylase solution to 5 ml of 50 mM Britton-Robinson buffer containing one Phadebas® tablet suspended therein, and measuring the absorbance at 620 nm; and

(b) the amino acid sequence shown in SEQ ID NO:1 or at least 90% identity with the amino acid sequence shown in SEQ ID NO:1, or

(c) the amino acid sequence shown in SEQ ID NO:2 or at least 90% identity with the amino acid sequence shown in SEQ ID NO:2;

wherein a cell according to any one of claims 21-25 is cultured under conditions conducive to the production of the  $\alpha$ -amylase, and the  $\alpha$ -amylase is subsequently recovered from the culture.

29. A method of producing an  $\alpha$ -amylase obtained from a *Bacillus* strain selected from the group consisting of NCIB 12289, NCIB 12512, NCIB 12513, and DSM 9375, and characterized by having:

(a) a specific activity at least 25% higher than the specific activity of Termamyl® at any temperature in the range of 25°C to 55°C and at pH 10, determined by diluting said  $\alpha$ -amylase in 50 mM Britton-Robinson buffer, adding 1 ml of this  $\alpha$ -amylase solution to 5 ml of 50



mM Britton-Robinson buffer containing one Phadebas® tablet suspended therein, and measuring the absorbance at 620 nm; and

(b) the amino acid sequence shown in SEQ ID NO:1 or at least 90% identity with the amino acid sequence shown in SEQ ID NO:1, or

(c) the amino acid sequence shown in SEQ ID NO:2 or at least 90% identity with the amino acid sequence shown in SEQ ID NO:2;

wherein a cell according to any one of claims 21-25 is cultured under conditions conducive to the production of the  $\alpha$ -amylase, and the  $\alpha$ -amylase is subsequently recovered from the culture.

1/5

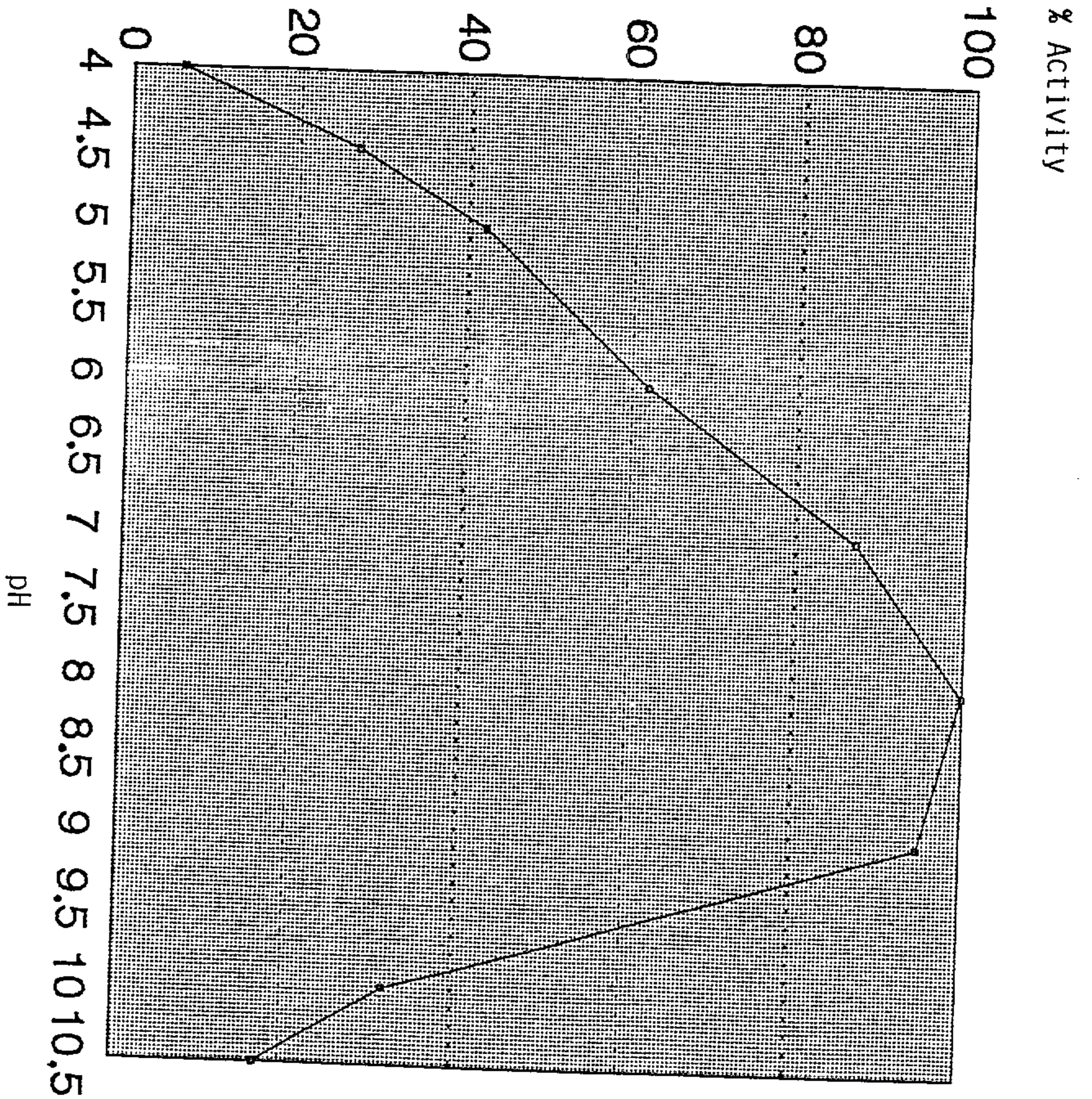


Fig. 1



2/5

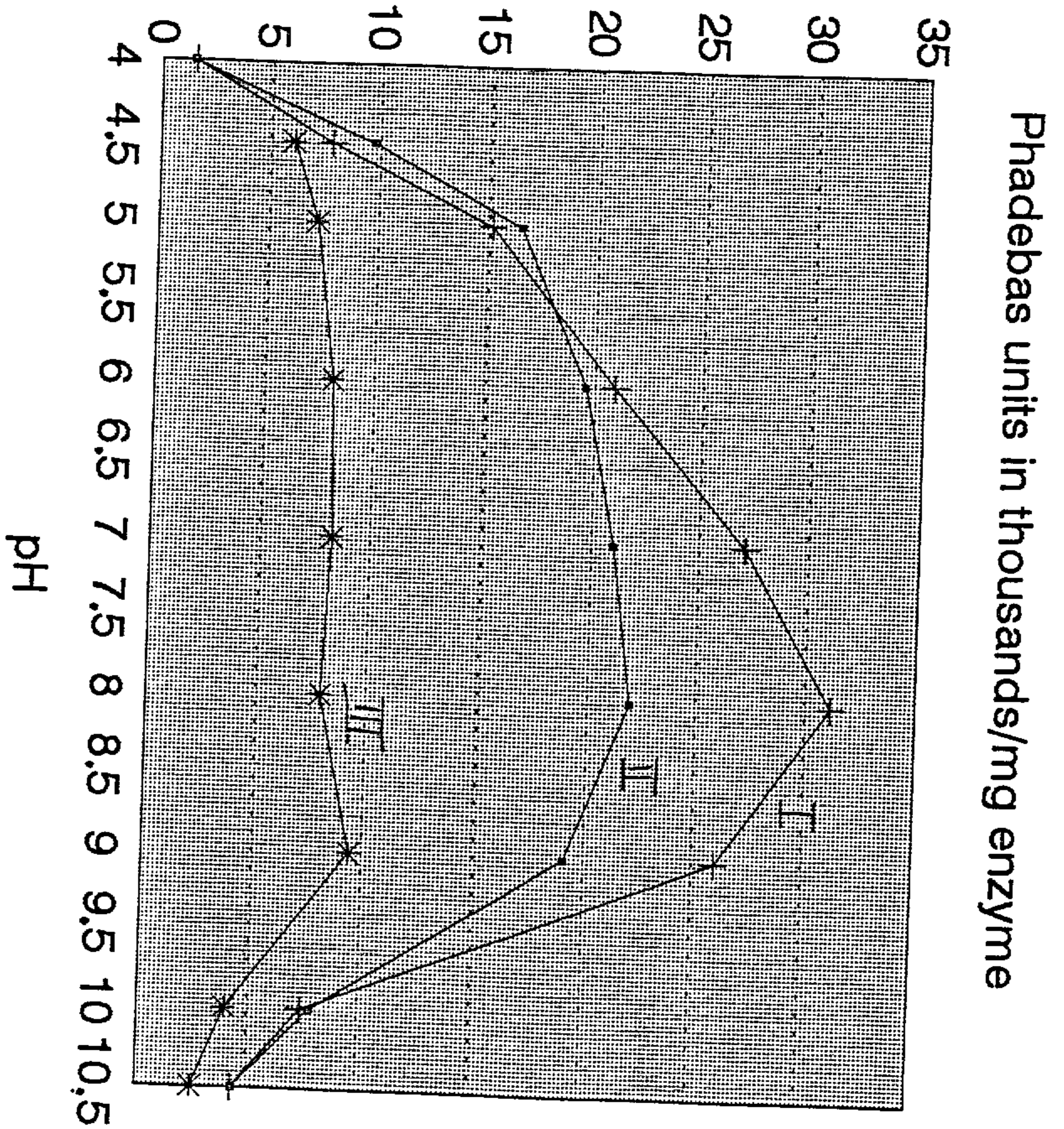


Fig. 2



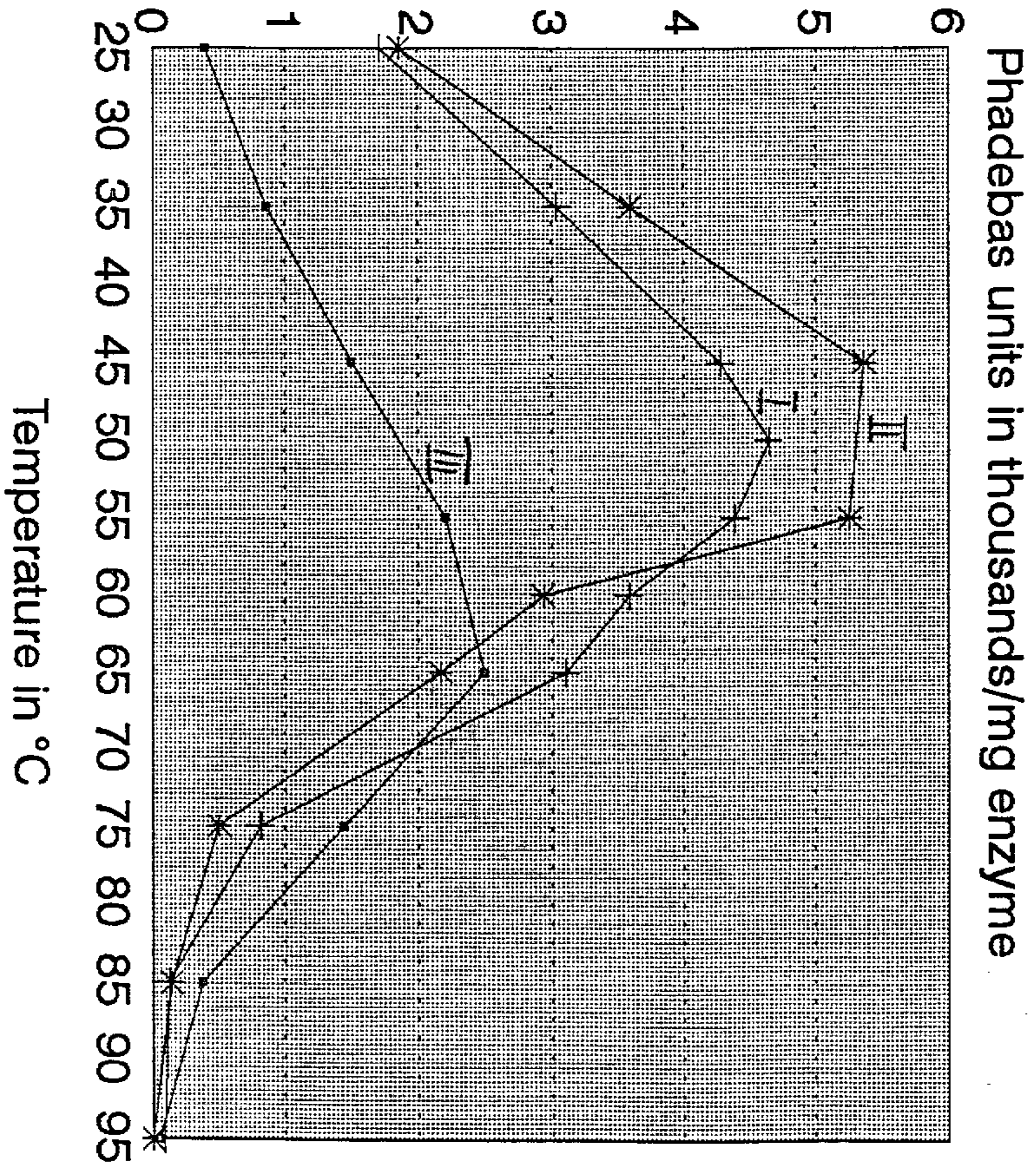


Fig. 3



4/5

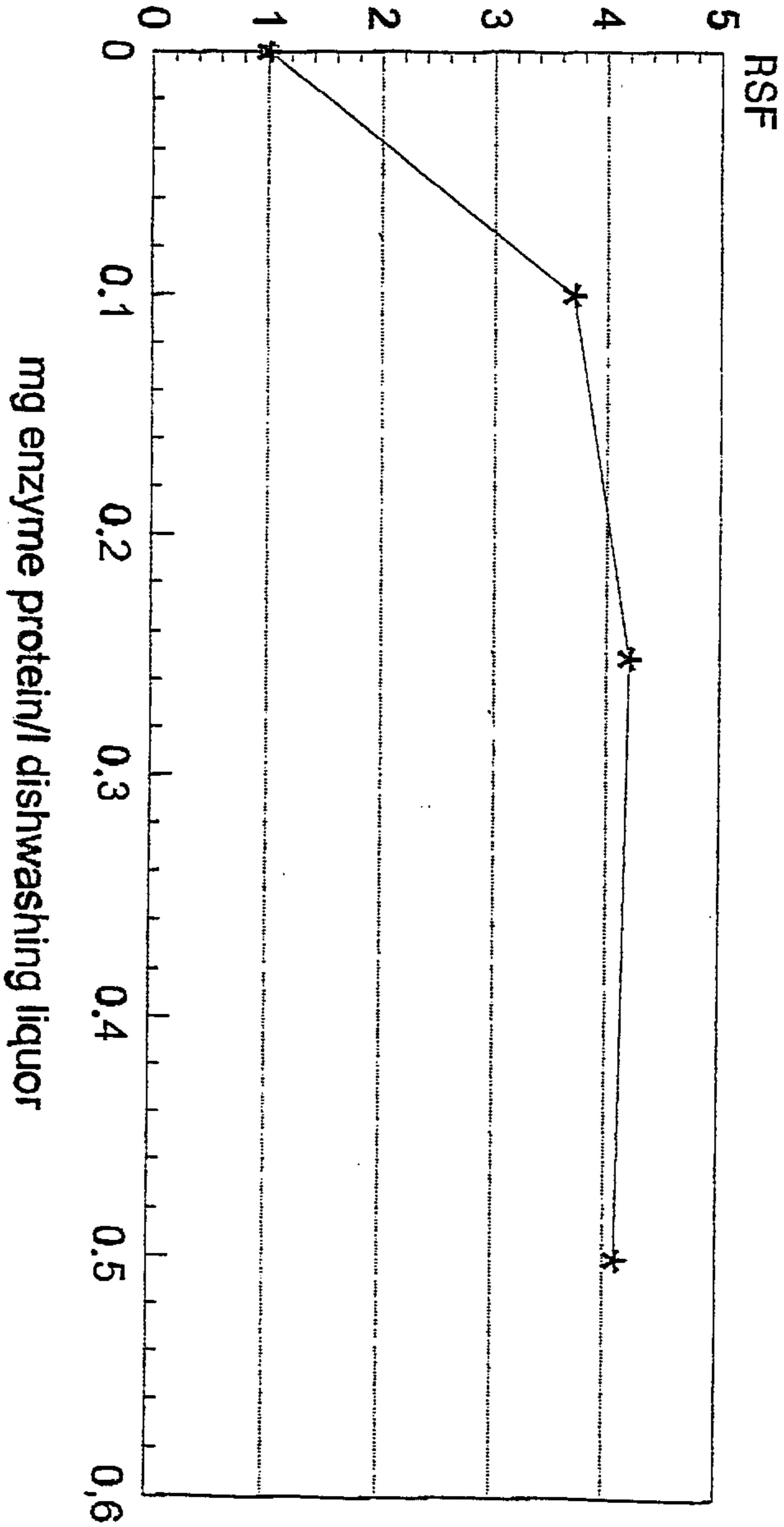


Fig. 4

5/5

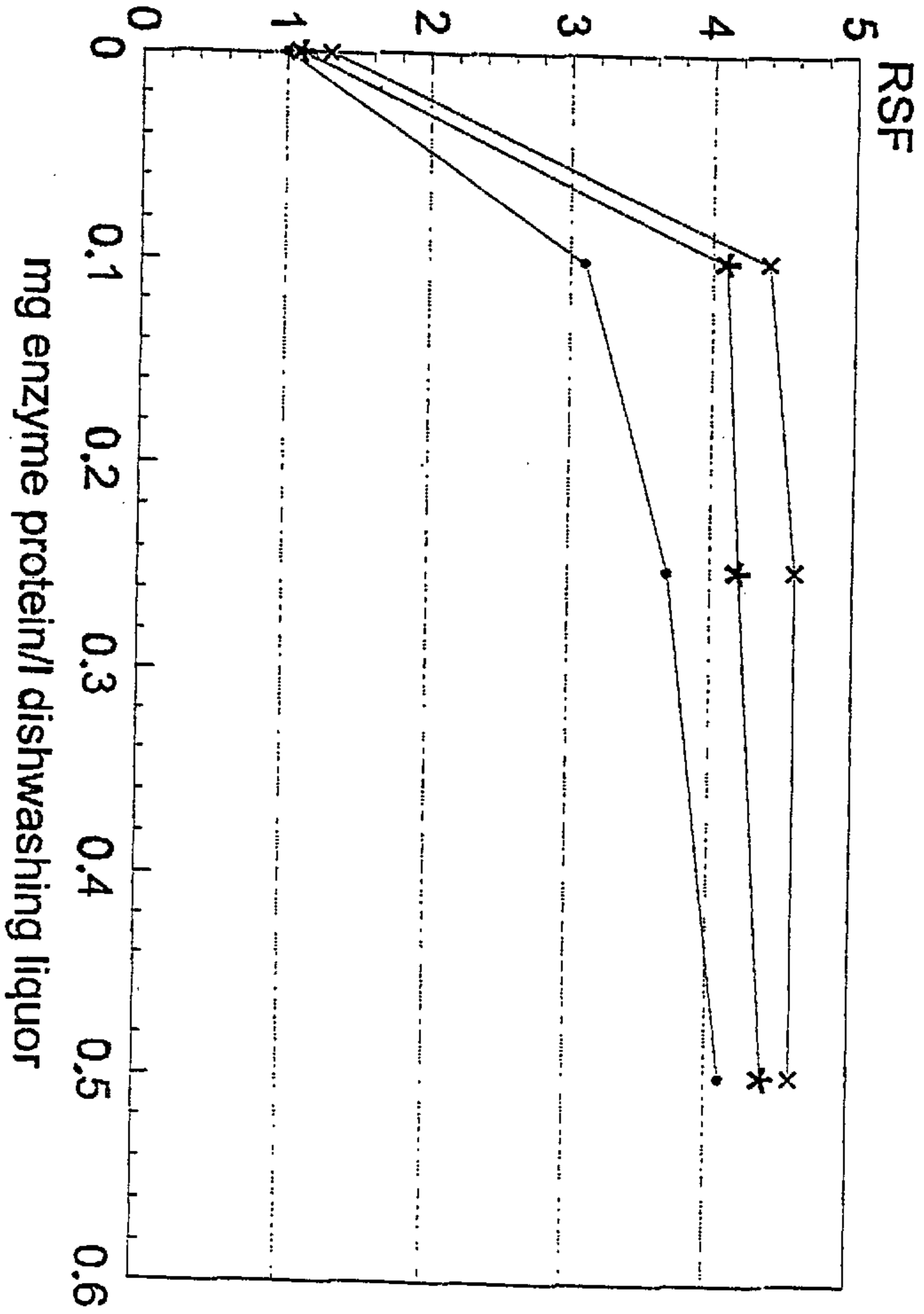


Fig. 5

% Activity

100

80

60

40

20

0

4 4.5 5 5.5 6 6.5 7 7.5 8 8.5 9 9.5 10 10.5

pH

