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(54) Title: AMYLASE VARIANTS

(57) Abrégé/Abstract:
A variant of a parent α-amylase enzyme having an improved washing and/or dishwashing performance as compared to the parent enzyme, wherein one or more amino acid residues of the parent enzyme have been replaced by a different amino acid residue and/or wherein one or more amino acid residues of the parent α-amylase have been deleted and/or wherein one or more amino acid residues have been added to the parent α-amylase enzyme, provided that the variant is different from one in which the methionine residue in position 197 of a parent B. licheniformis α-amylase has been replaced by alanine or threonine, as the only modification being made. The variant may be used for washing and dishwashing.

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(54) Title: AMYLASE VARIANTS

(57) Abstract

A variant of a parent α-amylase enzyme having an improved washing and/or dishwashing performance as compared to the parent enzyme, wherein one or more amino acid residues of the parent enzyme have been replaced by a different amino acid residue and/or wherein one or more amino acid residues of the parent α-amylase have been deleted and/or wherein one or more amino acid residues have been added to the parent α-amylase enzyme, provided that the variant is different from one in which the methionine residue in position 197 of a parent *B. licheniformis* α-amylase has been replaced by alanine or threonine, as the only modification being made. The variant may be used for washing and dishwashing.

AMYLASE VARIANTS

FIELD OF THE INVENTION

The present invention relates to amylase variants having an improved washing and/or dishwashing performance, to DNA constructs encoding the variants, and to vectors and cells harbouring the DNA constructs. Furthermore, the invention relates to methods of producing the amylase variants and to detergent additives and detergent compositions comprising the amylase variants. Finally, the invention relates to the use of the amylase variants for textile desizing.

BACKGROUND OF THE INVENTION

For a number of years α-amylase enzymes have been used for a variety of different purposes, the most important of which are starch liquefaction, textile desizing, starch modification in the paper and pulp industry, and for brewing and baking. A further use of α-amylase, which is becoming increasingly important, is the removal of starchy stains during washing or dishwashing.

In recent years attempts have been made to construct α-amylase variants having improved properties with respect to specific uses such as starch liquefaction and textile desizing.

For instance, US 5,093,257 discloses chimeric α-amylases comprising an N-terminal part of a *B. stearothermophilus* α-amylase and a C-terminal part of a *B. licheniformis* α-amylase. The chimeric α-amylases are stated to have unique properties, such as a different thermostability, as compared to their parent α-amylase. However, all of the specifically described chimeric α-amylases were shown to have a decreased enzymatic activity as compared to their parent α-amylases.

EP 252 666 describes hybrid amylases of the general formula Q-R-L, in which Q is a N-terminal polypeptide residue of from 55 to 60 amino acid residues which is at least 75% homologous to the 57 N-terminal amino acid residues of a specified α-amylase from *B. amyloliquefaciens*, R is a specified polypeptide, and L is a C-terminal polypeptide comprising from 390 to 400 amino acid residues which is at least 75% homologous to the 395 C-terminal amino acid residues of a specified *B. licheniformis* α-amylase.

Suzuki et al. (1989) disclose chimeric α-amylases, in which specified regions of a *B. amyloliquefaciens* α-amylase have been substituted for the corresponding regions of a *B. licheniformis* α-amylase. The chimeric α-amylases were constructed with the purpose of identifying regions responsible for thermostability. Such regions were found to include amino acid residues 177-186 and amino acid residues 255-270 of the *B. amyloliquefaciens* α-amylase. The alterations of amino acid residues in the chimeric α-amylases did not seem to affect properties of the enzymes other than their thermostability.

WO 91/00353 discloses α-amylase mutants which differ from their parent α-amylase in at least one amino acid residue. The α-amylase mutants disclosed in said patent application are stated to exhibit improved properties for application in the degradation of starch and/or textile desizing due to their amino acid substitutions. Some of the mutants exhibit improved stability, but no improvements in enzymatic activity were reported or indicated. The only mutants exemplified are prepared from a parent *B. licheniformis* α-amylase and carry one of the following mutations: H133Y or H133Y + T149I. Another suggested mutation is A111T.

FR 2,676,456 discloses mutants of the *B. licheniformis* α-amylase, in which an amino acid residue in the proximity of His 133 and/or an amino acid residue in the proximity of Ala 209 have been replaced by a more hydrophobic amino acid residue. The resulting α-amylase mutants are stated to have an improved

thermostability and to be useful in the textile, paper, brewing and starch liquefaction industry.

EP 285 123 discloses a method of performing random mutagenesis of a nucleotide sequence. As an example of such sequence a nucleotide sequence encoding a *B. stearothermophilus* α-amylase is mentioned. When mutated, an α-amylase variant having improved activity at low pH values is obtained.

In none of the above references is it mentioned or even suggested that α-amylase mutants may be constructed which have improved properties with respect to the detergent industry.

EP 525 610 relates to mutant enzymes having an improved stability towards ionic tensides. The mutant enzymes have been produced by replacing an amino acid residue in the surface part of the parent enzyme with another amino acid residue. The only mutant enzyme specifically described in EP 525 610 is a protease. Amylase is mentioned as an example of an enzyme which may obtain an improved stability towards ionic tensides, but the type of amylase, its origin or specific mutations have not been specified.

WO 94/02597 which was unpublished at the priority dates of the present invention, discloses novel α-amylase mutants which exhibit an improved stability and activity in the presence of oxidizing agents. In the mutant α-amylases, one or more methionine residues have been replaced with amino acid residues different from Cys and Met. The α-amylase mutants are stated to be useful as detergent and/or dishwashing additives as well as for textile desizing.

WO 94/18314 (published only after the priority dates of the present invention) discloses oxidatively stable α-amylase mutants, including mutations in the M197 position of *B. licheniformis* α-amylase.

EP 368 341 describes the use of pullulanase and other amylolytic enzymes optionally in combination with an α-amylase for washing and dishwashing.

The object of the present invention is to provide α-amylase variants which exert an improved washing and/or dishwashing performance compared to their parent α-amylase. Such variant α-amylases have the advantage that they may be employed in a lower dosage than their parent α-amylase. Furthermore, the α-amylase variants may be able to remove starchy stains which cannot or can only with difficulty be removed by α-amylase detergent enzymes known today.

BRIEF DISCLOSURE OF THE INVENTION

The present inventors have surprisingly found that it is possible to improve the washing and/or dishwashing performance of α-amylases by modifying one or more amino acid residues thereof. The present invention is based on this finding.

Accordingly, in a first aspect the present invention relates to a variant of a parent α-amylase enzyme having an improved washing and/or dishwashing performance as compared to the parent enzyme, wherein one or more amino acid residues of the parent enzyme have been replaced by a different amino acid residue and/or wherein one or more amino acid residues of the parent α-amylase have been deleted and/or wherein one or more amino acid residues have been added to the parent α-amylase enzyme, provided that the variant is different from one in which the methionine residue in position 197 of a parent *B. licheniformis* α-amylase has been replaced by alanine or threonine, as the only modification being made.

Except for the disclosure of WO 94/02597, in which replacement of the methionine residue located in position 197 of a *B. licheniformis* α-amylase known as Termamyl® (available from Novo Nordisk A/S, Denmark) by alanine or threonine have been shown to result in an improved performance, as far as the present inventors are aware, no prior disclosure exists which suggests or

discloses that washing and/or dishwashing performance of α-amylases may be improved by modifying one or more amino acid residues of the native α-amylase.

In the present context the term "performance" as used in connection with washing and dishwashing is intended to mean an improved removal of starchy stains, i.e. stains containing starch, during washing or dishwashing, respectively. The performance may be determined in conventional washing and dishwashing experiments and the improvement evaluated as a comparison with the performance of the parent unmodified α-amylase. Examples of suitable washing and dishwashing tests are given in the Materials and Methods section and in the examples below. It will be understood that a variety of different characteristics of the α-amylase variant, including specific activity, substrate specificity, Km, Vmax, pI, pH optimum, temperature optimum, thermoactivation, stability towards detergents, etc. taken alone or in combination are involved in providing the improved performance. The skilled person will be aware that the performance of the variant cannot, alone, be predicted on the basis of the above characteristics, but would have to be accompanied by washing and/or dishwashing performance tests.

In the present context the term "variant" is used interchangeably with the term "mutant". The term "variant" is intended to include hybrid α-amylases, i.e. α-amylases comprising parts of at least two different parent α-amylases.

In further aspects the invention relates to a DNA construct comprising a DNA sequence encoding an α-amylase variant of the invention, a recombinant expression vector carrying the DNA construct, a cell which is transformed with the DNA construct or the vector, as well as a method of producing the α-amylase variant by culturing said cell under conditions conducive to the production of the α-amylase variant, after which the α-amylase variant is recovered from the culture.

In a further aspect the invention relates to a method of preparing a variant of a parent α-amylase having improved washing and/or dishwashing performance as compared to the parent α-amylase, which method comprises

a) constructing a population of cells containing genes encoding variants of said parent α-amylase,

b) screening said population of cells for α-amylase activity under conditions simulating at least one washing and/or dishwashing condition,

c) isolating a cell from said population containing a gene encoding a variant of said parent α-amylase which has improved activity as compared with said parent α-amylase under the conditions selected in step b),

d) culturing the cell isolated in step c) under suitable conditions in an appropriate culture medium, and

e) recovering the α-amylase variant from the culture obtained in step d).

In the present context, the term "simulating at least one washing and/or dishwashing condition" is intended to indicate a simulation of, e.g., the temperature or pH prevailing during washing or dishwashing, as well as the chemical composition of a detergent composition to be used in the washing or dishwashing treatment. The term "chemical composition" is intended to include one, or a combination of two or more, constituents of the detergent composition in question. The constituents of a number of different detergent compositions are listed further below.

The "population of cells" referred to in step a) may suitably be constructed by cloning a DNA sequence encoding a parent α-amylase and subjecting the DNA to site-directed or random mutagenesis as described herein.

In a still further aspect the invention relates to a method of producing a hybrid α-amylase having an improved washing and/or dishwashing performance as compared to any of its parent enzymes, which method comprises

a) recombining *in vivo* or *in vitro* the N-terminal coding region of an α-amylase gene or corresponding cDNA of one of the parent α-amylases with the C-terminal coding region of an α-amylase gene or corresponding cDNA of another parent α-amylase to form recombinants,

b) selecting recombinants that produce a hybrid α-amylase having an improved washing and/or dishwashing performance as compared to any of its parent α-amylases,

c) culturing recombinants selected in step b) under suitable conditions in an appropriate culture medium, and

d) recovering the hybrid α-amylase from the culture obtained in step c).

In final aspects the invention relates to the use of an α-amylase variant of the invention as a detergent enzyme, in particular for washing or dishwashing, to a detergent additive and a detergent composition comprising the α-amylase variant, and to the use of an α-amylase variant of the invention for textile desizing.

DETAILED DISCLOSURE OF THE INVENTION

Nomenclature

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

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

Ala 30 Asn or A30N

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

Ala 30 * or A30*

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

Ala 30 AlaLys or A30AK

A deletion of a consecutive stretch of amino acid residues, such as amino acid residues 30-33, is indicated as (30-33)*

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

* 36 Asp or *36D

for insertion of an aspartic acid in position 36

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

Ala 30 Asp + Glu 34 Ser or A30N+E34S

representing mutations in positions 30 and 34 substituting alanine and glutamic acid for asparagine and serine, respectively.

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

A30N,E or

A30N or A30E

Furthermore, when a position suitable for modification is identified herein without any specific modification being suggested, it is to be understood that any amino acid residue may be substituted for the amino acid residue present in the position. Thus, for instance, when a modification of an alanine in position 30 is mentioned, but not specified, it is to be understood that the alanine may be

deleted or substituted for any other amino acid, i.e. any one of R,N,D,A,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V

The parent α-amylases and variants thereof

The α-amylase variant of the invention is preferably prepared on the basis of a parent α-amylase of microbial origin. Thus, the parent α-amylase may be of bacterial origin or may be derived from a fungus including a filamentous fungus or a yeast. The parent α-amylase may be one conventionally used as a detergent enzyme, or one for which such use has never been suggested.

Of particular interest is a parent α-amylase which is derived from a strain of a gram-positive bacterium, such as a strain of *Bacillus*. *Bacillus* α-amylases have, in general, been found to have desirable properties with respect to detergent use.

More specifically, the parent bacterial α-amylase may be selected from an α-amylase derived from a strain of *B. licheniformis*, an α-amylase derived from a strain of *B. amyloliquefaciens*, an α-amylase derived from a strain of *B. stearothermophilus* or an α-amylase derived from a strain of *B. subtilis*. In the present context, "derived from" is intended not only to indicate an α-amylase produced or producible by a strain of the organism in question, but also an α-amylase encoded by a DNA sequence isolated from such strain and produced in a host organism transformed with said DNA sequence. Finally, the term is intended to indicate an α-amylase which is encoded by a DNA sequence of synthetic and/or cDNA origin and which has the identifying characteristics of the α-amylase in question.

It has been found that a number of α-amylases produced by *Bacillus* spp. are highly homologous on the amino acid level. For instance, the *B. licheniformis* α-amylase comprising the amino acid sequence shown in SEQ ID No. 2 has been found to be about 89% homologous with the *B. amyloliquefaciens* α-amylase comprising the amino acid sequence shown in SEQ ID No. 4 and about 79%

homologous with the *B. stearothermophilus* α-amylase comprising the amino acid sequence shown in SEQ ID No. 6.

However, other properties of these enzymes are considerably different. Thus, in general the above mentioned *B. licheniformis* α-amylase has been found to have a high pH optimum, a different specificity compared to other *Bacilllus* α-amylases and a low Km which usually is indicative of an excellent substrate binding, whereas the *B. amyloliquefaciens* and the *B. stearothermophilus* α-amylase have a high specific activity and a different starch degradation pattern compared to that of the *B. licheniformis* α-amylase. The *B. stearothermophilus* α-amylase exerts a better washing and/or dishwashing performance than the *B. amyloliquefaciens* α-amylase, but not a performance comparable to the very satisfactory performance of the *B. licheniformis* α-amylase.

In the present invention it has surprisingly been found that the washing and/or dishwashing performance of the satisfactorily performing *B. licheniformis* α-amylase may be further and considerably improved by modifying certain amino acid residues or regions in the amino acid sequence of the α-amylase so as to correspond to a homologous amino acid region in one of the other, more poorly performing *Bacillus* α-amylases mentioned above.

Thus, in accordance with the present invention it has surprisingly been found possible to use the high degree of amino acid sequence homology observed between the α-amylases produced by the *Bacillus* spp. *B. licheniformis*, *B. amyloliquefaciens* and *B. stearothermophilus* to prepare α-amylase variants having improved washing and/or dishwashing performance. More, specifically the variants are prepared on the basis of modification of one or more specific amino acid residues to one or more amino acid residues present in a corresponding or homologous position of the other homologous α-amylases.

For ease of reference, an alignment of the amino acid sequences shown in SEQ ID Nos. 2, 4 and 6, respectively, is shown below. The amino acid numbering of

each of the α-amylase sequences is also given. From this alignment homologous positions (and thus homologous amino acid residues) in the sequences may easily be identified.

	SEQUENCE	Res#
SEQ ID 6	AAPFNGTMMQYFEWYLPDDGTLWTKVANEANNLSSLGITA	40
SEQ ID 4	---VNGTLMQYFEWYTPNDGQHWKRLQNDAEHLSDIGITA	37
SEQ ID 2	-ANLNGTLMQYFEWYMPNDGQHWRRLQNDSAYLAEHGITA	39

	SEQUENCE	Res#
SEQ ID 6	LWLPPAYKGTSRSDVGYGVYDLYDLGEFNQKGTVRTKYGT	80
SEQ ID 4	VWIPPAYKGLSQSDNGYGPYDLYDLGEFQQKGTVRTKYGT	77
SEQ ID 2	VWIPPAYKGTSQADVGYGAYDLYDLGEFHQKGTVRTKYGT	79

	SEQUENCE	Res#
SEQ ID 6	KAQYLQAIQAAHAAGMQVYADVVFDHKGGADGTEWVDAVE	120
SEQ ID 4	KSELQDAIGSLHSRNVQVYGDVVLNHKAGADATEDVTAVE	117
SEQ ID 2	KGELQSAIKSLHSRDINVYGDVVINHKGGADATEDVTAVE	119

	SEQUENCE	Res#
SEQ ID 6	VNPSDRNQEISGTYQIQAWTKFDFPGRGNTYSSFKWRWYH	160
SEQ ID 4	VNPANRNQETSEEYQIKAWTDFRFPGRGNTYSDFKWHWYH	157
SEQ ID 2	VDPADRNRVISGEHLIKAWTHFHFPGRGSTYSDFKWHWYH	159

	SEQUENCE	Res#
SEQ ID 6	FDGVDWDESRKLSRIYKFRGIGKAWDWEVDTENGNYDYLM	200
SEQ ID 4	FDGADWDESRKISRIFKFRGEGKAWDWEVSSENGNYDYLM	197
SEQ ID 2	FDGTDWDESRKLNRIYKFQ--GKAWDWEVSNENGNYDYLM	197

	SEQUENCE	Res#
SEQ ID 6	YADLDMDHPEVVTELKNWGKWYVNTTNIDGFRLDAVKHIK	240
SEQ ID 4	YADVDYDHPDVVAETKKWGIWYANELSLDGFRIDAAKHIK	237
SEQ ID 2	YADIDYDHPDVAAEIKRWGTWYANELQLDGFRLDAVKHIK	237

SEQUENCE		Res#
SEQ ID 6	FSFFPDWLSYVRSQTGKPLFTVGEYWSYDINKLHNYITKT	280
SEQ ID 4	FSFLRDWVQAVRQATGKEMFTVAEYWQNNAGKLENYLNKT	277
SEQ ID 2	FSFLRDWVNHVREKTGKEMFTVAEYWQNDLGALENYLNKT	277

SEQUENCE		Res#
SEQ ID 6	DGTMSLFDAPLHNKFYTASKSGGAFDMRTLMTNTLMKDQP	320
SEQ ID 4	SFNQSVFDVPLHFNLQAASSQGGGYDMRRLLDGTVVSRHP	317
SEQ ID 2	NFNHSVFDVPLHYQFHAASTQGGGYDMRKLLNGTVVSKHP	317

SEQUENCE		Res#
SEQ ID 6	TLAVTFVDNHDTEPGQALQSWVDPWFKPLAYAFILTRQEG	360
SEQ ID 4	EKAVTFVENHDTQPGQSLESTVQTWFKPLAYAFILTRESG	357
SEQ ID 2	LKSVTFVDNHDTQPGQSLESTVQTWFKPLAYAFILTRESG	357

SEQUENCE		Res#
SEQ ID 6	YPCVFYGDYYGI---PQYNIPSLKSKIDPLLIARRDYAYG	397
SEQ ID 4	YPQVFYGDMYGTKGTSPKEIPSLKDNIEPILKARKEYAYG	397
SEQ ID 2	YPQVFYGDMYGTKGDSQREIPALKHKIEPILKARKQYAYG	397

SEQUENCE		Res#
SEQ ID 6	TQHDYLDHSDIIGWTREGGTEKPGSGLAALITDGPGGSKW	437
SEQ ID 4	PQHDYIDHPDVIGWTREGDSSAAKSGLAALITDGPGGSKR	437
SEQ ID 2	AQHDYFDHHDIVGWTREGDSSVANSGLAALITDGPGGAKR	437

SEQUENCE		Res#
SEQ ID 6	MYVGKQHAGKVFYDLTGNRSDTVTINSDGWGEFKVNGGSV	477
SEQ ID 4	MYAGLKNAGETWYDITGNRSDTVKIGSDGWGEFHVNDGSV	477
SEQ ID 2	MYVGRQNAGETWHDITGNRSEPVVINSEGWGEFHVNGGSV	477

SEQUENCE		Res#
SEQ ID 6	SVWVPRKTTVSTIARPITTRPWTGEFVRWTEPRLVAWP	515
SEQ ID 4	SIYVQK	483
SEQ ID 2	SIYVQR	483

Although the present invention is illustrated on the basis of modifications of the *B. licheniformis* α-amylase having the amino acid sequence shown in SEQ ID No. 2 (commercially available from Novo Nordisk A/S, Denmark as Termamyl®), it will be understood that analogues of said α-amylase may be modified correspondingly to create variants with improved washing and/or dishwashing performance. Thus, whenever reference is made to a specific modification of the *B. licheniformis* α-amylase it will be understood that an analogous α-amylase may be modified analogously.

In the present context, the term "analogue" is intended to indicate an α-amylase which

i) is at least 60% homologous with the sequence shown in SEQ ID No. 2, and/or

ii) exhibits immunological cross-reactivity with an antibody raised against the said α-amylase, and/or

iii) is encoded by a DNA sequence which hybridizes with the same probe as the DNA sequence encoding the said α-amylase, which latter DNA sequence is shown in SEQ ID No. 1.

Property i) of said analogue of the *B. licheniformis* α-amylase having the sequence shown in SEQ ID No. 2 is intended to indicate the degree of identity between the analogue and the *B. licheniformis* α-amylase indicating a derivation of the first sequence from the second. In particular, a polypeptide is considered to be homologous with the *B. licheniformis* α-amylase if a comparison of the respective amino acid sequences reveals a degree of sequence identity of greater than about 60%, such as above 70%, 80%, 85%, 90% or even 95%. Sequence comparisons can be performed via known algorithms, such as the one described by Lipman and Pearson (1985).

Said analogues of the *B. licheniformis* α-amylase comprising the amino acid sequence shown in SEQ ID No. 2 as defined by property i) above are therefore intended to comprise a homologous α-amylase derived from other *Bacillus* spp. than *B. licheniformis*, e.g. from *B. amyloliquefaciens* or *B. stearothermophilus*. Furthermore, the analogue may be a *B. licheniformis* α-amylase having an amino acid sequence different from, but homologous with, that shown in SEQ ID No. 2. An example of such an α-amylase is that produced by the *B. licheniformis* described in EP 252 666 (ATCC 27811), and those identified in WO 91/00353 and WO 94/18314. Other specific examples of analogues of the *B. licheniformis* α-amylase comprising the amino acid sequence shown in SEQ ID No. 2 are Optitherm® and Takatherm® (available from Solvay), Maxamyl® (available from Gist-Brocades), Spezym AA® (available from Genencor), and Keistase® (available from Daiwa).

Finally, the α-amylase analogue may be a genetically engineered α-amylase, e.g. any of those mentioned in the above described prior art references or a variant of any of the above specified *B. licheniformis* α-amylases. Typically, a genetically engineered α-amylase will have been prepared in order to improve one or more properties such as thermostability, acid/alkaline stability, temperature, pH optimum, and the like.

The properties ii) and iii) of said analogue of the *B. licheniformis* α-amylase comprising the amino acid sequence shown in SEQ ID No. 2 may be determined as follows:

Property ii) of said analogue, i.e. the immunological cross reactivity, may be assayed using an antibody raised against or reactive with at least one epitope of the *B. licheniformis* α-amylase comprising the amino acid sequence shown in SEQ ID No. 2. The antibody, which may either be monoclonal or polyclonal, may be produced by methods known in the art, e.g. as described by Hudson et al., 1989. The immunological cross-reactivity may be determined using assays known in the art, examples of which are Western Blotting or radial immunodiffusion

assay, e.g. as described by Hudson et al., 1989. In this respect, immunological cross-reactivity between the α-amylases having the amino acid sequences SEQ ID Nos. 2, 4 and 6, respectively, has been found.

The oligonucleotide probe used in the characterization of the analogue in accordance with property iii) defined above may suitably be prepared on the basis of the full or partial nucleotide or amino acid sequence shown in SEQ ID No. 1 and 2, encoding or constituting, respectively, the *B. licheniformis* α-amylase. Suitable conditions for testing hybridization involve presoaking in 5xSSC and prehybridizing for 1h at ~40°C in a solution of 20% formamide, 5xDenhardt's solution, 50mM sodium phosphate, pH 6.8, and 50μg of denatured sonicated calf thymus DNA, followed by hybridization in the same solution supplemented with 100μM ATP for 18h at ~40°C, or other methods described by e.g. Sambrook et al., 1989.

The present inventors have surprisingly found that modification of one or more amino acid residues in the N-terminal part of the *B. licheniformis* α-amylase comprising the amino acid sequence shown in SEQ ID No. 2 results in improved washing and/or dishwashing performance of the resulting variant α-amylase.

This finding is surprising in that the N-terminal part of the α-amylase in a spatial model has been found to be located at a position remote from the active site of the molecule, indicating little importance of this region for activity. The spatial model of *B. licheniformis* α-amylase was built using, as scaffold, the *Aspergillus oryzae* α-amylase X-ray structure, 2TAA.PDB, from the protein databank, Brookhaven National Laboratories. Only regions around the B-barrel "domain" were built. The model was made by incorporating two minor deletions in the N-terminal part and a large insertion (30 residues) in the middle part of the *B. licheniformis* α-amylase sequence compared to that of the *A. oryzae* α-amylase.

In accordance with the above finding, and in a specific embodiment, the invention relates to a variant of a parent α-amylase comprising the amino acid sequence

shown in SEQ ID No. 2, or a variant of an analogue of said parent α-amylase, which variant has improved washing and/or dishwashing performance and which comprises at least one substitution, deletion or addition in the N-terminal end of the parent α-amylase, in particular within the first 50 N-terminal amino acid residues of amino acid sequence of the mature α-amylase.

More particularly, a variant of the parent *B. licheniformis* α-amylase comprising the amino acid sequence shown in SEQ ID No. 2, or a variant of an analogue of said parent α-amylase, in which at least one amino acid residue located in position 17-35, such as position 20-35, of said parent α-amylase has been substituted or deleted, or in which at least one amino acid has been added to said parent α-amylase within the amino acid segment located in position 17-35 (such as 20-35), has been found to be of interest.

This segment constitutes a region of a relatively low degree of homology in the otherwise highly conserved N-terminal part of the α-amylases derived from *B. licheniformis*, *B. amyloliquefaciens* and *B. stearothermophilus*. It has been found that amino acid substitutions within this region of the *B. licheniformis* α-amylase, in particular to amino acid residues located in the homologous position in *B. amyloliquefaciens* and *B. stearothermophilus* α-amylase, lead to α-amylase variants with improved properties.

In particular, the region defined by amino acid residues 29-35 of the *B. licheniformis* α-amylase comprising the amino acid sequence shown in SEQ ID No. 2 comprises a large number of positions in which no homology exists between the various *Bacillus* α-amylases. Accordingly, the *B. licheniformis* α-amylase variant of the invention may be a variant in which at least one amino acid residue located in position 29-35 of the parent α-amylase has been substituted or deleted, or in which at least one amino acid has been added to the parent α-amylase within the amino acid segment located in position 29-35.

More specifically, the *B. licheniformis* α-amylase variant of the invention may be one in which the amino acid residue(s) located in one or more of the following positions have been modified, i.e. deleted or replaced by any other amino acid residue as explained above:

N17, R23, S29, A30, Y31, A33, E34, H35

As a preferred example of a *B. licheniformis* α-amylase variant of the invention may be mentioned a variant which comprises at least one of the following mutations:

R23K,T

S29A

A30E,N

Y31H,N

A33S

E34D,S

H35I,L;

or any combination of these mutations.

In example 1 below, the construction of a number of different *B. licheniformis* α-amylase variants is described, which variants have been modified by one or more amino acid substitutions or deletions within the N-terminal end region of the *B. licheniformis* α-amylase. All of these variants have been found to have an improved washing and/or dishwashing performance as compared to their parent α-amylase.

Furthermore, other specific amino acid residues or regions of interest of the *B. licheniformis* α-amylase comprising the amino acid sequence shown in SEQ ID No. 2 or an analogue thereof are listed below, together with preferred modifications of these amino acid residues or regions. Accordingly, in a further embodiment the present invention relates to a *B. licheniformis* α-amylase variant which comprises at least one modification of an amino acid residue or region

listed below. The variant comprises at least one, or a combination of two or more, of the specific amino acid modifications mentioned below:

a) modification of an amino acid residue located in position 1, 2, 3 and/or 15; accordingly, a *B. licheniformis* α-amylase variant of interest is one which comprises a mutation in position A1, N2, L3 or M15 of the parent α-amylase, preferably one or more of the mutations A1V, M15T,L, N2*, L3V or A1*+N2*;

b) modification of amino acid residues located in the region spanning amino acid residues 51-58, in particular an amino acid residue located in position 51, 52 and/or 58 thereof, e.g. at least one of the following mutations: Q51R, A52S, A58P,V;

c) modification of the amino acid residue H68, in particular one of the following mutations: H68N,Q;

d) modification of amino acid residues located in position 85 and/or 88, in particular at least one of the mutations S85Q, K88Q;

e) modification of amino acid residues located in the region 94-104, in particular an amino acid residue located in position 94, 95, 96, 99, 103 and/or 104 thereof, e.g. at least one of the following mutations: N96Q, G99A, I103F, N104D;

f) modification of amino acid residues located in the region 121-136, in particular an amino acid residue located in position 121, 127, 128, 131, 132, 133 and/or 134 thereof, e.g. at least one of the mutations D121N, R127Q, V128E, G131E, E132T, H133Y, L134Q, K136Q;

g) modification of amino acid residues located in position 140, 142, 148 and/or 152, e.g. at least one of the following mutations: H140K, H142D, D152S, S148N;

h) modification of amino acid residues located in the region 142-182, in particular a deletion of all or a substantial part of the amino acid residues in the said region;

i) modification of amino acid residues located in the region 172-178, in particular an amino acid residue located in the position 172, 175, 177 and/or 178, e.g. at least one of the following mutations: N172S, F177FRG, Q178I,E;

j) modification of amino acid residues S187, A209 and/or T217, in particular the mutation S187D, A209V and/or T217K;

k) modification of amino acid residue R242, in particular the mutation R242P;

l) modification of an amino acid residue located in the region 246-251, in particular an amino acid residue located in the position 246, 247, 250 and/or 251, e.g. H247A,Y, E250Q,S, K251A,Q

m) modification of amino acid residue E255, in particular the mutation E255P;

n) modification of an amino acid residue located in the region 260-269, in particular an amino acid residue located in position 260, 264, 265, 267, 268, and/or 269, e.g. at least one of the following mutations: A260G, N265Y, A269K;

o) modification of an amino acid residue located in the region 290-293, in particular an amino acid residue located in position 290, 291 and/or 293, e.g. at least one of the following mutations: Y290F,N, Q291K, H293Q,Y;

p) modification of an amino acid residue located in the region 314-320, in particular an amino acid residue in position 315, 318 and/or 320, e.g. the following mutations: K315D, L318T and/or S320A;

q) modification of amino acid residues T341 and/or Q360, in particular the mutation T341P and/or Q360C;

r) modification of an amino acid residue located in the region 369-383, in particular an amino acid residue in position 370, 371, 372, 373, 374, 375, 376, 379 and/or 382, e.g. at least one of the following mutations: 370*, 371*, 372*, (370-372)*, S373P, Q374P, R375Y, A379S, H382S;

s) modification of an amino acid residue located in position 393, 398 and/or 409, e.g. the mutations Q393D, A398T,P and/or V409I;

t) modification of an amino acid residue located in the region 416-421, in particular an amino acid residue located in position 419, 420 and/or 421, e.g. at least one of the following mutations: V419K, A420P, N421G;

u) modification of amino acid residues A435 and/or H450, in particular the mutations A435S and/or H450Y;

v) modification of an amino acid residue located in the region 458-465, in particular an amino acid residue located in position 458, 459 and/or 461, e.g. at least one of the following mutations: P459T, V461K,T;

w) modification of the amino acid residue M197 in combination with at least one further mutation, including a deletion or replacement, of an additional amino acid residue of the amino acid sequence and/or an addition of at least one amino acid residue within the sequence, or at the C-terminal and/or N-terminal end of the amino acid sequence.

Specific examples of α-amylase variants as defined in w) above include variants comprising one of the mutations M197T,G,I,L,A,S,N,C in combination with any other mutation defined herein.

Based on the spatial model of the *B. licheniformis* α-amylase referred to above, it is presently contemplated that the deletion mentioned in h) above may result in an improved accessability to the active site, thereby improving the substrate

specificity without, however, changing the thermoactivation to any substantial extent.

Normally, it is found that insertion of additional proline residues in enzymes results in a stabilization of the enzyme at elevated temperatures, possibly due to the fact that a high number of proline residues makes the structure of the enzyme more rigid at elevated temperatures. In the present invention it has surprisingly been found that insertion of additional proline residues in the *B. licheniformis* α-amylase results in a destabilization of the resulting variant at elevated temperatures. Thus, by insertion of proline residues the temperature optimum of the resulting variant is lowered.

It has surprisingly been found that proline-substituted variants of the *B. licheniformis* α-amylase with a lowered temperature optimum show considerably improved washing and/or dishwashing performance.

When the parent α-amylase is a *B. licheniformis* α-amylase, the non-proline amino acid residue to be replaced with proline is preferably located in a position which in other α-amylases, such as a *B. amyloliquefaciens* or *B. stearothermophilus* α-amylase, is occupied by proline.

Accordingly, in an important embodiment the variant of the invention is one in which one or more non-proline residues have been substituted for proline residues. When the parent α-amylase is the *B. licheniformis* α-amylase, mutations of interest include:

R242P, E255P, T341P, S373P, Q374P, A420P, Q482P.

Finally, on the basis of the spatial model of the *B. licheniformis* α-amylase referred to above, it is contemplated that the variants prepared by the following amino acid substitutions in the substrate binding area have an improved (higher) pH optimum with respect to dishwashing/washing performance:

R23E,D, K106E,D, I135E,D, K156E,D, V186E,D, Y198E,D, Y193E,D, Q178E,D, K234E,D, K237E,D and/or Q360E,D.

As mentioned above, one example of an analogous amylase is a *B. amyloliquefaciens* α-amylase. Another is a *B. stearothermophilus* α-amylase. The amino acid sequences of a *B. amyloliquefaciens* α-amylase and a *B. stearothermophilus* α-amylase are shown in SEQ ID No. 4 and SEQ ID No. 6, respectively. The terms *B. amyloliquefaciens* α-amylase and *B. stearothermophilus* α-amylase, respectively, are intended to include analogues of these α-amylases which

i) have an amino acid sequence which is at least 60% homologous, such as at least 70%, 75%, 80%, 85%, 90% or 95% homologous, with the sequences shown in SEQ ID No. 4 and 6, respectively, and/or

ii) exhibit immunological cross-reactivity with an antibody raised against said α-amylase, and/or

iii) are encoded by a DNA sequence which hybridizes with the same probe as the DNA sequence encoding said α-amylase, which latter DNA sequence is shown in SEQ ID No. 3 and 5, respectively.

Properties i)-iii) are to be understood in the same manner as explained above in connection with the *B. licheniformis* α-amylase. Specific examples of analogues of the *B. amyloliquefaciens* α-amylase comprising the amino acid sequence shown in SEQ ID No. 4 are BAN® (available from Novo Nordisk A/S), Optiamyl® (available from Solvay), Dexlo® and Rapidase® (available from Gist-Brocades) and Kazuzase® (a mixed α-amylase and protease product available from Showa Denko). Specific examples of analogues of the *B. stearothermophilus* α-amylase comprising the amino acid sequence shown in SEQ ID No. 6 are Liquozyme 280L® (available from Novo Nordisk A/S) and G-zyme 995® (available from Enzyme BioSystems).

It is contemplated that the principles disclosed herein for preparation of variants with improved washing and/or dishwashing performance may be used for preparing variants of the closely related *B. amyloliquefaciens* and the *B. stearothermophilus* α-amylases. Thus, for instance, amino acid residues located in positions in the *B. amyloliquefaciens* or *B. stearothermophilus* α-amylase homologous to the *B. licheniformis* amino acid residues mentioned above may be substituted with similar amino acid residues, thereby giving rise to novel variants with improved properties.

Homologous positions may be identified by a comparison of the primary structures (cf. the comparison between SEQ ID Nos. 2, 4 and 6 given hereinbefore) or of the tertiary structures of the α-amylases in question.

Homologous positions in the tertiary structure may be determined by comparison with the established crystal structure of other α-amylases, such as the *A. oryzae* α-amylase structure (referred to above) or the *A. niger* α-amylase structure (Boel et al., 1990, Biochemistry 29, pp. 6244-6249.

Furthermore, it is contemplated that the above described principles for preparing α-amylase variants having improved washing and/or dishwashing performance may be used for preparing variants of other α-amylases such as an α-amylase derived from *B. subtilis* or from a strain of *Aspergillus* such as a strain of *A. niger*, e.g. the α-amylase described in Danish Patent Application DK 5126/87, or *A. oryzae*, e.g. the commercially available Fungamyl® (Novo Nordisk A/S) having the amino acid sequence shown in SEQ ID No. 7, Mycolase® (Gist-Brocades), Clarase (Solvay), and Phlowzyme® (Enzyme BioSystems).

As mentioned above, the α-amylase variant of the invention may be a hybrid α-amylase. Accordingly, in a further embodiment the variant of the invention having an improved washing and/or dishwashing performance is a hybrid α-amylase comprising a combination of partial amino acid sequences derived from at least two parent α-amylases. In the context of hybrid amylases, the term

"improved washing and/or dishwashing performance" is intended to indicate that the performance of the hybrid is better than that of any of the parent amylases when tested under similar conditions.

As far as the present inventors are aware, no prior disclosure or suggestion of hybrid α-amylases having improved washing and/or dishwashing performance exists. In fact, hybrid α-amylases have never previously been described or suggested for use in washing or dishwashing.

Preferably, at least one of the parent α-amylases of the hybrid is a microbial α-amylase (the other parent, e.g., being of mammalian origin); more preferably, all of the parent α-amylases are of microbial origin. In one embodiment it is preferred that the hybrid α-amylase comprises a combination of partial amino acid sequences derived from at least two bacterial α-amylases, from at least one bacterial and one fungal α-amylase, or from at least two fungal α-amylases.

A preferred example of a hybrid α-amylase of the invention is one which comprises a C-terminal part of an α-amylase derived from a strain of *B. licheniformis,* and a N-terminal part of an α-amylase derived from a strain of *B. amyloliquefaciens* or from a strain of *B. stearothermophilus*.

Preferably, the *B. licheniformis* α-amylase and/or the *B. amyloliquefaciens* and/or *B. stearothermophilus* α-amylases are those comprising the amino acid sequences shown in SEQ ID Nos. 2, 4 and 6, respectively, or an analogue of any of said α-amylases as defined in further detail hereinbefore. It will be understood that the hybrid α-amylase of the invention may comprise partial sequences of two parent α-amylases, as well as of three or more parent α-amylases. Furthermore, the hybrid α-amylase of the invention may comprise one, two or more parts of each of the parent α-amylases, such as, e.g., an N-terminal part of a first parent α-amylase, intermediate parts of a second parent α-amylase and optionally further intermediate parts of the first, third or further parent α-amylases, and finally a C-terminal part of any of these parent α-amylases.

A particularly preferred hybrid α-amylase of the invention is one which comprises at least 410, e.g. 415, such as at least 430, at least 445, e.g. 446, or at least 460 amino acid residues of the C-terminal part of the *B. licheniformis* α-amylase comprising the amino acid sequence shown in SEQ ID No. 2 or an analogue thereof as defined herein. The N-terminal part of the hybrid α-amylase is preferably derived from the *B. amyloliquefaciens* or *B. stearothermophilus* α-amylase.

In a further embodiment the invention relates to a hybrid α-amylase as defined above which in addition comprises one or more mutations, e.g. prepared by site-specific or random mutagenesis. Of particular interest is a hybrid α-amylase as described above comprising a C-terminal part of the α-amylase having the amino acid sequence shown in SEQ ID No. 2, in which the methionine residue in position 197 has been replaced with another amino acid residue. Specific examples of desirable mutations are M197T, M197G, M197L, M197A, M197N and M197S.

It should be noted that, according to the invention, any one of the modifications of the amino acid sequence indicated above for the α-amylase variants (and hybrid α-amylases) may be combined with any one of the other modifications mentioned above, where appropriate.

The present inventors have found that an apparent relationship exists between the washing and/or dishwashing performance of a given enzyme and the hydrolysis velocity obtained in a given reaction.

More specifically, it has been found that the higher the hydrolysis velocity, the better the washing and/or dishwashing performance which is obtained. Thus, without being limited to any theory it is contemplated that the improvement of washing and/or dishwashing performance obtained with an α-amylase variant of the invention as compared to that of the parent α-amylase may be directly predicted by comparing the hydrolysis velocity obtained for the variant and the

parent α-amylase, respectively, when tested under similar conditions. The hydrolysis velocity may be calculated by use of the Michaelis-Menten equation, c.f. Example 11 below.

From the equation given in Example 11 it will be apparent that at low substrate concentrations, the hydrolysis velocity is directly proportional to Vmax and is inversely proportional to Km.

Accordingly, the α-amylase variant of the invention is preferably one which at low substrate concentrations has a higher hydrolysis velocity than the parent α-amylase. Alternatively, the α-amylase variant of the invention is preferably one which has a higher Vmax and/or a lower Km than the parent α-amylase when tested under the same conditions. In the case of a hybrid α-amylase, the parent α-amylase to be used for the comparison should be the one of the parent enzymes having the best performance.

The Vmax, Km and V may be determined by well-known procedures, e.g. by the method described in Example 11 below.

Methods of preparing α-amylase variants

Several methods for introducing mutations into genes are known in the art. After a brief discussion of the cloning of α-amylase-encoding DNA sequences (which for instance encode functional analogues of the *Bacillus* α-amylases disclosed herein), methods for generating mutations at specific sites within the α-amylase-encoding sequence will be discussed.

Cloning a DNA sequence encoding an α-amylase

The DNA sequence encoding a parent α-amylase may be isolated from any cell or microorganism producing the α-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 α-amylase to be studied. Then, if the amino acid sequence of the

α-amylase is known, homologous, labelled oligonucleotide probes may be synthesized and used to identify α-amylase-encoding clones from a genomic library prepared from the organism in question. Alternatively, a labelled oligonucleotide probe containing sequences homologous to a known α-amylase gene could be used as a probe to identify α-amylase-encoding clones, using hybridization and washing conditions of lower stringency.

Yet another method for identifying α-amylase-encoding clones would involve inserting fragments of genomic DNA into an expression vector, such as a plasmid, transforming α-amylase-negative bacteria with the resulting genomic DNA library, and then plating the transformed bacteria onto agar containing a substrate for α-amylase, thereby allowing clones expressing the α-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 (1981) or the method described by Matthes et al. (1984). 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 genomic and cDNA origin, prepared by ligating fragments of synthetic, genomic or cDNA origin (as appropriate, the fragments corresponding to various parts of the entire DNA sequence), in accordance with standard techniques. The DNA sequence may also be prepared by polymerase chain reaction (PCR) using specific primers, for instance as described in US 4,683,202 or R.K. Saiki et al. (1988).

Site-directed mutagenesis

Once an α-amylase-encoding DNA sequence has been isolated, and desirable sites for mutation identified, mutations may be introduced using synthetic

oligonucleotides. These oligonucleotides contain nucleotide sequences flanking the desired mutation sites; mutant nucleotides are inserted during oligonucleotide synthesis. In a specific method, a single-stranded gap of DNA, bridging the α-amylase-encoding sequence, is created in a vector carrying the α-amylase gene. Then the synthetic nucleotide, bearing the desired mutation, is annealed to a homologous portion of the single-stranded DNA. The remaining gap is then filled in with DNA polymerase I (Klenow fragment) and the construct is ligated using T4 ligase. A specific example of this method is described in Morinaga et al. (1984). US 4,760,025 discloses the introduction of oligonucleotides encoding multiple mutations by performing minor alterations of the cassette. However, an even greater variety of mutations can be introduced at any one time by the Morinaga method, because a multitude of oligonucleotides, of various lengths, can be introduced.

Another method of introducing mutations into α-amylase-encoding DNA sequences is described in Nelson and Long (1989). It involves the 3-step generation of a PCR fragment containing the desired mutation introduced by using a chemically synthesized DNA strand as one of the primers in the PCR reactions. From the PCR-generated fragment, a DNA fragment carrying the mutation may be isolated by cleavage with restriction endonucleases and reinserted into an expression plasmid.

Random mutagenesis

Random mutations may be introduced in a DNA sequence encoding a parent α-amylase by subjecting the DNA sequence to a suitable physical or chemical mutagenic agent such as UV irradiation, ethyl methanesulfonate (EMS), sodium bisulphite or any other mutagenic agent known in the art, or by subjecting the DNA sequence to directed random mutagenesis by use of PCR using degenerate oligonucleotides for the introduction of mutations in a specified region.

Methods of preparing hybrid α-amylases

As an alternative to site-specific mutagenesis, α-amylase variants which are hybrids of at least two of parent α-amylases may be prepared by combining the relevant parts of the respective genes in question.

Naturally occurring enzymes may be genetically modified by random or site directed mutagenesis as described above. Alternatively, part of one enzyme may be replaced by a part of another to obtain a chimeric enzyme. This replacement can be achieved either by conventional *in vitro* gene splicing techniques or by *in vivo* recombination or by combinations of both techniques. When using conventional *in vitro* gene splicing techniques, a desired portion of the α-amylase gene coding sequence may be deleted using appropriate site-specific restriction enzymes; the deleted portion of the coding sequence may then be replaced by the insertion of a desired portion of a different α-amylase coding sequence so that a chimeric nucleotide sequence encoding a new α-amylase is produced. Alternatively, α-amylase genes may be fused, e.g. by use of the PCR overlay extension method described by Higuchi et al. 1988.

The *in vivo* recombination techniques depend on the fact that different DNA segments with highly homologous regions (identity of DNA sequence) may recombine, i.e. break and exchange DNA, and establish new bonds in the homologous regions. Accordingly, when the coding sequences for two different but homologous amylase enzymes are used to transform a host cell, recombination of homologous sequences *in vivo* will result in the production of chimeric gene sequences. Translation of these coding sequences by the host cell will result in production of a chimeric amylase gene product. Specific *in vivo* recombination techniques are described in US 5,093,257 and EP 252 666.

The α-amylase genes from *B. licheniformis* and from *B. amyloliquefaciens* are approximately 70 percent homologous at the DNA level and suitable for hybrid formation by *in vivo* gene splicing.

In an alternative embodiment, the hybrid enzyme may be synthesized by standard chemical methods known in the art. For example, see Hunkapiller et al. (1984). Accordingly, peptides having the amino acid sequences described above may be synthesized in whole or in part and joined to form the hybrid enzymes of the invention.

Screening for or selection of variants of the invention

The screening for or selection of variants (including hybrids) of the invention may suitably be performed by determining the starch-degrading activity of the variant, for instance by growing host cells transformed with a DNA sequence encoding a variant on a starch-containing agarose plate and identifying starch-degrading host cells. Furthermore, the selection or screening may suitably involve testing of one or more parameters of importance in connection with washing and/or dishwashing performance. Such parameters may, e.g., include the specific activity, the substrate specificity, the thermoactivation, the pH optimum, the temperature optimum, the tolerance towards constituents of conventionally used detergent compositions (e.g. of the types mentioned further below) and any other parameter considered to be of importance for washing and/or dishwashing performance. All of these parameters may be determined in accordance with well-known principles. Finally, the performance of the variant may be tested by use of a suitable washing and/or dishwashing assay, e.g. as described in the Materials and Methods section below.

Expression of α-amylase variants

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

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

The expression vector of the invention may also comprise a suitable transcription terminator and, in eukaryotes, polyadenylation sequences operably connected to the DNA sequence encoding the α-amylase variant of the invention. Termination

and polyadenylation sequences may suitably be derived from the same sources as the promoter.

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

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

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

The procedures used to ligate the DNA construct of the invention encoding an α-amylase variant, the promoter, terminator and other elements, respectively, and to insert them into suitable vectors containing the information necessary for replication, are well known to persons skilled in the art (cf., for instance, Sambrook et al. (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 α-amylase variant of the invention. The cell may

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

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

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

In a yet further aspect, the present invention relates to a method of producing an α-amylase variant of the invention, which method comprises cultivating a host cell

as described above under conditions conducive to the production of the variant and recovering the variant from the cells and/or culture medium.

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

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

Detergent Additive and Composition for Dishwashing and Washing

Due to their improved washing and/or dishwashing performance, α-amylase variants (including hybrids) of the invention are particularly well suited for incorporation into detergent compositions, e.g. detergent compositions intended for performance in the range of pH 7-13, particularly the range of pH 8-11.

According to the invention, the α-amylase variant may be added as a component of a detergent composition. As such, it may be included in the detergent composition in the form of a detergent additive. The detergent composition as well as the detergent additive may additionally comprise one or more other enzymes conventionally used in detergents, such as proteases, lipases, amylolytic enzymes, oxidases (including peroxidases), or cellulases.

It has been found that substantial improvements in washing and/or dishwashing performance may be obtained when α-amylase is combined with another amylolytic enzyme, such as a pullulanase, an iso-amylase, a beta-amylase, an

amyloglucosidase or a CTGase. Examples of commercially available amylolytic enzymes suitable for the given purpose are AMG®, Novamyl® and Promozyme®, all available from Novo Nordisk A/S.

Accordingly, in a particular embodiment the invention relates to a detergent additive comprising an α-amylase variant of the invention in combination with at least one other amylolytic enzyme (e.g. chosen amongst those mentioned above).

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

Non-dusting granulates may be produced, e.g., as disclosed in US 4,106,991 and US 4,661,452, and may optionally be coated by methods known in the art. The detergent enzymes may be mixed before or after granulation.

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.

In a still further aspect, the invention relates to a detergent composition comprising an α-amylase variant (including hybrid) of the invention.

The detergent composition of the invention may be in any convenient form, e.g. as powder, granules or liquid. A liquid detergent may be aqueous, typically containing up to 90% of water and 0-20% of organic solvent, or non-aqueous, e.g. as described in EP Patent 120,659.

Washing detergent composition

The washing detergent composition (i.e. a composition useful for laundry washing) comprises a surfactant which may be anionic, non-ionic, cationic, amphoteric or a mixture of these types. The detergent will usually contain 0-50% of anionic surfactant such as linear alkylbenzene sulfonate, α-olefinsulfonate, alkyl sulfate, alcohol ethoxy sulfate or soap. It may also contain 0-40% of non-ionic surfactant such as nonyl phenol ethoxylate or alcohol ethoxylate. Furthermore, it may contain an N-(polyhydroxyalkyl)-fatty acid amide surfactant (e.g. as described in WO 92/06154).

The detergent may contain 1-40% of detergent builders such as zeolite, di- or triphosphate, phosphonate, citrate, NTA, EDTA or DTPA, alkenyl succinic anhydride, or silicate, or it may be unbuilt (i.e. essentially free of a detergent builder).

The detergent composition of the invention may be stabilized using conventional stabilizing agents for the enzyme(s), e.g. a polyol such as e.g. propylene glycol, a sugar or sugar alcohol, lactic acid, boric acid, or a boric acid derivative, e.g. an aromatic borate ester, and the composition may be formulated as described in e.g. WO 92/19709 or WO 92/19708. Other enzyme stabilizers are well known in the art.

The detergent composition of the invention may contain bleaching agents, e.g. perborate, percarbonate and/or activator, tetraacetyl ethylene diamine, or nonanoyloxybenzene sulfonate, and may be formulated as described in, e.g., WO 92/07057.

The detergent composition of the invention may also contain other conventional detergent ingredients, e.g. deflocculating polymers, fabric conditioners, foam boosters, foam depressors, anti-corrosion agents, soil-suspending agents, sequestering agents, anti-soil redeposition agents, dyes, bactericides, optical brighteners and perfumes, as well as enzymes as mentioned above.

Particular forms of detergent composition within the scope of the invention and containing an α-amylase variant of the invention include:

a) A detergent composition formulated as a detergent powder containing phosphate builder, anionic surfactant, nonionic surfactant, silicate, alkali to adjust to desired pH in use, and neutral inorganic salt.

b) A detergent composition formulated as a detergent powder containing zeolite builder, anionic surfactant, nonionic surfactant, acrylic or equivalent polymer, silicate, alkali to adjust to desired pH in use, and neutral inorganic salt.

c) A detergent composition formulated as an aqueous detergent liquid comprising anionic surfactant, nonionic surfactant, organic acid, alkali, with a pH in use adjusted to a value between 7 and 11.

d) A detergent composition formulated as a nonaqueous detergent liquid comprising a liquid nonionic surfactant consisting essentially of linear alkoxylated primary alcohol, phosphate builder, alkali, with a pH in use adjusted to a value between about 7 and 11.

e) A compact detergent composition formulated as a detergent powder in the form of a granulate having a bulk density of at least 600 g/l, containing anionic surfactant and nonionic surfactant, phosphate builder, sodium silicate, and little or substantially no neutral inorganic salt.

f) A compact detergent composition formulated as a detergent powder in the form of a granulate having a bulk density of at least 600 g/l, containing anionic surfactant and nonionic surfactant, zeolite builder, sodium silicate, and little or substantially no neutral inorganic salt.

g) A detergent composition formulated as a detergent powder containing anionic surfactant, nonionic surfactant, acrylic polymer, fatty acid soap, sodium carbonate, sodium sulfate, clay particles, and sodium silicate.

h) A liquid compact detergent comprising 5-65% by weight of surfactant, 0-50% by weight of builder and 0-30% by weight of electrolyte.

i) A compact granular detergent comprising linear alkyl benzene sulphonate, tallow alkyl sulphate, C_{4-5} alkyl sulphate, C_{4-5} alcohol 7 times ethoxylated, tallow alcohol 11 times ethoxylated, dispersant, silicone fluid, trisodium citrate, citric acid, zeolite, maleic acid acrylic acid copolymer, DETMPA, cellulase, protease, lipase, an amylolytic enzyme, sodium silicate, sodium sulphate, PVP, perborate and accelerator.

j) A granular detergent comprising sodium linear C_{1-2} alkyl benzene sulfonate, sodium sulfate, zeolite A, sodium nitrilotriacetate, cellulase, PVP, TAED, boric acid, perborate and accelerator.

k) A liquid detergent comprising C_{12-14} alkenyl succinic acid, citric acid monohydrate, sodium C_{12-15} alkyl sulphate, sodium sulfate of C_{12-15} alcohol 2 times ethoxylated, C_{12-15} alcohol 7 times ethoxylated, C_{12-15} alcohol 5 times ethoxylated, diethylene triamine penta (methylene phosphonic acid), oleic acid, ethanol, propanediol, protease, cellulase, PVP, suds supressor, NaOH, perborate and accelerator.

Furthermore, examples of suitable detergent compositions in which α-amylase variants of the invention may advantageously be included comprise the detergent

compositions described in EP 373 850, EP 378 261, WO 92/19709, EP 381 397, EP 486 073, WO 92/19707, EP 407 225, and WO 92/13054.

Dishwashing Composition

The dishwashing detergent composition comprises a surfactant which may be anionic, non-ionic, cationic, amphoteric or a mixture of these types. The detergent will contain 0-90% of non-ionic surfactant such as low- to non-foaming ethoxylated propoxylated straight-chain alcohols.

The detergent composition may contain detergent builder salts of inorganic and/or organic types. The detergent builders may be subdivided into phosphorus-containing and non-phosphorus-containing types. The detergent composition usually contains 1-90% of detergent builders.

Examples of phosphorus-containing inorganic alkaline detergent builders, when present, include the water-soluble salts especially alkali metal pyrophosphates, orthophosphates, polyphosphates, and phosphonates. Examples of non-phosphorus-containing inorganic builders, when present, include water-soluble alkali metal carbonates, borates and silicates as well as the various types of water-insoluble crystalline or amorphous alumino silicates of which zeolites are the best-known representatives.

Examples of suitable organic builders include the alkali metal, ammonium and substituted ammonium, citrates, succinates, malonates, fatty acid sulfonates, carboxymethoxy succinates, ammonium polyacetates, carboxylates, polycarboxylates, aminopolycarboxylates, polyacetyl carboxylates and polyhydroxysulfonates.

Other suitable organic builders include the higher molecular weight polymers and co-polymers known to have builder properties, for example appropriate polyacrylic acid, polymaleic and polyacrylic/polymaleic acid copolymers and their salts.

The dishwashing detergent composition may contain bleaching agents of the chlorine/bromine-type or the oxygen-type. Examples of inorganic chlorine/bromine-type bleaches are lithium, sodium or calcium hypochlorite and hypobromite as well as chlorinated trisodium phosphate. 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 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 and glycerol triacetate.

The dishwashing detergent composition of the invention may be stabilized using conventional stabilizing agents for the enzyme(s), e.g. a polyol such as e.g. propylene glycol, a sugar or a sugar alcohol, lactic acid, boric acid, or a boric acid derivative, e.g. an aromatic borate ester.

The dishwashing detergent composition of the invention may also contain other conventional detergent ingredients, e.g. deflocculant material, filler material, foam depressors, anti-corrosion agents, soil-suspending agents, sequestering agents, anti-soil redeposition agents, dehydrating agents, dyes, bactericides, fluorescers, thickeners and perfumes.

Finally, the α-amylase variants of the invention may alone or in combination with at least one amylolytic enzyme, e.g. one of those defined above, be used in conventional dishwashing detergents, e.g. any of the detergents described in any of the following patent publications:

EP 551670, EP 533239, WO 9303129, EP 507404, US 5141664, GB 2247025, EP 414285, GB 2234980, EP 408278, GB 2228945, GB 2228944, EP 387063, EP 385521, EP 373851, EP 364260, EP 349314, EP 331370, EP 318279, EP 318204, GB 2204319, EP 266904, US 5213706, EP 530870, CA 2006687, EP 481547, EP 337760, WO 93/14183, US 5223179, WO 93/06202, WO 93/05132, WO 92/19707, WO 92/09680, WO 92/08777, WO 92/06161, WO 92/06157, WO 92/06156, WO 91/13959, EP 399752, US 4941988, US 4908148.

Textile desizing

In the textile processing industry, α-amylases are traditionally used as auxiliaries in the desizing process to facilitate the removal of starch-containing size which has served as a protective coating on weft yarns during weaving.

Complete removal of the size coating after weaving is important to ensure optimum results in the subsequent processes, in which the fabric is scoured, bleached and dyed. Enzymatic starch break-down is preferred because it does not involve any harmful effect on the fibre material.

In order to reduce processing cost and increase mill throughput, the desizing processing is sometimes combined with the scouring and bleaching steps. In such cases, non-enzymatic auxiliaries such as alkali or oxidation agents are typically used to break down the starch, because traditional α-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 α-amylase enzymes having an improved resistance towards or compatible with oxidation (bleaching) agents at elevated

pH, in order to retain the advantages of enzymatic size break down in a time-saving simultaneous desizing/scouring/bleaching process.

It is contemplated that α-amylase variants of the invention may be found to have an improved resistance towards oxidation agents and thus be useful in desizing processes as described above, in particular for substitution of non-enzymatic alkali or oxidation agents used today.

The present invention is further described with reference to the appended drawing in which

Fig. 1A is a restriction map of plasmid pDN1380,

Fig. 1B a restriction map of plasmid pDN1528,

Fig. 2 is a graph showing the improved dishwashing performance of M197T and *amyL* variant III compared to the parent α-amylase when tested at pH 10.5 and 55°C,

Fig. 3 is a graph showing the temperature/activity profile of Termamyl® compared to E255P and S373P in an automatic dishwashing detergent (5 g/l) (pH 10.1) as a function of the temperature (0.41 Phadebas Units = 1 NU).

Fig. 4 shows the delta reflection for different concentrations of enzyme obtained during laundry washing as described in Example 8. The delta reflection has been calculated from the reflection obtained for a swatch having been washed with the relevant enzyme and the reflectance obtained for a swatch washed without enzyme,

Fig. 5 shows the pH/activity profiles (activity/mg enzyme) of the *amyL* variant III and the *amyL* variant III + M197T of the invention as compared to that of Termamyl® measured at 60°C,

Fig. 6 is a graph showing the performance dose/response curves of E255P, S373P and Q374P compared to Termamyl® in full-scale dishwash performance evaluation (55°C, 4 g/l of standard European-type automatic dishwashing detergent),

Fig. 7 shows the temperature/activity profile of *amyL* variant III + M197T compared to Termamyl® according to mg enzyme (50mM Britton-Robinson buffer, 0.1 mM $CaCl_2$, 55°C),

Fig. 8 shows the temperature/activity profile of the *amyL* variant III + M197T of the invention compared to Termamyl® (pH 9.0, 100mM Glycine buffer, 0.1 mM $CaCl_2$),

Fig. 9 shows the dishwashing performance of the *amyL* variant III + M197T of the invention compared to Termamyl® (pH 10.3, 4 g/l of a standard European-type automatic dishwashing detergent), and

Figs. 10 and 11 show the results obtained following storage in a standard European-type automatic dishwashing detergent at 30°C/60 r.h. of *amyL* variant III + M197T compared to Termamyl® in a detergent composition.

The following examples further illustrate the present invention, and they are not intended to be in any way limiting to the scope of the invention as claimed.

MATERIALS AND METHODS

Determination of α-amylase activity

α-Amylase activity is given herein in terms of Novo Units (NU). One thousand NU [i.e. one Kilo Novo α-amylase Unit (KNU)] is the amount of enzyme which, per hour, under standard conditions (37 ± 0.05°C; Ca content 0.0003 M; pH 5.6) dextrinizes 5.26 grams of starch dry substance (Merck Amylum solubile, Erg. B.6 Batch No. 9947275). Further details concerning the definition of NU are given in a

brochure ("AF 9/6") which is available from Novo Nordisk A/S, Novo Allé, DK-2880 Bagsvaerd, Denmark.

The determination of α-amylase activity is performed by a method - developed by Novo Nordisk A/S for determination of Termamyl® activity - in which Phadebas tablets (Phadebas® Amylase Test, supplied by Pharmacia Diagnostics) are used as substrate. This substrate is a cross-linked insoluble blue-coloured starch polymer which is mixed with bovine serum albumin and a buffer substance and tabletted. After suspension in water, the starch is hydrolysed by the α-amylase giving soluble blue fragments. The absorbance of the resulting blue solution, measured at 620 nm, is a function of the α-amylase activity; the enzyme activity is compared to that of an enzyme standard. Standard conditions for the method are:

Temperature:	37°C
pH:	7.3
Reaction time:	15 minutes
Calcium:	0.15 nM

Further details concerning this method are given in a brochure ("AF 207/1") which is available from Novo Nordisk A/S, Novo Allé, DK-2880 Bagsvaerd, Denmark.

Somogyi Method for the Determination of Reducing Sugars

The method is based on the principle that the sugar 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.

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

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

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

REAGENTS

1. Somogyi's copper reagent

35.1 g of $Na_2HPO_4.2H_2O$, and
40.0 g of potassium sodium tartrate ($KNaC_4H_4O_2.4H_2O$)
are dissolved in
700 ml of deionized water.
100 ml of 1 N sodium hydroxide and
80 ml of 10% cupric sulphate ($CuSO_4.5H_2O$) 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
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.

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)

Determination of Km

The kinetics of hydrolysis catalyzed by the amylases at various substrate concentrations were determined using the Somogyi-Nelson method with soluble starch as substrate (Merck 1252.). The hydrolysis velocities were measured under different substrate concentrations (1%, 0.5%, 0.3%, 0.25% and 0,2% starch solution). The number of reducing sugars were measured using the Somogyi-Nelson method, and determined as glucose eqv. made/mg of amylase x h giving the hydrolysis velocity. The data were plotted according to the Michaelis-Menten and Lineweaver-Burk equations. From these equations Vmax and Km can easily be calculated.

Laundry washing

Detergent:	Commercial European heavy duty liquid compact detergent (HDL)
Detergent dosage:	5 g/l
Soil:	Potato starch colored with Cibacron Blue 3GA
Water hardness:	18°dH
Time:	20 minutes
pH (during wash):	approx. 7.8
Evaluation:	Reflectance at 660 nm.

Automatic dishwashing

1) Washing conditions

Amylases:	*B. licheniformis* α-amylase (SEQ ID No.2) M 197T
	QL37
Amylase dosage:	0 - 0.72 mg enzyme protein/l washing liquor
Detergent:	standard European-type automatic dishwashing detergent
Detergent dosage:	4.2 g/l washing liquor
Soil:	Corn starch on plates and glasses
Dishwashing:	55°C program, Baucknecht GS 1272
pH:	10.3 during dishwashing

2) Evaluation

Removal of starch film (RSF) from plates and glasses is evaluated after colouring with iodine on the following scale from 0 to 6:

Rating	Dishware	Glassware
6	clean	clean
5	spots	thin
4	thin	moderate
3	moderate	heavy
2	heavy	very heavy
1	very heavy	extremely heavy
0	blind (unwashed)	blind (unwashed)

Mini dishwashing assay

A suspension of starchy material is boiled and cooled to 20°C. The cooled starch suspension is 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 are then weighed. For assay purposes, a solution of standard European-type automatic dishwashing detergent (5 g/l) having a temperature of 55°C is prepared. The detergent is allowed a dissolution time of 1 minute, after which the amylase variant in question is added to the detergent solution (contained in a beaker equipped with magnetic stirring) so as to give an enzyme concentration of 0.5 mg/ml. At the same time, the weighed glass plates, held in small supporting clamps, are immersed in a substantially vertical position in the amylase/detergent solution, which is then stirred for 15 minutes at 55°C. The glass plates are 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 variant in question [expressed as an index relative to Termamyl® (index 100)] is then determined from the difference in weight of the glass plates before and after treatment, as follows:

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

EXAMPLES

EXAMPLE 1

In this example the construction of DNA encoding a number of different *B. licheniformis* variants are described. Each variant is referred to by its amino acid modifications compared to the parent *B. licheniformis* α-amylase.

Plasmid pDN1528 (Fig. 1B) has been used for these constructions. The plasmid is a derivative of the *B. subtilis* plasmid pUB110 (Gryczan et al., 1978) and contains the pUB110 origin of replication, the cat gene conferring

chloramphenicol resistance, and the gene encoding the *B licheniformis* α-amylase having the DNA sequence shown in SEQ ID No. 1 (=*amyL*). The *B. licheniformis* α-amylase promoter (*amyL* promoter) transcribes the *amyL* gene.

Construction of *amyL* variant I: (1-2)*+L3V

The deletion of residues 1 and 2, and the substitution of leucine 3 with a valine were introduced simultaneously in *amyL* by PCR amplification of a fragment of DNA using the *amyL* gene (located on plasmid pDN1528) as a template and two oligonucleotides as primers. The 5' primer #6079 covers the region of residues 1-3 and the unique PstI restriction site. The sequence of this primer is given in Table 1:

The other primer 1C (Table 1) is located 3' to the mutagenic primer and has a sequence identical to *amyL*.

PCR was carried out as 30 cycles of (30 seconds at 94°C, 30 seconds at 50°C, and 60 seconds at 73°C) followed by 600 seconds at 73°C.

The amplified DNA fragment was purified and digested with restriction enzymes PstI and SacII. The resulting PstI-SacII DNA fragment was ligated with plasmid pDN1528 digested with the same unique restriction enzymes. The resulting plasmid carries a variant *amyL* gene with the desired mutations, and the variant protein can be expressed from this construct.

Construction of *amyL* variant II: (1-2)*+L3V+M15T

The substitution of methionine 15 with a threonine was carried out by overlap-extension mutagenesis (Higuchi et al., 1988) using the *amyL* variant ((1-2)*+L3V) as a template and the mutagenic primers #6164 and #6173 listed in Table 1. Thus, the resulting gene contains the deletion of residues 1 and 2, L3V and M15T.

In a PCR reaction (reaction A) a 480 bp DNA fragment was amplified by the use of two DNA primers, viz. #6164 containing the desired nucleotide alterations (Table 1) and one flanking primer, 1C. A separate PCR reaction (reaction B) amplified a 140 bp DNA fragment to the opposite site of the mutation site by the use of primer 1B and primer #6173. These PCR reactions were 25 cycles of (30 seconds at 94°C, 30 seconds at 50°C, and 60 seconds at 73°C) followed by 600 seconds at 73°C. The amplified fragments from reactions A and B overlap around the mutation site and a longer fragment of DNA was amplified in a third PCR reaction C: 20 cycles of (30 seconds at 94°C, 30 seconds at 50°C, and 60 seconds at 73°C) followed by 600 seconds at 73°C, by the use of only the two flanking primers, 1B and 1C. Reaction C DNA was digested with PstI and SacII restriction endoneucleases, and the resulting 360 bp PstI-SacII DNA fragment was subcloned into plasmid pDN1528, digested with the same unique restriction enzymes.

Construction of *amyL* variant III: (1-2)*+ L3V+M15T+R23K+S29A+A30E+Y31H+A33S+E34D+H351

A) By site-specific mutagenesis

In the DNA sequence encoding the *amyL* variant II ((1-2)*+L3V+M15T) constructed as described above, the following amino acid substitutions were introduced simultaneously: R23K, S29A, A30E, Y31H, A33S, E34D, and H35I by the overlap extension method as previously described.

Primers 1C and Reg 1A were used in reaction A, and primers 1B and Reg 1B were used in reaction B. The conditions for the PCR reactions were identical to those described above, and a PCR reaction C was carried out in a similar way. All the mutations were cloned on the 360 bp PstI-SacII fragment into pDN1528 as mentioned above.

This *amyL* variant may be prepared by the following alternative method:

B) Preparation of amyL variant III by α-amylase gene fusion

The plasmids useful for carrying out gene fusions are very similar and are all based on the *Bacillus* expression vector, pDN1380 (cf. Fig. 1A).

pDN1380 contains an origin of replication from plasmid pUB110, the maltogenic α-amylase promoter (P-beta promoter) described by Diderichsen and Christiansen (1988) located in front of a polylinker, and the *cat* gene encoding chloramphenicol acetyl transferase from the cloning vector pC194 (see, e.g., Erlich, 1977).

Amylase encoding genes should be cloned in pDN1380 in such a way that the amylase gene is transcribed from the P-beta promoter. A resulting plasmid pDN1681 containing the *B. amyloliquefaciens* α-amylase gene having the DNA sequence shown in SEQ ID No. 3 (*amyQ*), a plasmid pDN1750 containing the *B. stearothermophilus* α-amylase gene having the DNA sequence shown in SEQ ID No. 5 (*amyS*) and a plasmid pDN1700 containing the *B. licheniformis* α-amylase gene having the DNA sequence shown in SEQ ID No. 1 (*amyL*) may be obtained.

Primers:

pUB110ori:	5' CACTTCAACGCACCTTTCAGC 3'
cat1:	5' CATGGACTTCATTTACTGGG 3'
QA:	5' CACTGCCGTCTGGATTCCCC 3'
QB:	5' GGGAATCCAGACGGCAGTG 3'
SA:	5' GAATTCAATCAAAAAGGGACGGTTCGG 3'
SB:	5' CCGTCCCTTTTTGATTGAATTCGCC 3'

The amylase gene fusions may be constructed by the PCR overlap-extension method as described by Higuchi et al. 1988.

The polymerase Chain Reaction (PCR) may be used to amplify the fragment of pDN1681 (5'-end of the *amyQ*) located between primer QB and pUB110ori (reaction A). In a separate PCR (reaction B), the 3'-end of *amyL* may be amplified as the fragment between primer QA and primer cat1 in plasmid pDN1700. The two purified fragments may be used in a third PCR (reaction C) in the presence of the primers flanking the total region, i.e. pUB110ori and cat1.

The fragment amplified in the third reaction may be purified, digested with restriction endonucleases *Eco*RI and *Sph*I and ligated with the 2.6 kb fragment obtained from plasmid pDN1380 by a digestion with restriction endonucleases *Eco*RI and *Sph*I. A protease- and amylase-weak *B. subtilis* strain (e.g. strain SHA273 mentioned in WO 92/11357) may be transformed with the ligated plasmids, starch degrading transformants may be selected on starch-containing agarose plates and the amplified DNA sequence may be verified.

Polymerase Chain Reactions may be carried out under standard conditions, as described by Higuchi et al. 1988.

Reaction A and B are 15 cycles of (60 seconds at 94°C, 60 seconds at 45°C, and 90 seconds at 73°C) followed by 600 seconds at 73°C. Reaction C is 15 cycles of (60 seconds at 94°C, 60 seconds at 50°C, and 90 seconds at 73°C) followed by 600 seconds at 73°C.

The amino acid sequence in the mature protein from the construct described in Example B) is identical to the sequence of the mature protein from Example A), but the DNA sequences are different in the 5' end of the genes. Furthermore, the construct in Example A) has the *amyL* signal sequence whereas the construct B) has the signal sequence of the *B. amyloliquefaciens* α-amylase.

EXAMPLE 2

The *amyL* variant III prepared as described in A) or B) in Example 1 above and the site-specific mutation M197T were combined by subcloning a KpnI-SalI fragment containing M197T into the DNA sequence encoding *amyL* variant III ((1-2)*+L3V+M15T+R23K+S29A+A30E+Y31H+A33S+E34D+H35I) described above.

KpnI and SalI are unique restriction sites found in the *B. licheniformis* α-amylase encoding sequence and the KpnI-SalI fragment constitutes a 534bp fragment containing the M197T mutation prepared by Nelson and Long mutagenesis as described in WO 94/02597. The same sites, KpnI and SalI, are also unique in the *B. licheniformis* α-amylase variant III described above and therefore the 534 bp fragment can be cloned directly into the vector fragment KpnI/SalI obtained from *amyL* variant III. The resulting DNA encodes *amyL* variant III with the additional mutation M197T.

In an alternative method, the M197T mutation may be introduced in the *B. licheniformis* α-amylase encoding DNA sequence SEQ ID No. 1 by the method described by Nelson and Long (1981) and further exemplified in WO 94/02597 with the following sequences of the mutagenic primer

5'-CGGCATACGTCAAATAATCATAGTTGC-3'

where the underlined nucleotide introduce the mutation M197T.

EXAMPLE 3

A number of other mutations were introduced in the DNA sequence shown in SEQ ID No. 1 encoding the *B. licheniformis* α-amylase by similar methods, using the oligonucleotides listed in Table 1 below. Combinations of mutations were

done by subcloning, if possible, or by mutagenesis carried out on a Termamyl® variant template.

E255P was constructed by the method described by Higuchi et al., 1988:
template: *amyL* in pDN1528.
PCR A: primers E255P,A and 2C. Standard conditions: 25 cycles of (30 seconds at 94°C, 30 seconds at 50°C, and 60 seconds at 73°C) followed by 600 seconds at 73°C.
PCR B: primers E255P,B and 2B. Standard conditions.
PCR C: standard C reaction: 20 cycles of (30 seconds at 94°C, 30 seconds at 50°C, and 60 seconds at 73°C) followed by 600 seconds at 73°C.
The mutation was subcloned as a 330 bp KpnI-BssHII fragment into pDN1528.

T341P was constructed similarly to *amyL* variant I. One PCR reaction was carried out on *amyL* variant III by the use of primers T341P and 3C. A 210 bp SalI-Tth111I fragment was subcloned into pDN1528.

S373P was constructed by the method described by Higuchi et al., 1988:
template: *amyL* in pDN1528.
PCR A: primers S373P,A and 3C. Standard conditions: 25 cycles of (30 seconds at 94°C, 30 seconds at 50°C, and 60 seconds at 73°C) followed by 600 seconds at 73°C.
PCR B: primers S373,B and 3B. Standard conditions.
PCR C: standard C reaction: 20 cycles of (30 seconds at 94°C, 30 seconds at 50°C, and 60 seconds at 73°C) followed by 600 seconds at 73°C.
The mutation was subcloned as a 210 bp SalI-Tth111I fragment into pDN1528.

Q374P was constructed by the method described by Higuchi et al., 1988:
template: *amyL* in pDN1528.
PCR A: primers Q374P,A and 3C. Standard conditions: 25 cycles of (30 seconds at 94°C, 30 seconds at 50°C, and 60 seconds at 73°C) followed by 600 seconds at 73°C.

PCR B: primers Q374P,B and 3B. Standard conditions.

PCR C: standard C reaction: 20 cycles of (30 seconds at 94°C, 30 seconds at 50°C, and 60 seconds at 73°C) followed by 600 seconds at 73°C.

The mutation was subcloned as a 210 bp SalI-Tth111I fragment into pDN1528.

S148N was constructed by the method described by Higuchi et al., 1988:

template: *amyL* in pDN1528.

PCR A: primers S148N,A and 2C. Standard conditions: 25 cycles of (30 seconds at 94°C, 30 seconds at 50°C, and 60 seconds at 73°C) followed by 600 seconds at 73°C.

PCR B: primers S148N,B and 1B. Standard conditions as above

PCR C: standard C reaction: 20 cycles of (30 seconds at 94°C, 30 seconds at 50°C, and 60 seconds at 73°C) followed by 600 seconds at 73°C.

The mutation was subcloned as a 120 bp KpnI-SacII fragment into pDN1528.

L230I,V233A was constructed by the method described by Higuchi et al., 1988:

template: *amyL* in pDN1528.

PCR A: primers L230I+V233A, A and 2C. Standard conditions: 25 cycles of (30 seconds at 94°C, 30 seconds at 50°C, and 60 seconds at 73°C) followed by 600 seconds at 73°C.

PCR B: primers L230I+V233A, B and 2B. Standard conditions as above.

PCR C: standard C reaction: 20 cycles of (30 seconds at 94°C, 30 seconds at 50°C, and 60 seconds at 73°C) followed by 600 seconds at 73°C.

The mutation was subcloned as a 330 bp KpnI-BssHII fragment into pDN1528.

A209V was constructed by the method described by Higuchi et al., 1988:

template: *amyL* in pDN1528.

PCR A: primers A209V,A and 2C. Conditions: 25 cycles of (30 seconds at 94°C, 30 seconds at 50°C, and 60 seconds at 73°C) followed by 600 seconds at 73°C.

PCR B: primers A209V,B and 1B. Conditions: 25 cycles of (30 seconds at 94°C, 30 seconds at 50°C, and 60 seconds at 73°C) followed by 600 seconds at 73°C.

PCR C: standard C reaction with only flanking primers: 20 cycles of (30 seconds at 94°C, 30 seconds at 50°C, and 60 seconds at 73°C) followed by 600 seconds at 73°C.

The mutation was subcloned as a 330 bp KpnI-BssHII fragment into pDN1528.

Table 1

The following primers have been used for the construction of various variants described above. The 3' end of these primers have identical sequence to parts of pDN1528, and they have all a melting temperature above 50°C.

1B: Corresponds to amino acids: (-20) - (-13), i.e. signal sequence.

5' GGT ACT ATC GTA ACA ATG GCC GAT TGC TGA CGC TGT TAT TTG C 3'

2B: Corresponds to amino acids: 149-155.

5' GGG GTA CTA GTA ACC CGG GCC ATA CAG CGA TTT TAA ATG G 3'

3B: Corresponds to amino acids: 320-326

5' GGG GTA CTA GTA ACC CGG GCC GGT TAC ATT TGT CGA TAA CC 3'

1C: Corresponds to amino acids: 167-161.

5' CTC GTC CCA ATC GGT TCC GTC 3'

2C: Corresponds to amino acids: 345-339.

5' GGC TTA AAC CAT GTT TGG AC 3'

3C (=pUB110ori): Anneals 3' to *amyL*.

5' CAC TTC AAC GCA CCT TTC AGC 3'

<u>(1-2)*+L3V</u>

#6079

5' CCT CAT TCT GCA GCA GCG GCG GTT AAT GGG ACG CTG ATG CAG 3'

M15T

#6164: 5' GAA TGG TAC ACG CCC AAT GAC GG 3'

#6173: 5' CC GTC ATT GGG CGT GTA CCA TTC 3'

amyL variant III: (1-2)*+L3V+M15T+R23K+S29A+A30E+Y31H+A33S+ E34D+H35I

Reg 1A: 5' GCG GAA CAT TTA TCG GAT ATC GGT ATT ACT GCC GTC TGG ATT C 3'

Reg 1B: 5' ATT ACC GAT ATC CGA TAA ATG TTC CGC GTC GTT TTG CAA ACG TTT CCA ATG TTG 3'

E255P

A: GAA AAA ACG GGG AAG CCA ATG TTT ACG GTA GC

B: GC TAC CGT AAA CAT TGG CTT CCC CGT TTT TTC

T341P

CG CTT GAG TCG ACT GTC CAA CCA TGG TTT AAG CCG CTT GC

S373P

A: GG ACG AAA GGA GAC CCC CAG CGC GAA ATT C

B: G AAT TTC GCG CTG GGG GTC TCC TTT CGT CCC G

Q374P

A: CG AAA GGA GAC TCC CCT CGC GAA ATT CCT GCC TTG

B: CAA GGC AGG AAT TTC GCG AGG GGA GTC TCC TTT CG

S148N

A: 5' GGG CGC GGC AAC ACA TAC AGC 3'

B: 5' GCT GTA TGT GTT GCC GCG CCC 3'

L230I,V233A

A: 5' C CGG ATT GAT GCT GCG AAA CAC ATT AAA TTT TCT TTT TTG 3'

B: 5' T GTG TTT CGC AGC ATC AAT CCG GAA ACC GTC CAA TTG C 3'

A209V

A: 5' GAC CAT CCT GAC GTC GTA GCA GAA ATT AAG 3'

B: 5' TTC TGC TAC GAC GTC AGG ATG GTC ATA ATC 3'

EXAMPLE 4

Preparation of the hybrid α-amylase SL68 by DNA fusion

The plasmid used is constructed in a similar way as described for *amyL* variant III Example 1B) above, except that:

1) reaction A contains plasmid pDN1750, primer SB and primer pUB110ori,

2) reaction B contains plasmid pDN1700, primer SA and primer cat1.

3) reaction A and reaction B are 15 cycles of (60 seconds at 93°C, 60 seconds at 50°C, and 90 seconds at 73°C) followed by 600 seconds at 73°C. Reaction C is as mentioned above (see Example 1B)).

4) The purified fragment from PCR C is digested consecutively with *Sph*I and partially with *Eco*RI and the purified 3.3 kb fragment is subcloned into pDN1380 digested to completion with the same restriction endonucleases.

Restriction endonuclease digestion, purification of DNA fragments, ligation, transformation of *B. subtilis*, and DNA sequencing are performed in accordance with well-known techniques. Transformation of *B. subtilis* was performed as described by Dubnau et al. (1971).

EXAMPLE 5

Fermentation and purification of α-amylase variants

The α-amylase variants encoded by the DNA sequences constructed as described in Examples 1-4 above are produced as follows:

The *B. subtilis* strain harbouring the expression plasmid is streaked on a LB-agar plate with 25 mg/ml chloramphenicol from -80°C stock, and grown overnight at 37°C.

The colonies are transferred to 100ml BPX media supplemented with 25 mg/ml chloramphenicol in a 500 ml shaking flask.

Composition of BPX medium:

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

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

100-200 ml of the fermentation broth are filtered using a pressure filter with filter aid. After filtration the amylase is precipitated using 80% saturated ammonium sulfate. The precipitate is washed and solubilized and desalted using an Amicon ultrafiltration unit and 25mM Tris pH 5.6. The desalted sample is subjected to an ion exchange using S-sepharose F.F.*. The amylase is eluted using a linear gradient of NaCl from 0 to 200mM. The eluate is desalted using an Amicon unit and applied on a Q-sepharose F.F.* at pH 9 in a 25mM Tris buffer. The elution of the amylase is performed using a gradient of 0-200mM NaCl.

EXAMPLE 6

Properties of the *amyL* variant III and *amyL* variant III + M197T constructed as described in Examples 1 and 2, respectively, were compared.

Determination of oxidation stability

Raw filtered culture broths with *amyL* variant III and *amyL* variant III + M197T were diluted to an amylase activity of 100 NU/ml (determined by the α-amylase activity assay described in the Materials and Methods section above) in 50 mM of a Britton-Robinson buffer at pH 9.0 and incubated at 40°C. Subsequently H_2O_2

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was added to a concentration of 200 mM, and the pH value was re-adjusted to 9.0. The activity was measured after 15 seconds and after 5, 15, and 30 minutes. The *amyL* variant III + M197T mutant was found to exhibit an improved resistance towards 200 mM H_2O_2, pH 9.0 compared to *amyL* variant III.

Specific activity

The specific activity of Termamyl®, the *amyL* variant III and the *amyL* variant III + M197T was determined as described in the Materials and Methods section above. It was found that the specific activity of *amyL* variant III + M197T was improved by 20% compared to that of *amyL* variant III. *amyL* variant III was found to exhibit a 40% higher specific activity compared to Termamyl®.

Furthermore, the specific acitivity was determined as a function of temperature and pH, respectively. From Figs. 7 and 8 it is apparent that the *amyL* variant III + M197T has increased specific activity compared to the parent enzyme (Termamyl®) in the range from pH 4.5 to pH 9.0. Furthermore, the temperature profile has been displaced 10°C downwards at pH 9. Even though the activity at pH 10.1 is reduced compared to Termamyl®, the performance of *amyL* variant III in ADD (automatic dishwashing detergent) at 45°C is highly improved (Fig. 9). This is probably due to the downwards displacement of the temperature profile.

pH/activity profile

of *amyL* variant III and *amyL* variant III + M197T was determined as described in the Materials and Methods section above, the only difference being that the incubation was performed at 60°C and at the relevant pH values. The results are apparent from Fig. 5, in which the activity is given as activity per mg enzyme.

Determination of storage stability

The storage stability of α-amylase variant *amyL* variant III + M197T was determined by adding the variant and its parent α-amylase, respectively, to the detergent in an amount corresonding to a dosage of 0.5 mg enzyme protein per litre of washing liquor (3 litres in the main wash) together with 12 g of detergent in

each wash (1.5 mg enzyme protein). The mixtures were stored at 30°C/60% relative humidity (r.h.) for 0, 1, 2, 3, 4, and 6 weeks. After storage the analytical activity of the samples were determined as well as the performance. The performance was tested by using the whole content of each storage glass (containing enzyme and detergent) in each wash. The soil was corn starch on plates and glasses, and the dishwashing was carried out at 55°C, using a Cylinda* 770 machine. The storage stability is illustrated in Figs. 10 and 11. *amyL* variant III + M197T was significantly more stable than its parent enzyme.

EXAMPLE 7

Automatic dishwashing

The dishwashing performance of α-amylase variants of the invention compared to that of their parent α-amylase was evaluated in an automatic dishwashing test.

The α-amylase variants were the *amyL* variant III, the preparation of which is described in Example 1 above, and the α-amylase variant M197T (prepared by replacing the methionine residue located in position 197 of the *B. licheniformis* α-amylase (SEQ ID No. 2)) with a threonine residue as described in WO 94/02597).

The automatic dishwashing test was performed as described in the Materials and Methods section above.

The results obtained are presented in Fig. 2, from which it is apparent that the *amyL* variant III and the α-amylase mutant M197T show a substantially improved starch removal, and thus dishwashing performance, relative to that of the the parent α-amylase.

* trade mark

EXAMPLE 8

Laundry washing

The washing performance of the *amyL* variant III prepared as described in Example 1 and its parent α-amylase was determined under the conditions described in the Material and Methods section above using the following amylase dosages: 0/0.21/0.43/0.86 mg enzyme protein/l.

The results obtained are apparent from Fig. 4. The delta reflectance shown in this figure has been 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 minus the reflectance obtained without enzyme.

From Fig. 4 it is evident that the α-amylase variant of the invention exerts a considerably improved starch removal relative to the parent α-amylase, in other words that the α-amylase variant has an improved washing performance compared to that of the parent α-amylase.

EXAMPLE 9

The dishwashing performance of a number of the *B. licheniformis* α-amylase variants described in Examples 1-5 was assayed in the mini dishwashing assay described in the Materials and Methods section above.

Some of the variants were tested on different days and, thus, the results obtained for the various α-amylase variants are not directly comparable. However, each variant has been tested against the parent α-amylase and the performance index relative to the parent α-amylase (Termamyl®, index 100) is thus experimentally verified.

It is evident that all variants have an improved dishwashing performance (as measured by their ability to remove starchy stains) as compared to their parent α-amylase.

B. licheniformis amylase variants	Index
Termamyl®	100
E255P	135
T341P	120
S373P	125
Q374P	126
(1-2)*+L3V	117
S148N	112
M15T	115
L230I+V233A	112
A209V	118
S29A+A30E+Y31H+A33S+E34D+H35I	100
Combinations	
T341P+Q374P	117
(1-2)*+L3V+M15T+R23K+S29A+A30E+Y31H+A33S+E34D+H35I	140
(1-2)*+L3V+M15T+R23K+S29A+A30E+Y31H+A33S+E34D+H35I +E255P	156
(1-2)*+L3V+M15T+R23K+S29A+A30E+Y31H+A33S+E34D+H35I+ M197T	124
(1-2)*+L3V+M15T+R23K+S29A+A30E+Y31H+A33S+E34D+H35I+ E255P+Q374P	143

(1-2)*+L3V+M15T+R23K+S29A+A30E+Y31H+A33S+E34D+H35I+ E255P+Q374P+T341P	127
(1-2)*+L3V+M15T+R23K+S29A+A30E+Y31H+A33S+E34D+H35I+ E255P+M197I	141
(1-2)*+L3V+M15T+R23K+S29A+A30E+Y31H+A33S+E34D+H35I+ E255P+M197N	124
(1-2)*+L3V+M15T+R23K+S29A+A30E+Y31H+A33S+E34D+H35I+ E255P+M197S	113
(1-2)*+L3V+M15T+R23K+S29A+A30E+Y31H+A33S+E34D+H35I+ E255P+M197T	71

EXAMPLE 10

The washing performance of a number of the *B. licheniformis* α-amylase variants described in Examples 1 - 5 was tested by means of the laundry washing assay described in the Materials and Methods section above, using the different commercially available detergents mentioned in the tables below.

The IX (dR at c=0.5) is the index (expressed as percentage) obtained by dividing the delta reflectance (see Example 8) for a swatch washed with 0.5 mg/l of the α-amylase variant in question by the delta reflectance for a swatch washed with 0.5 mg/l of Termamyl®. dR at c=0.2 and dR at c=0.1 are the corresponding index (IX) values for enzyme concentrations of 0.2 and 0.1 mg/l, respectively.

It is evident that all variants have an improved washing performance (as measured by their ability to remove starchy stains) relative to their parent α-amylase.

5 g/l Ariel Ultra Liquid* No presoak, 40°C, 20 minutes, pH 7	
Enzyme	IX (dR at c=0.5)
Termamyl®	100
amyL var. III+ M197T	140
S29A+A30E+Y31H+A33S+E34D+H35I	103
E458D+P459T+V461K+N463G+E465D	100
R242P	106
E255P	133
M15T	101

2 g/l Tide*with Bleach Feb 92 No presoak, 40°C, 15 minutes, pH 10	
Enzyme	IX (dR at c=0.2)
Termamyl®	100
S29A+A30E+Y31H+A33S+E34D+H35I	103
E458D+P459T+V461K+N463G+E465D	122
R242P	112
E255P	109
T341P	108
H450Y	109
Q374P	111
M15T	120

* trade marks

3 g/l of Bleach containing Commercial South American HDP DF-931001.1 16 hours presoak, 30°C, 15 minutes, pH 10	
Enzyme	IX (dR at c=0.1)
Termamyl®	100
amyL var. III + M197T	103
amyL var. III + M197L	111
S29A+A30E+Y31H+A33S+E34D+H35I	114
E458D+P459T+V461K+N463G+E465D	109
R242P	117
E255P	134
T341P	116
H450Y	106
Q374P	113
M15T	117
H68Q	115

EXAMPLE 11

Determination of Vmax, Km and V

Km and Vmax of the α-amylases comprising the amino acid sequences SEQ ID Nos. 2, 4 and 6, respectively, and the α-amylase variant III and the hybrid α-amylase SL68 described in Examples 1 - 4, respectively, were determined as described in the Materials and Methods section above.

The following Vmax and Km values were obtained:

	Vmax mg glucose eqv. mg enzyme x h	Km mg starch/ml
SEQ ID No. 6	45.0	1.47
SEQ ID No. 4	11.5	1.28
SEQ ID No. 2	6.4	0.18-0.25
amyL variant III	8.0	0.18-0.25
SL68	7.3	0.18-0.25

The hydrolysis velocity obtained for each of the enzymes may at low substrate concentrations be determined on the basis of the Michaelis-Menten equation

$$V = Vmax \times [S]/[S] + Km$$

which, when [S] << Km may be reduced to V= Vmax x [S]/Km.

From this equation it is apparent that a higher hydrolysis velocity (V) may be obtained when Km is reduced and/or Vmax is increased.

During washing it is reasonable to assume that the substrate concentration is considerable lower than Km and accordingly, based on the above stated values for Km and Vmax, it is possible to determine the hydrolysis velocity of each of the variants listed above. The following values are be found:

[S]	V, SEQ ID 2	V, *amyL* III	V, SL68
0.3	4.0	5	4.6
0.1	2.2	3	2.6
0.05	1.3	1.9	1.6

From the above table it is evident that the hydrolysis velocity of *amyL* variant III is higher than that of SL68, which again is higher than that of the *B. licheniformis* α-amylase having the amino acid sequence shown in SEQ ID No. 2 (the parent enzyme).

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SEQUENCE LISTING

In the following SEQ ID Nos. 1, 3, 5 the 5', coding sequence and 3' sequence of the relevant α-amylase genes are illustrated. The 5' sequence is the first separate part of the sequence written with lower case letters, the coding sequence is the intermediate part of the sequence, where the signal sequence is written with lower case letters and the sequence encoding the mature α-amylase is written with upper case letters, and the 3' sequence is the third separate part of the sequence written with lower case letters.

SEQ ID No. 1

cggaagattggaagtacaaaaataagcaaaagattgtcaatcatgtcatgagccatgcgg-
gagacggaaaaatcgtctta atgcacgatatttatgcaacgttcgcagatgctgctgaa-
gagattattaaaaagctgaaagcaaaaggctatcaattggt aactgtatctcagcttga-
agaagtgaagaagcagagaggctattgaataaatgagtagaagcgccatatcggcgctt-
ttc ttttggaagaaaatatagggaaaatggtacttgttaaaaattcggaatatttatac-
aacatcatatgtttcacattgaaa ggggaggagaatc

atgaaacaacaaaaacggctttacgcccgattgctgacgctgttatttgcgctcatctt-
cttgctgc ctcattctgcagcagcggcgGCAAATCTTAATGGGACGCTGATGCAGTATT-
TTGAATGGTACATGCCCAATGACGGCCAA CATTGGAGGCGTTTGCAAAACGACTCGGCA-
TATTTGGCTGAACACGGTATTACTGCCGTCTGGATTCCCCCGGCATATAA GGGAACGAG-
CCAAGCGGATGTGGGCTACGGTGCTTACGACCTTTATGATTTAGGGGAGTTTCATCAAA-
AAGGGACGGTTC GGACAAAGTACGGCACAAAAGGAGAGCTGCAATCTGCGATCAAAAGT-
CTTCATTCCCGCGACATTAACGTTTACGGGGAT GTGGTCATCAACCACAAAGGCGGCGC-
TGATGCGACCGAAGATGTAACCGCGGTTGAAGTCGATCCCGCTGACCGCAACCG CGTAA-
TTTCAGGAGAACACCTAATTAAAGCCTGGACACATTTTCATTTTCCGGGGCGCGGCAGCA-
CATACAGCGATTTTA AATGGCATTGGTACCATTTTGACGGAACCGATTGGGACGAGTCC-
CGAAAGCTGAACCGCATCTATAAGTTTCAAGGAAAG GCTTGGGATTGGGAAGTTTCCAA-
TGAAAACGGCAACTATGATTATTTGATGTATGCCGACATCGATTATGACCATCCTGA
TGTCGCAGCAGAAATTAAGAGATGGGGCACTTGGTATGCCAATGAACTGCAATTGGACGG-
TTTCCGTCTTGATGCTGTCA AACACATTAAATTTTCTTTTTTGCGGGATTGGGTTAATC-
ATGTCAGGGAAAAAACGGGGAAGGAAATGTTTACGGTAGCT GAATATTGGCAGAAT-
GACTTGGGCGCGCTGGAAAACTATTTGAACAAAACAAATTTTAATCATTCAGTGTTTGAC-

GTGCC GCTTCATTATCAGTTCCATGCTGCATCGACACAGGGAGGCGGCTATGATATGAG-
GAAATTGCTGAACGGTACGGTCGTTT CCAAGCATCCGTTGAAATCGGTTACATTTGTCG-
ATAACCATGATACACAGCCGGGGCAATCGCTTGAGTCGACTGTCCAA ACATGGTTTAAG-
CCGCTTGCTTACGCTTTTATTCTCACAAGGGAATCTGGATACCCTCAGGTTTTCTACGGG-
GATATGTA CGGGACGAAAGGAGACTCCCAGCGCGAAATTCCTGCCTTGAAACACAAAAT-
TGAACCGATCTTAAAAGCGAGAAAACAGT ATGCGTACGGAGCACAGCATGATTATTTC-
GACCACCATGACATTGTCGGCTGGACAAGGGAAGGCGACAGCTCGGTTGCA AATTCAGG-
TTTGGCGGCATTAATAACAGACGGACCCGGTGGGGCAAAGCGAATGTATGTCGGCCGGCA-
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ATTCGGAAGGCTGGGGAGAGTTTCACGTAAACG GCGGGTCGGTTTCAATTTATGTTC-
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SEQ ID No. 2

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VQR

SEQ ID No. 3

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SEQ ID No. 4

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SEQ ID No. 5

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tgcctgcga

SEQ ID No. 6

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SEQ ID No. 7

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61 WITPVTAQLP QTTAYGDAYH GYWQQDIYSL
91 NENYGTADDL KALSSALHER GMYLMVDVVA

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151 FIQNYEDQTQ VEDCWLGDNT VSLPDLDTTK
181 DVVKNEWYDW VGSLVSNYSI DGLRIDTVKH
211 VQKDFWPGYN KAAGVYCIGE VLDGDPAYTC
241 PYQNVMDGVL NYPIYYPLLN AFKSTSGSMD
271 DLYNMINTVK SDCPDSTLLG TFVENHDNPR
301 FASYTNDIAL AKNVAAFIIL NDGIPIIYAG
331 QEQHYAGGND PANREATWLS GYPTDSELYK
361 LIASANAIRN YAISKDTGFV TYKNWPIYKD
391 DITIAMRKGT DGSQIVTILS NKGASGDSYT
421 LSLSGAGYTA GQQLTEVIGC TTVTVGSDGN
451 VPVPMAGGLP RVLYPTEKLA GSKICSSS
```

CLAIMS

1. A variant of a parent *Bacillus licheniformis* α-amylase enzyme, wherein said variant:

has α-amylase activity; and

comprises a substitution in position 255 corresponding to a position of the amino acid sequence of the parent α-amylase enzyme, said parent having the amino acid sequence shown in SEQ ID NO:2.

2. The variant according to claim 1, which comprises mutation E255P.

3. The variant according to claim 1, which further comprises at least one substitution, deletion, or addition in the N-terminal end of the α-amylase.

4. The variant according to claim 3, in which at least one amino acid residue, located in any of positions 1, 2, 3, 15, and 17-35 of the α-amylase, has been substituted or deleted.

5. The variant according to claim 4, in which at least one amino acid residue, located in any of positions 29-35 of the α-amylase, has been substituted or deleted.

6. The variant according to claim 4 or 5, in which at least one of the following amino acid residues has been modified: A1, N2, L3, M15, N17, R23, S29, A30, Y31, A33, E34, and H35.

7. The variant according to claim 6, which comprises at least one of the following mutations: A1V; M15T,L; deletion of N in position 2; L3V; deletion of A in position 1+N2; S29A; A30E,N; Y31H,N; A33S; E34D,S; H35I,L; and R23K,T.

8. The variant according to claim 1 or 2, which further comprises at least one of the following mutations: Q51R; A52S; and A58P,V.

9. The variant according to claim 1 or 2, which further comprises one of the

following mutations: H68N and H68Q.

10. The variant according to claim 1 or 2, which further comprises at least one of the following mutations: S85Q and K88Q.

11. The variant according to claim 1 or 2, which further comprises at least one of the following mutations: N96Q, G99A, I103F, and N104D.

12. The variant according to claim 1 or 2, which further comprises at least one of the following mutations: D121N, R127Q, V128E, G131E, E132T, H133Y, L134Q, and K136Q.

13. The variant according to claim 1 or 2, which further comprises at least one of the following mutations: H140K, H142D, D152S, and S148N.

14. The variant according to claim 1 or 2, which further comprises at least one of the following mutations: N172S, F177FRG, and Q178I,E.

15. The variant according to claim 1 or 2, which further comprises at least one of the following mutations: S187D, A209V, and T217K.

16. The variant according to claim 1 or 2, which further comprises at least one of the following mutations: L230I, V233A, and R242P.

17. The variant according to claim 1 or 2, which further comprises at least one of the following mutations: H247A,Y; E250Q,S; and K251A,Q.

18. The variant according to claim 1 or 2, which further comprises at least one of the following mutations: A260G, N265Y, and A269K.

19. The variant according to claim 1 or 2, which further comprises at least one of the following mutations: Y290F,N; Q291K; and H293Q,Y.

20. The variant according to claim 1 or 2, which further comprises at least one of the following mutations: K315D, L318T, and S320A.

21. The variant according to claim 1 or 2, which further comprises at least one of the following mutations: T341P and Q360C.

22. The variant according to claim 1 or 2, which further comprises at least one of the following mutations: deletion in position 370; deletion in position 371; deletion in position 372; S373P; Q374P; R375Y; A379S; and H382S.

23. The variant according to claim 1 or 2, which further comprises at least one of the following mutations: Q393D; A398T,P; and V409I.

24. The variant according to claim 1 or 2, which further comprises at least one of the following mutations: V419K; A420P; and N421G.

25. The variant according to claim 1 or 2, which further comprises at least one of the following mutations: A435S and H450Y.

26. The variant according to claim 1 or 2, which further comprises at least one of the following mutations: P459T and V461K,T.

27. The variant according to claim 1 or 2, which further comprises one of the following mutations: M197T,G,I,A,L,S,N,C.

28. The variant according to any one of claims 1-27, which further comprises one of the following combinations of mutations:

deletion of A in position 1 + deletion of N in position 2 + L3V+M15T+R23K+S29A+A30E+Y31H+A33S+E34D+H35I;

deletion of A in position 1 + deletion of N in position 2 + L3V+M15T+R23K+S29A+A30E+Y31H+A33S+E34D+H35I+Q374P;

deletion of A in position 1 + deletion of N in position 2 +
L3V+M15T+R23K+S29A+A30E+Y31H+A33S+E34D+H35I+Q374P+
T341P;

deletion of A in position 1 + deletion of N in position 2 +
L3V+M15T+R23K+S29A+A30E+Y31H+A33S+E34D+H35I+M197I;

deletion of A in position 1 + deletion of N in position 2 +
L3V+M15T+R23K+S29A+A30E+Y31H+A33S+E34D+H35I+M197N;

deletion of A in position 1 + deletion of N in position 2 +
L3V+M15T+R23K+S29A+A30E+Y31H+A33S+E34D+H35I+M197S;

deletion of A in position 1 + deletion of N in position 2 +
L3V+M15T+R23K+S29A+A30E+Y31H+A33S+E34D+H35I+Q374P+
T341P+M197I; and

deletion of A in position 1 + deletion of N in position 2 +
L3V+M15T+R23K+S29A+A30E+Y31H+A33S+E34D+H35I+M197T.

29. A DNA construct comprising a DNA sequence encoding the α-amylase variant according to any one of claims 1-28.

30. A recombinant expression vector which carries the DNA construct according to claim 29.

31. A cell which is transformed with the DNA construct according to claim 29 or the vector according to claim 30.

32. The cell according to claim 31, which is a bacterium or a fungus.

33. The cell according to claim 32, 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*.

34. A method of producing the α-amylase variant according to any one of claims 1-28, wherein the cell according to any one of claims 31-33 is cultured under conditions conducive to the production of the α-amylase variant, and the α-amylase variant is subsequently recovered from the culture.

35. Use of the α-amylase variant according to any one of claims 1-28 for washing or dishwashing.

36. A detergent additive comprising the α-amylase variant according to any one of claims 1-28.

37. The detergent additive according to claim 36, which additionally comprises an enzyme selected from the group consisting of a protease, a lipase, a peroxidase, an amylotic enzyme, and a cellulase.

38. A detergent composition comprising the α-amylase variant according to any one of claims 1-28 and a surfactant.

39. The detergent composition according to claim 38, which additionally comprises an enzyme selected from the group consisting of a protease, a lipase, a peroxidase, an amylotic enzyme, and a cellulase.

40. A manual or automatic dishwashing detergent composition, comprising the α-amylase variant according to any one of claims 1-28 and a surfactant.

41. The dishwashing detergent composition according to claim 40, which additionally comprises an enzyme selected from the group consisting of a protease, a lipase, a peroxidase, an amylotic enzyme, and a cellulase.

42. A manual or automatic laundry washing composition comprising the α-

amylase variant according to any one of claims 1-28 and a surfactant.

43. The laundry washing composition according to claim 42, which additionally comprises an enzyme selected from the group consisting of a protease, a lipase, a peroxidase, an amylotic enzyme, and a cellulase.

44. Use of the α-amylase variant according to any one of claims 1-28 for textile desizing.

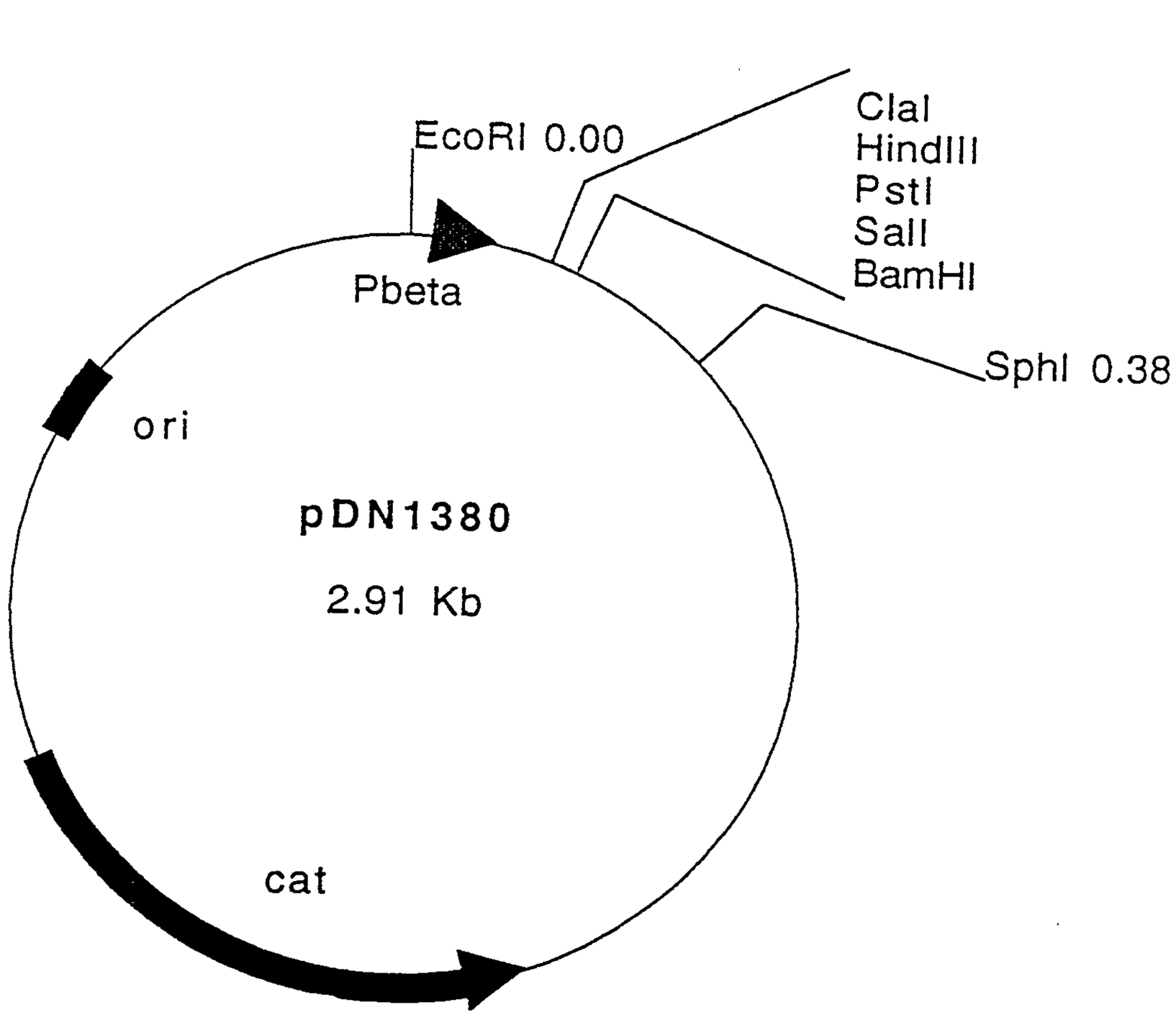

EcoRI 0.00
ClaI
HindIII
PstI
SalI
BamHI
Pbeta
SphI 0.38
ori
pDN1380
2.91 Kb
cat

Fig. 1A

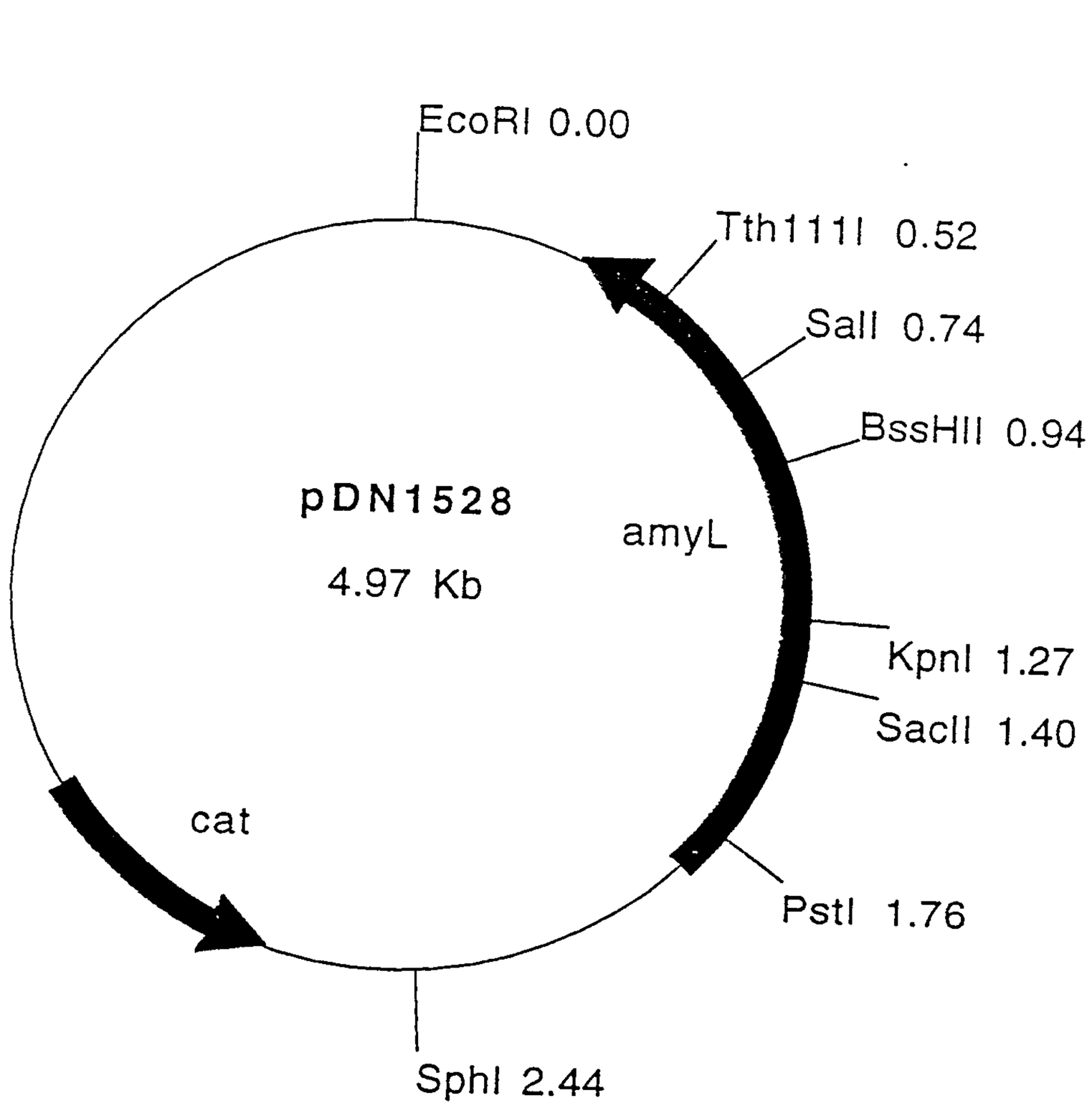

EcoRI 0.00
Tth111I 0.52
SalI 0.74
BssHII 0.94
KpnI 1.27
SacII 1.40
PstI 1.76
SphI 2.44
pDN1528
4.97 Kb
amyL
cat

Fig. 1B

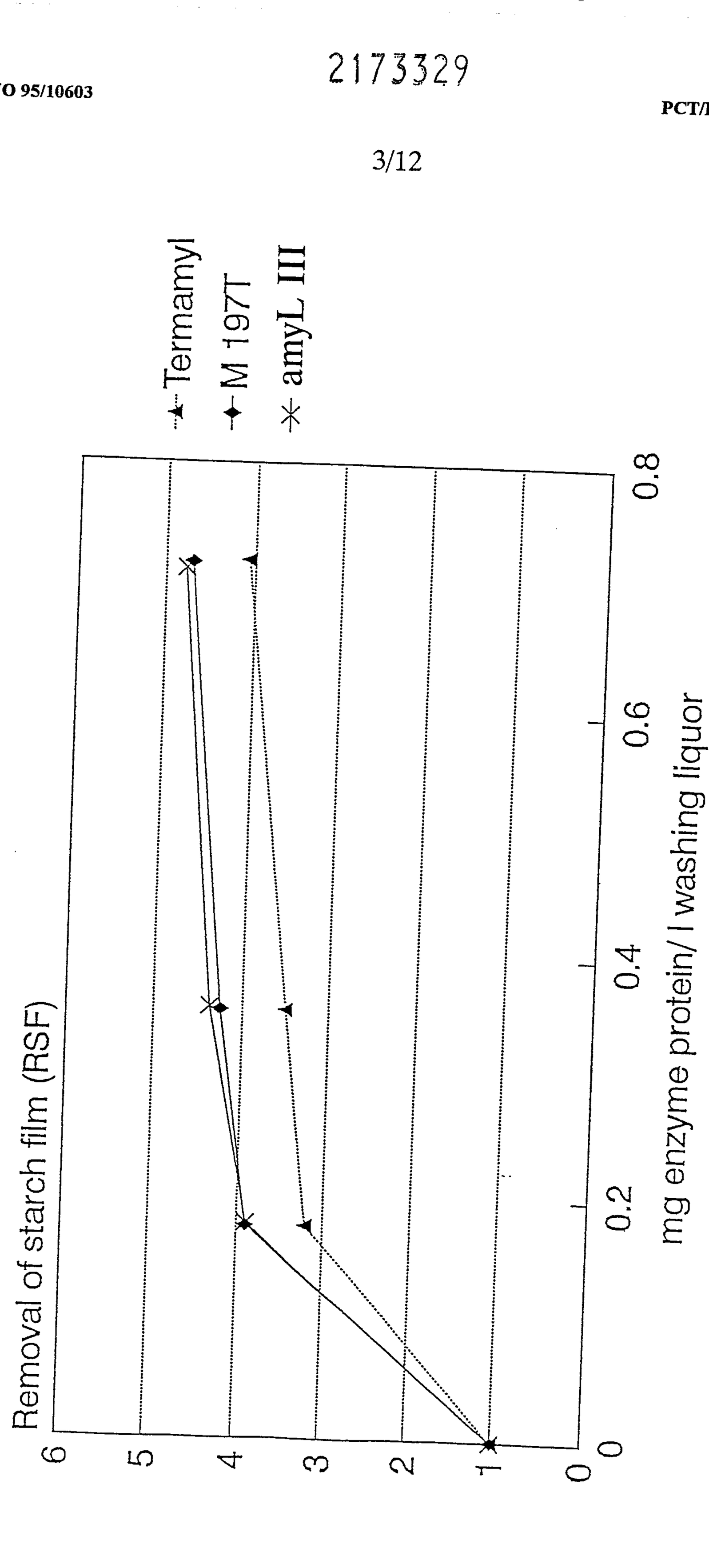

Termamyl
M 197T
amyL III
Removal of starch film (RSF)
6
5
4
3
2
1
0
0
0.2
0.4
0.6
0.8
mg enzyme protein/ l washing liquor

Fig. 2

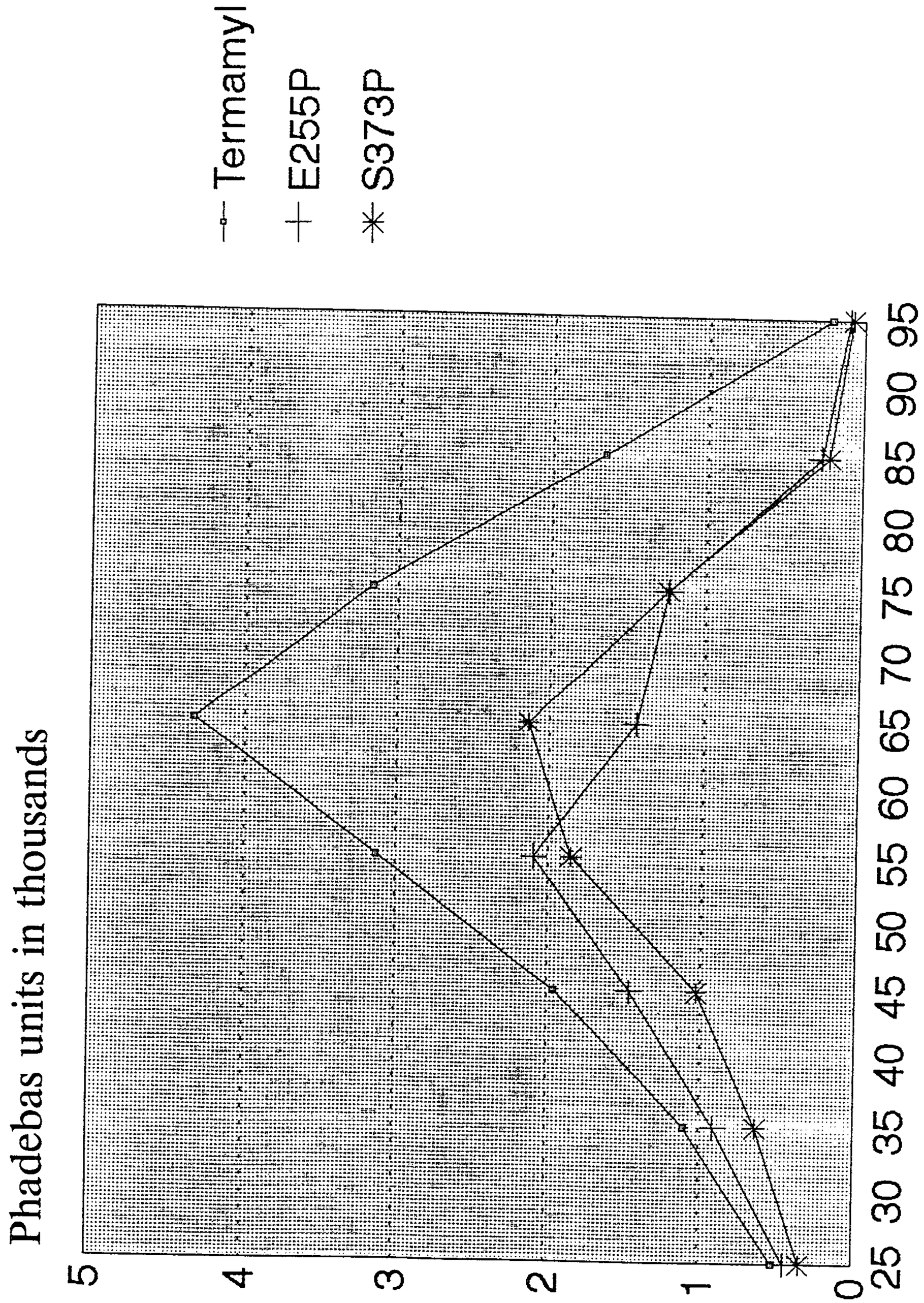

Term
E255
S373
Phadebas units in thousands
0
1
2
3
4
5
25 30 35 40 45 50 55 60 65 70 75 80 85 90 95
Temperature in °C

Fig. 3

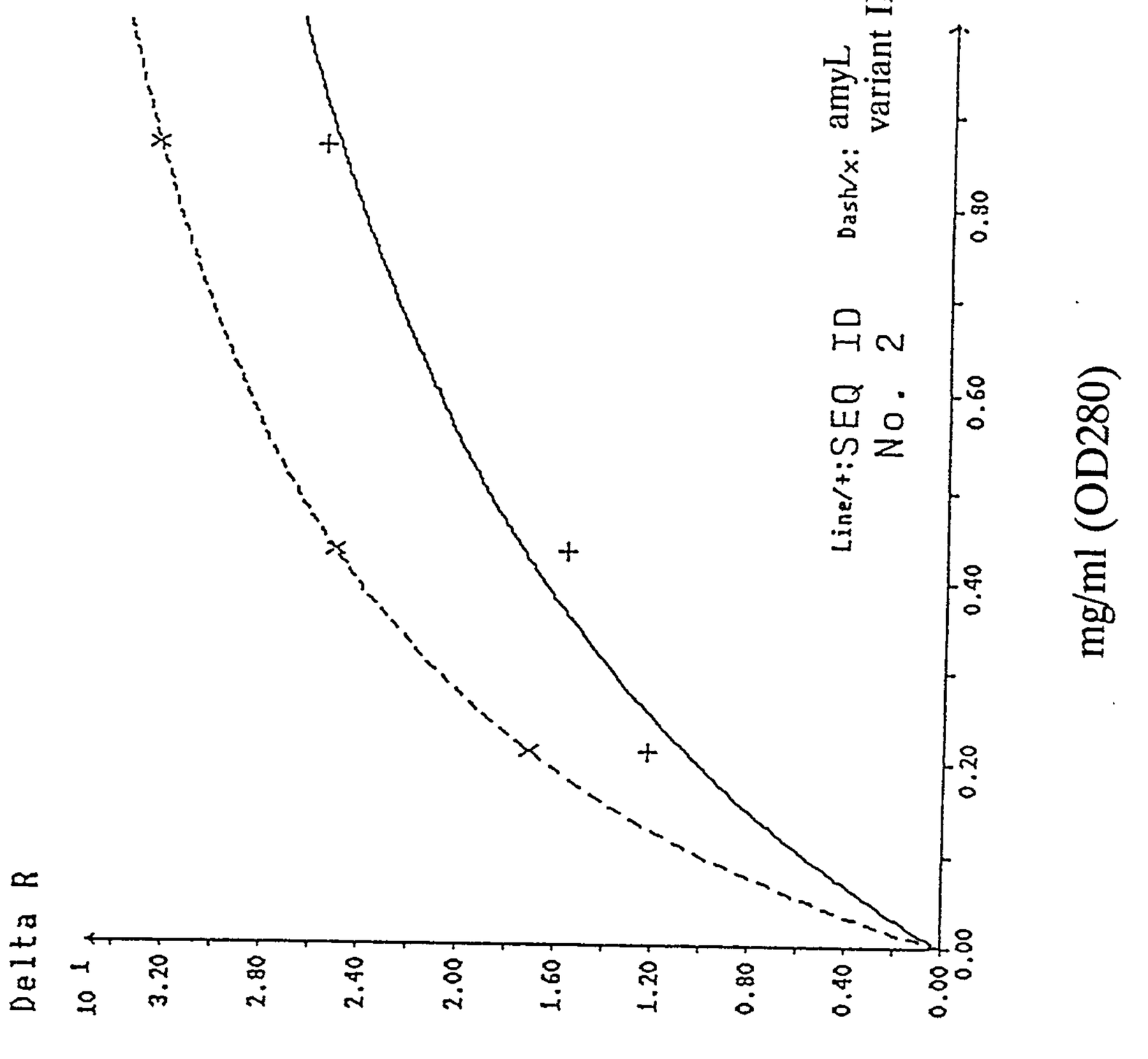

Delta R
10 1
3.20
2.80
2.40
2.00
1.60
1.20
0.80
0.40
0.00
0.00
0.20
0.40
0.60
0.80
mg/ml (OD280)
Line/+:SEQ ID No. 2
Dash/x: amyL variant III

Fig. 4

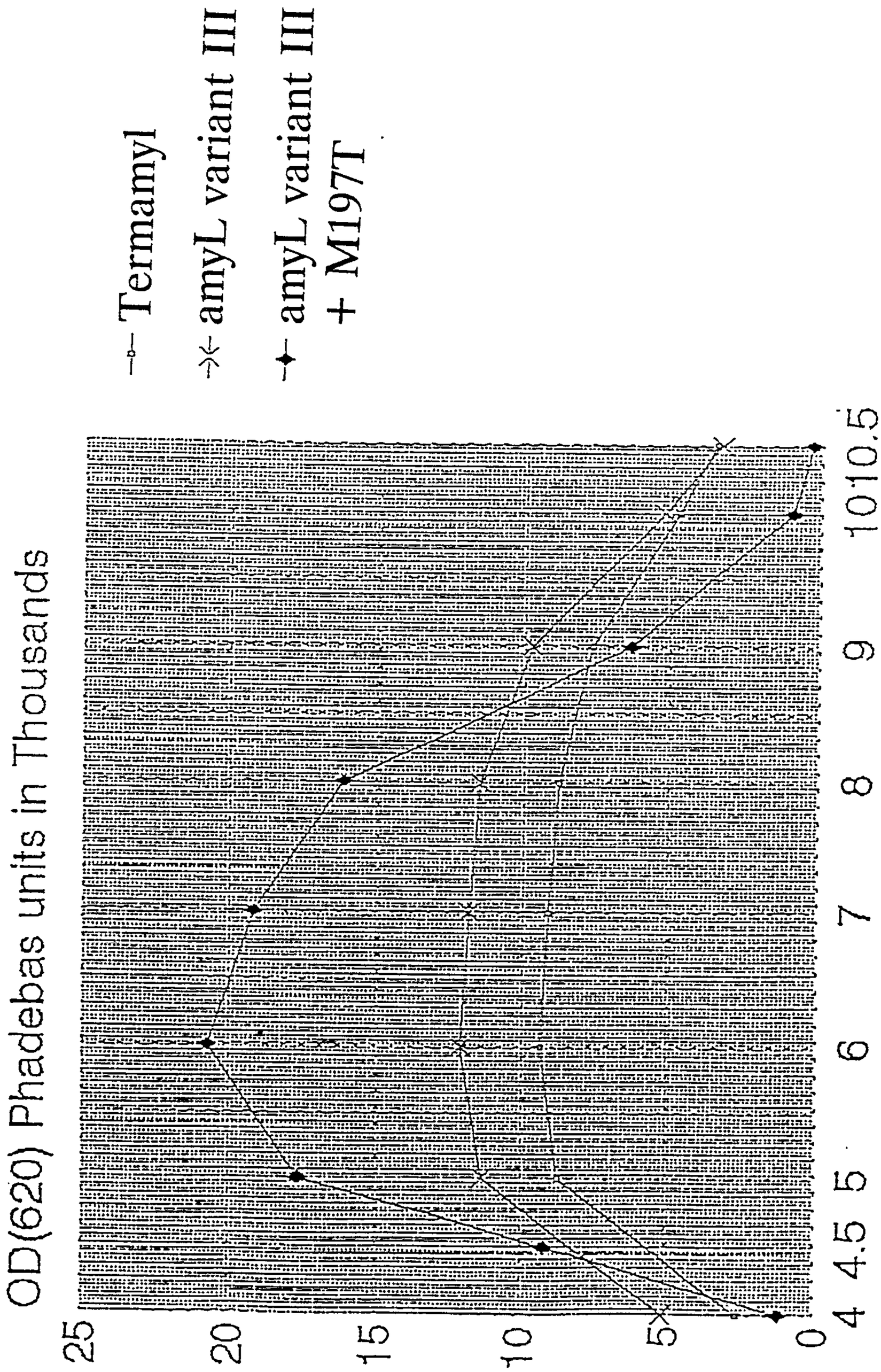

Term amyl
amyL variant III
amyL variant III
+ M197T
OD(620) Phadebas units in Thousands
25
20
15
10
5
0
4
4.5
5
6
7
8
9
10
10.5
pH

Fig. 5

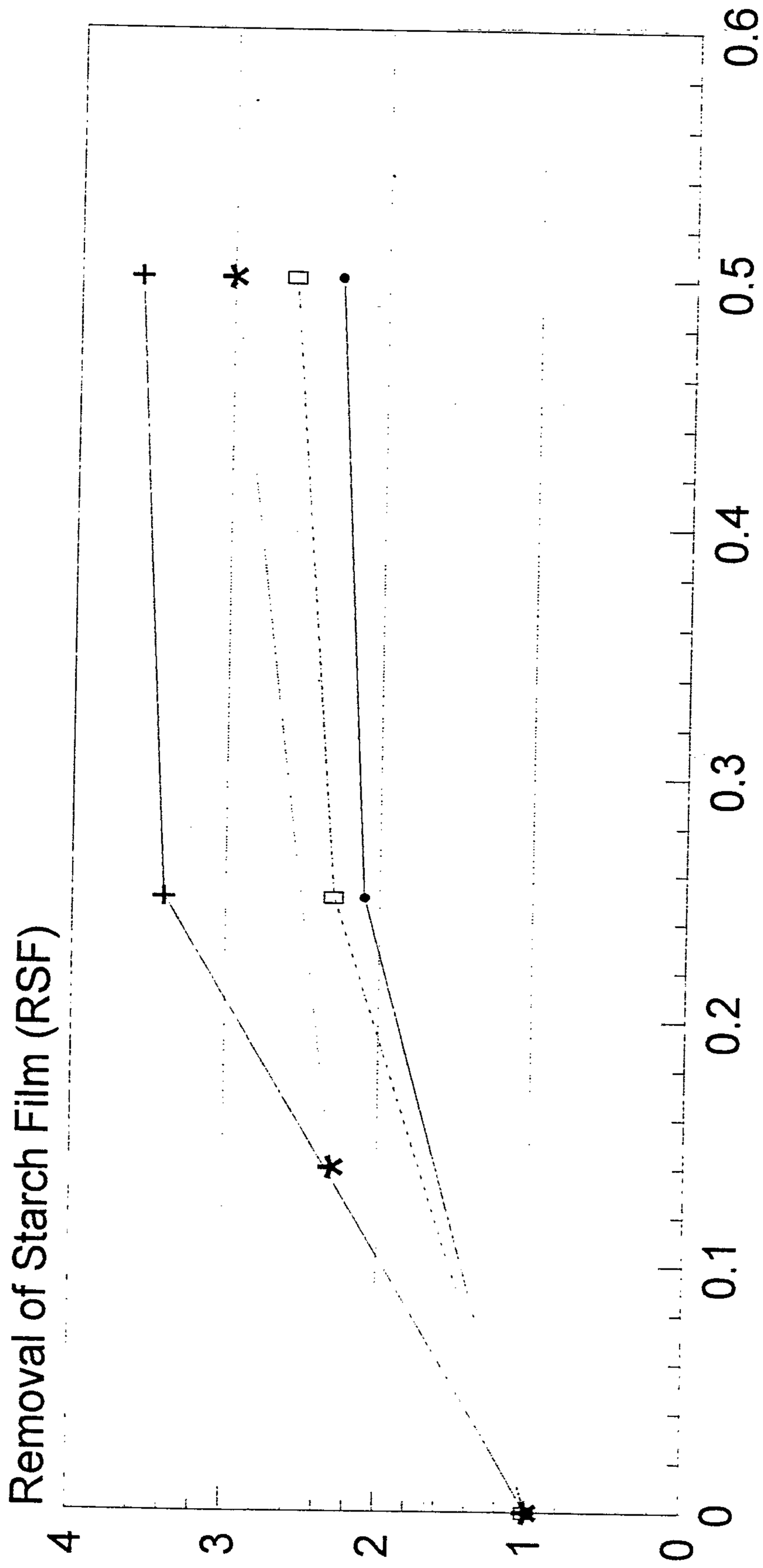

Removal of Starch Film (RSF)
4
3
2
1
0
0
0.1
0.2
0.3
0.4
0.5
0.6
mg enzyme protein/l dishwashing liquor
• Termamyl + E255P * S373P □ Q374P

Fig. 6

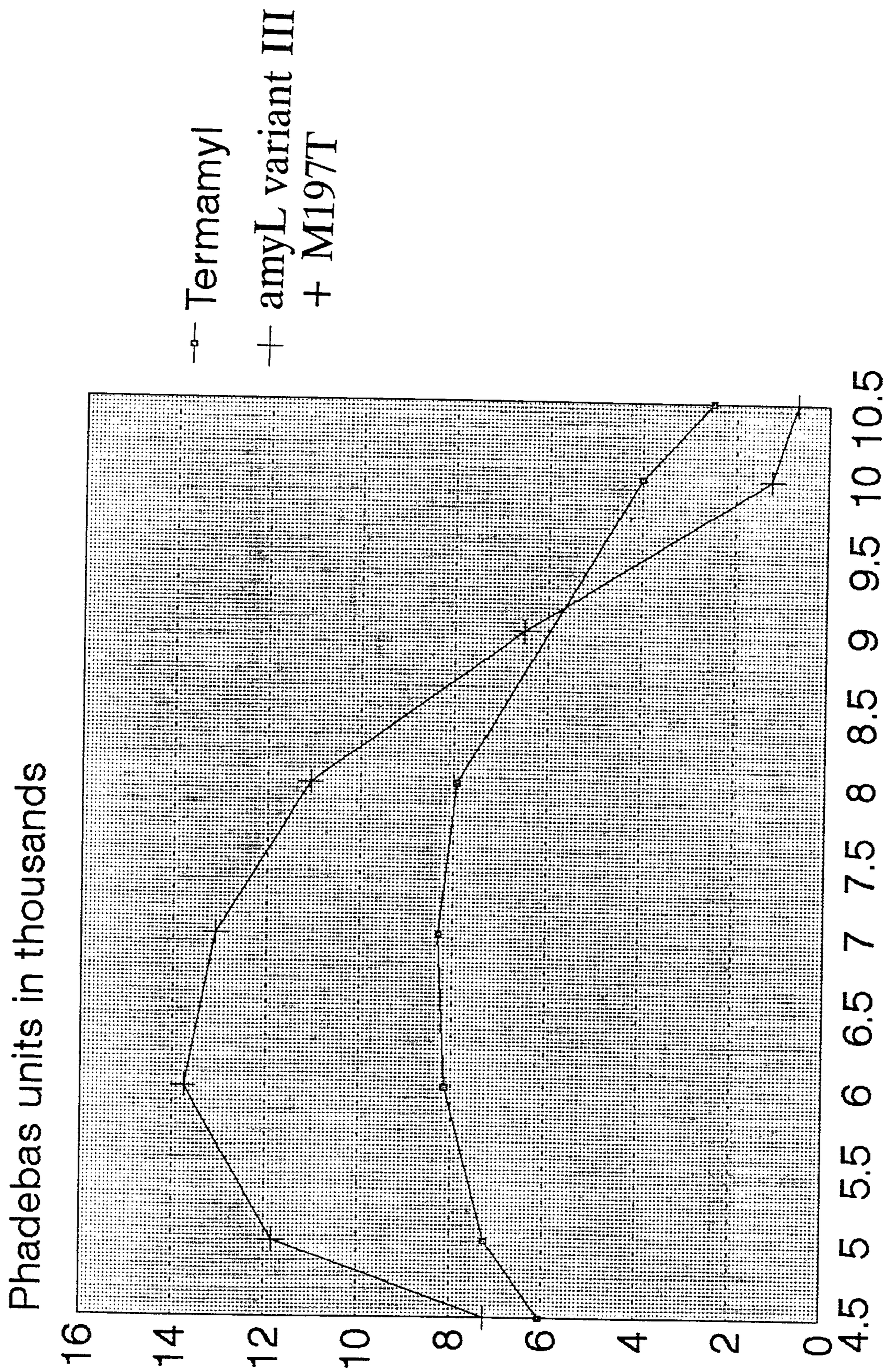

Termamyl
amyL vari
+ M197T
Phadebas units in thousands
16
14
12
10
8
6
4
2
0
4.5 5 5.5 6 6.5 7 7.5 8 8.5 9 9.5 10 10.5
pH

Fig. 7

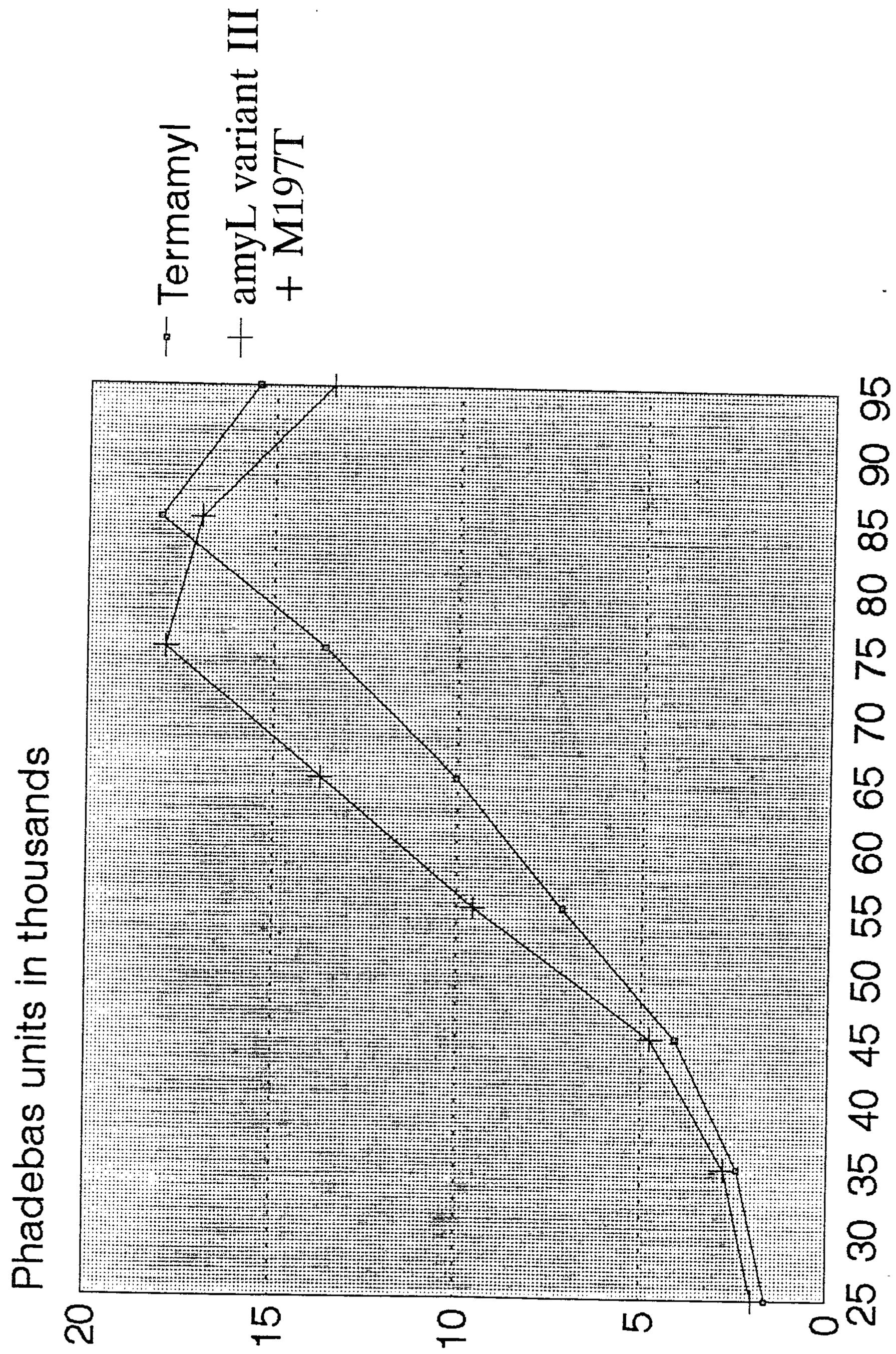

Termamyl
amyL variant III
+ M197T
Phadebas units in thousands
20
15
10
5
0
25 30 35 40 45 50 55 60 65 70 75 80 85 90 95
Temperature

Fig. 8

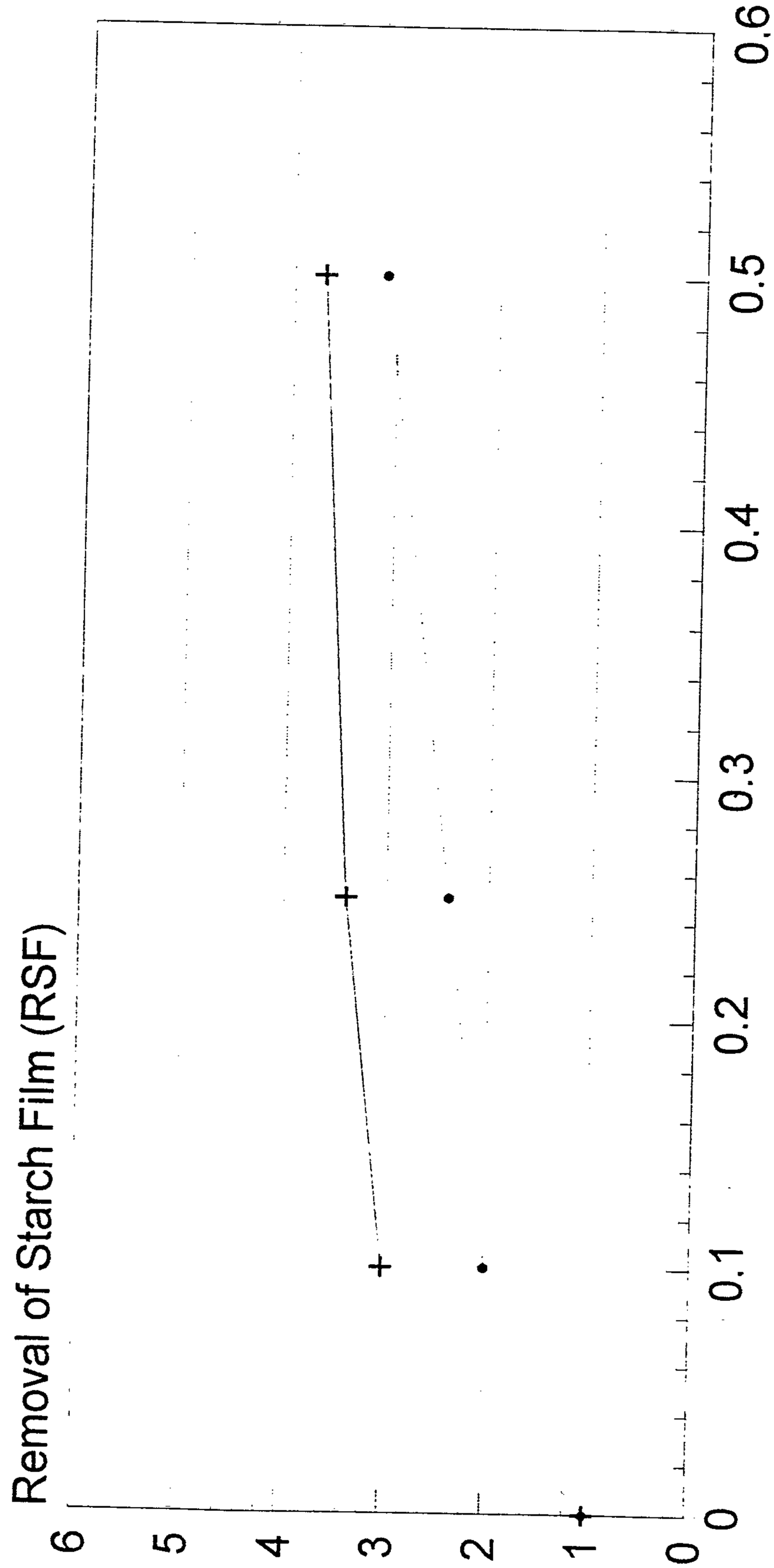

Removal of Starch Film (RSF)
6
5
4
3
2
1
0
0
0.1
0.2
0.3
0.4
0.5
0.6
mg enzyme protein/l dishwashing liquor
+ Termamyl + amyL variant III M197T
• + NA

Fig. 9

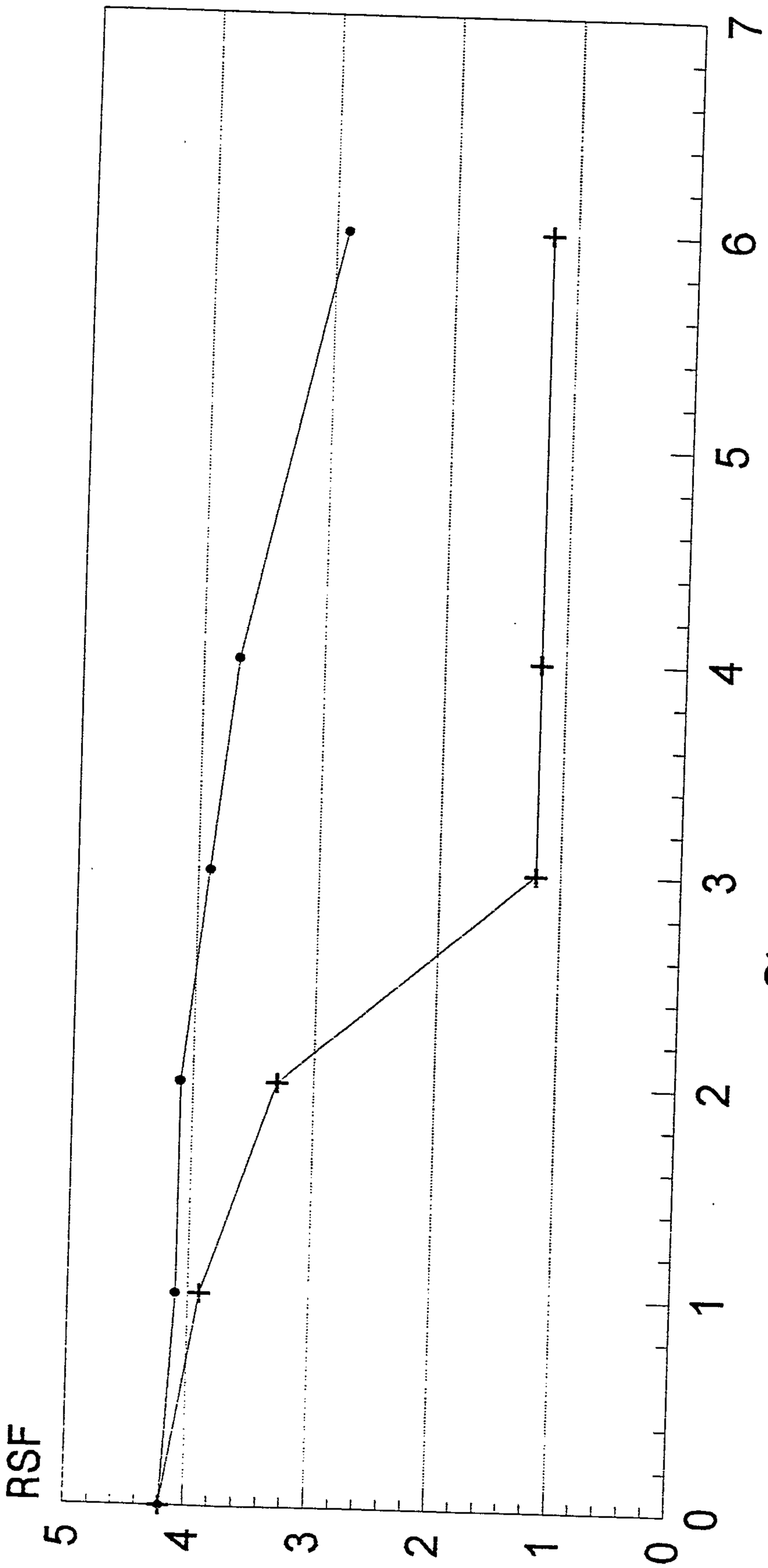

RSF
Storage Time (weeks)
amyL variant III
+ M197T
+ Termamyl
0
1
2
3
4
5
6
7

Fig. 10

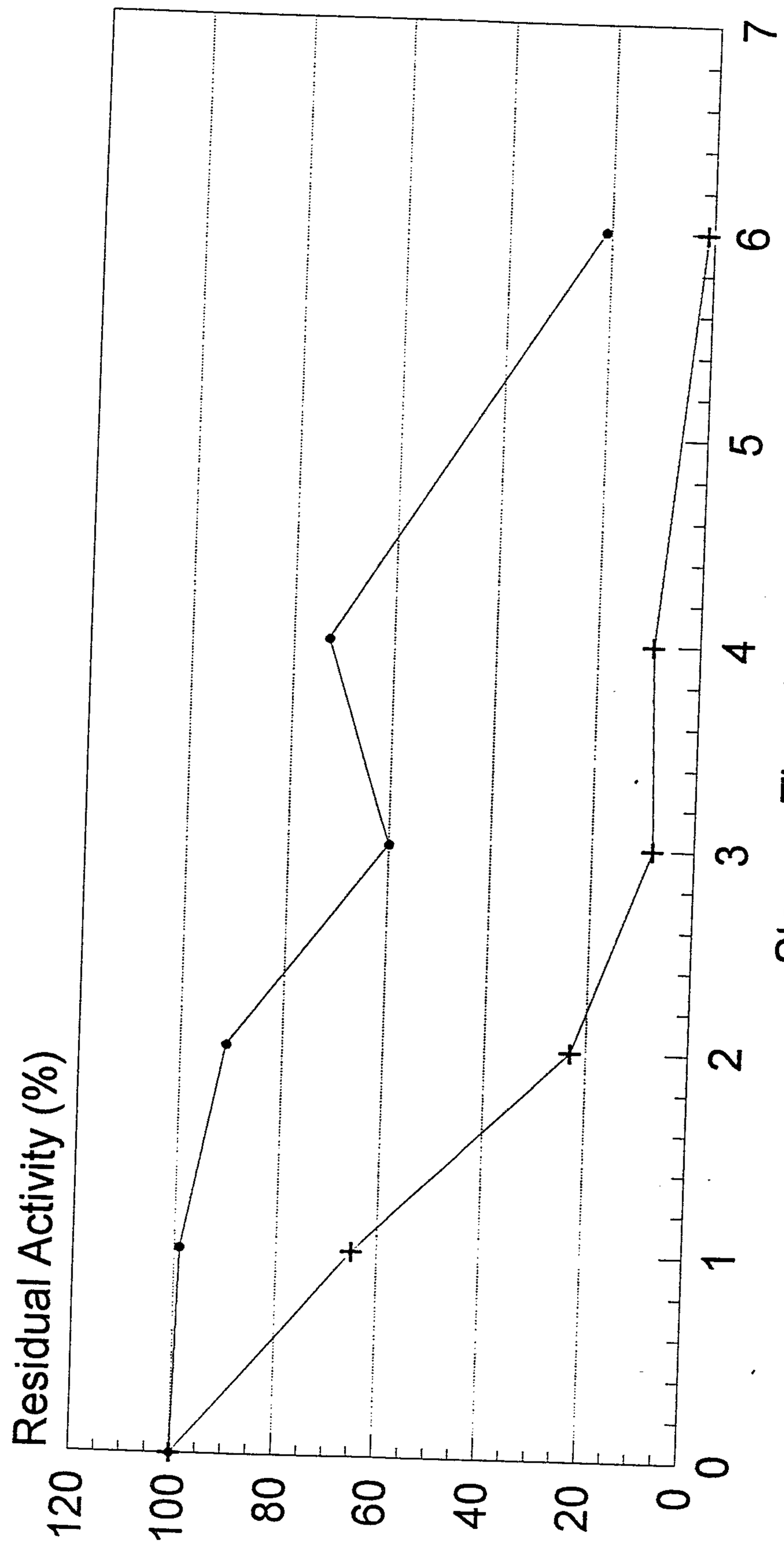

Residual Activity (%)
120
100
80
60
40
20
0
0
1
2
3
4
5
6
7
Storage Time (weeks)
amyL variant III + Termamyl
+ M197T

Fig. 11