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(54) **Titre : UTILISATION D'ENZYMES PECTINOLYTIQUES DANS LE TRAITEMENT DE PUREE DE FRUIT OU DE LEGUME, ET SEQUENCES ENZYMATIQUES AFFERENTES**
(54) **Title: USE OF PECTINOLYTIC ENZYMES FOR THE TREATMENT OF FRUIT AND VEGETABLE MASH AND ENZYME SEQUENCES THEREFOR**

(57) **Abrégé/Abstract:**

The invention relates to the use of one or more pectinolytic enzyme(s) for the treatment of fruit or vegetable mash as well as a process for enzymatic treatment of fruit or vegetable mash comprising the step of adding one or more pectinolytic enzyme(s), wherein at least one pectinolytic enzyme is obtainable from *Trichoderma reesei*, as well as to a process for the preparation of a fruit or vegetable juice comprising the process for enzymatic treatment of fruit or vegetable mash. Moreover, the invention discloses recombinant DNA molecules encoding a polypeptide having endo-polygalacturonase activity, a polypeptide having exo-polygalacturonase activity, a polypeptide having exo-rhamnogalacturonase activity and a polypeptide having xylogalacturonase activity.

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(54) Title: USE OF PECTINOLYTIC ENZYMES FOR THE TREATMENT OF FRUIT AND VEGETABLE MASH AND ENZYME SEQUENCES THEREFOR

(57) Abstract: The invention relates to the use of one or more pectinolytic enzyme(s) for the treatment of fruit or vegetable mash as well as a process for enzymatic treatment of fruit or vegetable mash comprising the step of adding one or more pectinolytic enzyme(s), wherein at least one pectinolytic enzyme is obtainable from *Trichoderma reesei*, as well as to a process for the preparation of a fruit or vegetable juice comprising the process for enzymatic treatment of fruit or vegetable mash. Moreover, the invention discloses recombinant DNA molecules encoding a polypeptide having endo-polygalacturonase activity, a polypeptide having exo-polygalacturonase activity, a polypeptide having exo-rhamnogalacturonase activity and a polypeptide having xylogalacturonase activity.



WO 2009/141156 A3

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Use of Pectinolytic Enzymes for the Treatment of Fruit and Vegetable Mash
and Enzyme Sequences Therefor

10 The present invention relates to the use of pectinolytic enzymes or polypeptides
having pectinolytic activity for the treatment of fruit or vegetable mash. The inven-
tion further relates to the use of pectinolytic enzymes for the preparation of fruit or
vegetable juice. At least one of the enzymes is obtainable from *Trichoderma*
15 *reesei*. Moreover, the invention relates to polypeptide sequences having pecti-
nolytic activity suitable in the treatment of fruit or vegetable mash as well as to
polynucleotides encoding said polypeptide sequences. Particularly, the invention
relates to the use of a polygalacturonase from *Trichoderma reesei* in the treatment
of fruit or vegetable mash, particularly apple mash, as well as to the use of said
enzyme for the preparation of fruit or vegetable juice, in particular apple juice.

20

Pectin polymers are important constituents of plant cell walls. Pectin is the major
structural polysaccharide of fruit or vegetable lamella and cell walls. The texture of
fruit or vegetable depends on the quantity and properties of pectin. Generally, un-
ripe fruit contains insoluble protopectin, whereas ripe fruit contains more soluble
25 pectin. Pectin is a heteropolysaccharide with a backbone composed of alternating
homogalacturonans (smooth regions) and rhamnogalacturonans (hairy regions).
The smooth regions are linear polymers of 1,4-linked α -D-galacturonic acid. The
galacturonic acid residues can be methylesterified on the carboxyl group.

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A fruit contains pectinolytic enzymes, which participate in the natural maceration
process during and after ripening. Industrial pectinases are used in processing
fruit and vegetable in feed and food. In industrial processes enzymes are used,
e.g. in fruit or vegetable processing, in order to hydrolyse pectin and to increase

the juice when pressing fruit or vegetable, to lower the viscosity to be able to concentrate cloudy juices or to degrade pectin completely in order to clarify juices and to concentrate them.

5 Fruit and vegetable juices, especially juice made from apples, can be produced either by a pressing operation or by liquefaction processes. Both processes are supported by the use of pectinolytic enzymes. Basically, the whole fruits are milled and treated with pectinolytic enzymes prior to pressing to loosen cell walls and to promote the free run of the juice. After pressing, the juice usually is heated, which
10 inactivates all the enzymes in the juices. Afterwards the juice is transferred to clarification tanks where additional enzyme is added to the juice to depectinize and hydrolyse starch prior to filtration. Then the enzymes are inactivated during the later pasteurisation of the juice or in the evaporator during concentration. For example, in the production of apple juice a certain structure of the mash is required
15 for a good pressing result. Pectinases that provoke the degradation or maceration of so-called insoluble pectins are unfavourable, since they increase solids in the juice. If the structure is completely destroyed, the so-called apple sauce effect is attained and the juice is very cloudy after pressing. Pectinases act preliminarily on soluble pectins and, thus, result in a lower viscosity of the juice assay and a very
20 easy run off. In the preparation of purees macerative properties are preferred.

In the prior art fruit mashes/fruit juices have already been prepared by using pectinolytic enzymes, containing smooth and hairy region pectinases. "Smooth region" pectinases comprise pectin esterases (or pectin methyl-esterases), polygalacturonases and pectin lyases (or pectin trans-eliminases). "Hairy region" pectinases
25 comprise mainly endo-arabanases, arabinofuranosidases, rhamnogalacturonases, arabino-galactanases, among others. Both enzyme categories are present in standard pectinase preparations derived from *Aspergillus niger*. In the prior art process the pectinase is added during crushing of the apples in order to achieve
30 suitable distribution of the enzyme in the mash as stirring is not recommended. After a holding time of 30 – 120 minutes, the mash is pressed by horizontal or belt press systems. The obtained juice is sieved in order to separate coarse particles.

Afterwards the juice is pasteurized or essence-stripped in a vacuum evaporator. After re-cooling to about 48 – 52 °C, the juice treatment takes place in order to depectinize and degradate the starch. This treatment takes about 1 – 2 hrs followed by a filtration process, i.e. ultrafiltration.

5

Pectinase preparations (pectinase compositions) of the prior art consisting of "smooth region" and "hairy region" pectinases are not suitable for such described press processes, as they liquefy the mash and cause high amounts of solids in the juice. The exclusive application of a specific polygalacturonase in combination
10 with a high pectin esterase supplies much better press results, i.e. shorter press cycles, higher press yields and lower solids in the juice. Furthermore the juice contains less or no residual pectin, which improves subsequent depectinisation and filtering.

15 At the processing of clear juices, a 2nd processing step called "depectinisation" is required, in which usually both "smooth region" and "hairy region" pectinases are used. In principle, it is necessary to degrade all present high molecular substances (mainly pectins, starch, etc.) in order to achieve an optimized ultrafiltration process. The pectinases used in the prior art processes are not satisfactory as
20 regards their performance at higher temperatures or the quality of the obtained juice.

Currently pectinases that are active at higher temperatures (> 60 °C) are not available. Moreover, pectinases used for the mash treatment yielding directly clear
25 juice after pressing without residual pectin are not satisfactorily available at present. Pectinolytic enzymes are known from the prior art. *Aspergillus* pectinases are, for example, disclosed in WO 94/14952 and WO 94/14966. Carbohydrate Research 338 (2003), 515-524, describes the isolation and characterisation of two *Trichoderma reesei* (ATCC 26920) polygalacturonase isoforms belonging to the
30 glycosyl hydrolase family 28. The enzyme is characterised in terms of its pH and temperature properties. A particular use of said polygalacturonases is not described.

Consequently, there has been a need in the prior art for pectinolytic enzymes being suitable for the treatment of fruit or vegetable mash in terms of easier handling as regards the temperature properties and the conduct of the process.

5

Accordingly, it is an object of the present invention to provide an improved process for the preparation of fruit or vegetable juice. In particular, it is an object of the present invention to provide an improved process for the preparation of fruit or vegetable mash. The method of the invention is to lead to a better yield and quality in terms of the finally obtained juice. Moreover, the process of the invention should be practicable over a wide range of temperatures and should also lead to good results when the process is carried out at high temperatures. The process of the invention is to improve the extractability or degradability and, thus, the press capacity of the mash. It is to lead to juices with a low content of residual pectins after pressing, i.e. the clarity of the obtained juices is to be improved and, thus, avoids laborious filtrations. The process of the invention should be suitable for different fruits.

A further object of the invention is to provide genes encoding pectinolytic enzymes as well as to provide the sequences of polypeptides having pectinolytic activity being suitable in the above-mentioned process. In particular, the sequences of the invention are to encode pectinolytic enzymes having a broad application range and leading to improvements in the process of the treatment of mash and the preparation of fruit or vegetable juice.

25

It has now surprisingly been found that pectinases from *Trichoderma reesei* show excellent performance in the treatment of fruit or vegetable mash and specifically in the treatment of a mash from fruits that contain soluble or low esterified pectin. In particular, it has been found that the *Trichoderma reesei* polygalacturonase (PGA1) shows excellent performance in apple mash treatments. It has surprisingly been found that *Trichoderma reesei* polygalacturonase (PGA1) can be used as the sole enzyme for the treatment of a mash from fruits that contain soluble or low

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esterified pectin and the process can favourably be conducted at elevated temperatures. It has further been found that *Trichoderma reesei* polygalacturonase PGA1 can favourably be used in combination with further pectinolytic enzymes, like pectin methylesterases, polygalacturonases, pectin lyases, pectate lyases, arabinofuranosidases, endo-arabanases or rhamnogalacturonases to improve the treatment of fruit mash even from fruits having high esterified or insoluble pectin, whereby the process has to be conducted at a temperature that is compatible to the enzymes used.

The invention relates to the use of one or more pectinolytic enzymes for the treatment of fruit or vegetable mash, wherein at least one pectinolytic enzyme is obtainable from *Trichoderma reesei*. In particular, the invention relates to the use of a polygalacturonase from *Trichoderma reesei* in the treatment of apple mash. Moreover, the invention relates to the process for enzymatic treatment of fruit or vegetable mash comprising the step of adding one or more pectinolytic enzyme(s) as well as to a process for the preparation of a fruit or vegetable juice comprising said process for enzymatic treatment of fruit or vegetable mash, wherein at least one pectinolytic enzyme is obtainable from *Trichoderma reesei*. In particular, the invention relates to a process for enzymatic treatment of apple mash, whereby a polygalacturonase from *Trichoderma reesei* having SEQ ID NO: 2 is used.

The invention, moreover, relates to a recombinant DNA molecule that upon expression in a prokaryotic or eukaryotic host cell encodes a polypeptide having endo-polygalacturonase activity, said recombinant DNA molecule comprising a DNA sequence selected from a) DNA sequences having or comprising SEQ ID NO: 1 (*pga1*), b) DNA sequences hybridizing with the DNA sequences of a) under stringent conditions, c) DNA sequences having a degree of identity of 70% to 98% to the sequences of a) or d) DNA sequences being related to the sequences of a), b) or c) due to the degeneracy of the genetic code.

The invention, moreover, relates to a recombinant DNA molecule that upon expression in a prokaryotic or eukaryotic host cell encodes a polypeptide having exo-

polygalacturonase activity. The recombinant DNA molecule comprising a DNA sequence selected from a) DNA sequences having or comprising SEQ ID NO: 3 (*pgx1*), b) DNA sequences hybridizing with the DNA sequences of a) under stringent conditions, c) DNA sequences having a degree of identity of 60% to 98% to the sequences of a) or d) DNA sequences being related to the sequences of a), b) or c) due to the degeneracy of the genetic code.

Furthermore, the invention relates to a recombinant DNA molecule that upon expression in a prokaryotic or eukaryotic host cell encodes a polypeptide having exorhamnogalacturonase activity, the recombinant DNA molecule comprising a DNA sequence selected from a) DNA sequences having or comprising SEQ ID NO: 5 (*rgx1*), b) DNA sequences hybridizing with the DNA sequences of a) under stringent conditions, c) DNA sequences having a degree of identity of 60% to 98% to the sequences of a) or d) DNA sequences being related to the sequences of a), b) or c) due to the degeneracy of the genetic code.

The invention also relates to a recombinant DNA molecule that upon expression in a prokaryotic or eukaryotic host cell encodes a polypeptide having xylogalacturonase activity, the recombinant DNA molecule comprising a DNA sequence selected from a) DNA sequences having or comprising SEQ ID NO: 7 (*xga1*), b) DNA sequences hybridizing with the DNA sequences of a) under stringent conditions, c) DNA sequences having a degree of identity of 60% to 98% to the sequences of a) or d) DNA sequences being related to the sequences of a), b) or c) due to the degeneracy of the genetic code.

The invention also relates to a polypeptide having pectinolytic activity and comprising an amino acid sequence selected from: a) a polypeptide comprising an amino acid sequence having at least 77% identity, preferably at least 80% identity, more preferred at least 85% identity, still more preferred at least 90% identity, still more preferred at least 95% identity and still more preferred at least 98% identity to the sequence of the PGAI polypeptide (SEQ ID NO: 2); (b) a variant of a) com-

prising a fragment having pectinolytic activity; and c) a fragment of a) or b) having pectinolytic activity.

The invention further relates to a polypeptide having exo-polygalacturonase, exo-rhamnogalacturonase or xylogalacturonase activity and comprising an amino acid sequence selected from a) a polypeptide comprising an amino acid sequence having at least 60% identity, preferably at least 70% identity, more preferred at least 80% identity, still more preferred at least 90% identity and still more preferred at least 95% identity to the sequence of the polypeptides SEQ ID NO: 4, SEQ ID NO: 6 or SEQ ID NO: 8, and b) a variant of a).

For the purpose of the present invention the term "pectinolytic enzyme" is to comprise pectinases, pectin esterases (or pectin methyl-esterases), polygalacturonases, pectin lyases (or pectin trans-eliminases), pectate lyases (or pectate trans-eliminases), arabinofuranosidases, endo-arabanases or rhamnogalacturonases.

When preparing a fruit or vegetable mash according to the present invention, the fruit or vegetable in question is first crushed, then the mash is treated with the pectinolytic enzyme of the present invention, then the mash is pressed and the thus obtained juice is optionally pasteurised and optionally further treated with (the) pectinolytic enzyme(s) and/or with other enzymes suitable for the conduct of the process. In this connection the temperature characteristics of the further enzymes to be used should be taken into account as regards the overall conduct of the process at higher temperatures.

25

The pectinolytic enzyme is added directly during or after crushing and in amounts usual in the art. The preferable application is to use a pectinase preparation consisting of 50.000 – 100.000 PGU/mg in a 1 – 5 % solution. The recommended dosage of the enzyme is 50 – 100 g/t of fruits. The recommended reaction temperature is 10 – 30 °C, the reaction time is 30 – 120 minutes. The average pH of the mash is 3.2 – 3.6.

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The pectinolytic enzyme can be added in any form that is convenient and compatible with the conduct of the process.

5 The pectinolytic enzyme is preferably added as concentrated or diluted liquid solution.

Preferably, the pectinolytic enzyme is a pectinolytic enzyme having one of the sequences SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 6 and SEQ ID NO: 8. Most preferable is the polygalacturonase from *Trichoderma reesei* having SEQ ID NO:
10 2.

The process described above is suitable for the treatment of any fruit or vegetable mash. Suitable fruits are selected from apples, pears, grapes, white grapes, red grapes, berries and plums. The process is suitable both for fruits that are processed at cold temperatures (p.ex. 10-30 °C) and for fruits that are processed at
15 high temperatures (p.ex. 50 °C). Suitable vegetables are selected from carrots and tomatoes. Other processable material may include coffee or cacao beans and pepper.

20 Most favourable results are obtained when the fruit mash is an apple mash and the enzyme used is polygalacturonase from *Trichoderma reesei*. Particularly favourable results are obtained when the fruit mash is a mash from fruits that contain low esterified and soluble pectin like strawberries or plums. It has been found that in this case a juice in high yield and high quality can be obtained by use of
25 *Trichoderma* PGA1 as single enzyme. In the case of fruits that contain highly esterified and/or insoluble pectin the use of additional pectinolytic enzymes in the process of preparing a corresponding juice may be necessary.

The invention also relates to the DNA and protein sequences of novel pectinolytic
30 enzymes from *Trichoderma reesei*. Those sequences are an endo-polygalacturonase (*pga1*), an exo-polygalacturonase (*pgx1*), an exo-

rhamnogalacturonase (*rgx1*) and a xylogalacturonase (*xga1*). The sequences are given in the enclosed sequence listing as SEQ ID NO: 1 to SEQ ID NO: 8.

The invention also comprises variants and derivatives of said DNA sequences as long as they encode a polypeptide having the claimed activity. Specifically com-
5 prised by the invention are DNA sequences that hybridise to the respective sequence under stringent conditions. Examples of stringent conditions are hybridisation at 65°C, 18 h in dextran sulfate solution (GenescreenPlus, Dupont), washing of the filters for 30 min, first with 6 x SSC, twice with 2 x SSC, three times with 3 x
10 SSC, with 0.1% SDS and after that 0.2 x SSC at 65°C (membrane transfer and detection method, Amersham).

Preferably, the invention relates to a polynucleotide having a degree of identity of at least 70%, preferably at least 80%, more preferred at least 85%, still more preferred at least 90%, still more preferred at least 95%, still more preferred at least
15 98% to the sequence of *pga1* (SEQ ID NO: 1).

Preferably, the invention relates to a polynucleotide having a degree of identity of at least 60%, preferably at least 70%, more preferred at least 75%, still more preferred at least 80%, still more preferred at least 85%, still more preferred at least
20 90%, still more preferred at least 95% and still more preferred at least 98% to one of the sequences selected from *pgx1* (SEQ ID NO: 3), *rgx1* (SEQ ID NO: 5) and *xga1* (SEQ ID NO: 7).

Furthermore, the invention relates to DNA sequences that are related to the sequences according to the present invention due to the degeneracy of the genetic code as well as all their allelic variants. The degeneracy of the genetic code may result from a natural degeneracy or from a especially selected use of the codon. Naturally occurring allelic variants can be identified by using well-known techniques of molecular biology, such as the polymerase chain reaction (PCR), or hybridisation techniques.
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A DNA sequence encoding a polypeptide according to the present invention may be used to transform any host cell, such as cells of fungi, yeast, bacteria, plants or mammals.

5 The degree of identity is preferably determined by detecting the number of residues of the shorter sequence taking part in the comparison and having an "appropriate" counterpart in the other sequence. In this respect homology is defined as degree of identity. For the purposes of the present invention identity is preferably determined in the usual way by using standard algorithms. According to the pre-
10 sent invention, only the cDNAs of the respective proteins are used for the comparison, and similar, preferably identical, sequence counterparts were determined as homologous sequences by means of known computer programmes. An example of such a programme is Clone Manager Suite, a programme that includes the programme part Align Part and is sold by Scientific & Educational Software, Dur-
15 ham, NC, USA. Under the option "local alignment" this programme conducts a comparison of two DNA sequences as defined above by using either the FastScan – MaxScore method or the Needleman-Wunsch method and by retaining the default values. According to the present invention, the programme version "Clone Manager 7 Align Plus 5" including the functions "Compare Two Se-
20 quences/Global/Compare DNA sequences" was especially used for determining the degree of identity. In this case algorithms available from the following sources were used: Hirschberg, D.S. (1975) A linear space algorithm for computing longest common subsequences, Commun. Assoc. Comput. Mach. 18:341-343; Myers, E.W. and W. Miller. (1988) Optimal alignments in linear space, CABIOS 4:1, 11-
25 17; Chao, K-M, W.R. Pearson and W. Miller. (1992) Aligning two sequences within a specified diagonal band, CA-BIOS 8:5, 481-487.

Expression of the cloned gene sequence(s) results in the production of the desired protein, or in the production of a fragment of this protein. This expression can take
30 place in a continuous manner in the transformed cells, or in a controlled manner.

Fragments are understood to be parts of polypeptide or nucleic acid molecules long enough to have the desired enzymatic properties or to code for the described pectinolytic polypeptides or a biologically active fragment thereof. Preferably, fragment sequences are the respective mature polypeptide sequences without a signal sequence.

The invention also relates to polypeptides having sequences with a degree of identity of at least 60%, preferably at least 70%, more preferred at least 80%, still more preferred at least 90% and most preferred at least 95% with the above polypeptide sequences SEQ ID NOs: 2, 4, 6 or 8 or fragments thereof or parts of it as long as the polypeptide retains the respective pectinolytic activity. Preferably, the invention relates to a polypeptide having a degree of identity of at least 77%, preferably at least 80%, more preferred at least 85%, still more preferred at least 90%, still more preferred at least 95% and still more preferred at least 98% to the sequence of the the PGA1 polypeptide (SEQ ID NO: 2).

As used in the present context the term "identity" of polypeptides refers to the global identity between two amino acid sequences compared to each other from the first amino acid encoded by the corresponding gene to the last amino acid.

The identity of the full-length sequences is measured by using Needleman-Wunsch global alignment program at EMBOSS (European Molecular Biology Open Software Suite; Rice et al., 2000) program package, version 3.0.0, with the following parameters: EMBL0SUM62, Gap penalty 10.0, Extend penalty 0.5. The algorithm is described in Needleman and Wunsch (1970) Journal of Molecular Biology 48, 443-453.

The terms "protein", "peptide" and "polypeptide" are to be rendered interchangeable. A polypeptide or enzyme with endo-polygalacturonase, exo-polygalacturonase, exo-rhamnogalacturonase or xylogalacturonase activity denotes an enzyme having said activity according to established assays in the art. The invention also includes variants of the claimed enzymes as long as they retain their original activity. A variant according to the present invention includes variants

of polypeptides that are derived by deletion or addition of one or more amino acid(s) to the N-terminal and/or C-terminal end of the native protein; deletion or addition of one or more amino acid(s) to one or more sites in the native protein; or substitution of one or more amino acid(s) to one or more sites in the enzyme. The production of such variants is generally well known to persons skilled in the art. Variants of amino acid sequences of polypeptides can, for example, be produced by mutations in the DNA. Methods of mutagenesis and changes in the nucleotide sequence are well known to persons skilled in the art (cf., for example, Kunkel, Proc. Natl. Acad. Sci. USA, 82:488 (1985), Kunkel et al., Methods in Enzymol., 154:367 (1987), US Patent No. 4,873,192, Walker and Gaastra, eds., Techniques in Molecular Biology, Mac Millan Publishing Company, New York (1983)). References on appropriate substitutions of amino acids, which do not negatively influence the biological activity of the protein of note, can be found in the model from Dayhoff et al., Atlas of Protein Sequence and Structure, Natl. Biomed. Res. Found., Washington, D.C. (1978). Conservative substitutions are preferred, such as exchanging one amino acid by another one with similar properties.

This kind of amino acids, which are interchangeable within a group, are listed in the following Table but not limited to it.

aliphatic	non-polar	G A P M
		I L V F W
	polar and uncharged	C S T N Q Y
	polar and charged	D E
		K R H
aromatic		H F W Y

The invention also relates to isolated or essentially purified nucleic acid preparations (compositions) or protein preparations (compositions). In this respect an isolated and purified polynucleotide/polypeptide or its segment refers to a polynucleotide or polypeptide or its segment that occurs isolated from its natural environment. An isolated segment of a polynucleic acid or polypeptide may occur in a

purified form or may occur in a non-native environment, such as in a transgenic host cell.

The present invention also relates to expression cassettes, which can be used to
5 introduce an open reading frame, which encodes a pectinolytic enzyme according to the invention, into a host cell. They preferably include a promoter with a transcription start region, which is linked to the open reading frame of the desired DNA sequence. Such an expression cassette may include a variety of restriction cleavage sites for the insertion of the open reading frame and/or other DNAs, e.g. a
10 transcription regulator region and/or selectable marker genes. In the 5'→3' direction of the transcription, the expression cassette includes a promoter with a transcription and translation start region, the DNA desired sequence and a translation and transcription termination regions. The expression cassette of such is functional in a microbial cell. The termination region may be native to the promoter or
15 the DNA in question or may be derived from any different source.

The term "open reading frame" (ORF) refers to the amino acid sequence that is coded between the translation start and stop codons of an encoding sequence. The terms "start codon" and "stop codon" refer to a unit of three contiguous nucleotides (codons) in a coding sequence, which specify the chain start and chain
20 stop of the protein synthesis (mRNA translation).

In connection with a nucleic acid "functional linkage" refers to a compound as a part of the same nucleic acid molecule in an appropriate position and with an appropriate orientation to the transcription start of the molecule. DNA functionally
25 linked to a promoter is under the transcription initiation regulation of the promoter. Coding sequences may be functionally linked to a regulator sequence in sense orientation or antisense orientation. With reference to polypeptides "functional linkage" refers to the connection as a part of the same polypeptide, i.e. by means
30 of peptidyl bonds.

According to the present invention any promoter may be used. Usually, promoter refers to the upstream of the nucleotide sequence in regard to the coding sequence and controls the expression of the coding sequence by recognition of the RNA polymerase and other factors that are necessary for a correct transcription.

5 The promoter used according to the present invention may include a minimal promoter, i.e. a short DNA sequence from a TATA box and other sequences that specify the transcription start site to which regulator elements are bound for the expression.

10 The promoter according to the present invention may also include a nucleotide sequence that comprises a minimal promoter and regulator elements; this minimal promoter may check the expression of a coding sequence or functional RNA.

The invention also relates to vectors including the DNA according to the present
15 invention. These vectors comprise any plasmid, cosmid, phage and other vector in a double-stranded or single-stranded, linear or circular form; these vectors themselves might be transmitted or mobilised and can transform a prokaryotic or eukaryotic host via integration into the cellular genome or they occur extrachromosomally (e.g. autonomously replicating plasmids with a replication origin).

20

The construction of vectors that can be used according to the present invention is known to the skilled person due to the aforementioned disclosure (cf., e.g., Sambrook et al., Molecular Cloning: A Laboratory manual (2nd edition, Coldspring Harbor Laboratory Press, Plainview, N.Y. (1989)). The expression cassette according
25 to the present invention may include one or more restriction enzyme cleavage site(s) to inserting the nucleotide sequence, which encodes a pectinolytic enzyme, under the regulation of a regulator sequence. The expression cassette may also include a termination signal functionally linked to the polynucleotide as well as regulator sequences, which are necessary for the proper translation of the polynu-
30 cleotide.

Selecting an appropriate expression vector depends on the host cells. Expression vectors of yeast or fungi may include a replication origin, an appropriate promoter and enhancer as well as any necessary ribosome binding site, polyadenylation site, splice donor and acceptor site, transcription termination sequence and non-transcribed 5'-flanking sequences.

Examples of appropriate host cells are: fungal cells of the genus *Aspergillus*, *Rhizopus*, *Trichoderma*, *Hypocrea*, *Neurospora*, *Mucor*, *Penicillium*, *Chrysosporium*, *Myceliophthora*, *Fusarium* etc., such as yeasts of the genera *Kluyveromyces*, *Saccharomyces*, *Schizosaccharomyces*, *Trichosporon*, *Schwanniomyces*, *Hansenula*, *Pichia* and others of this category. Appropriate host systems are, for example, fungi like *Aspergilli*, e.g. *Aspergillus niger* (ATCC 9142) or *Aspergillus ficuum* (NRL 3135) or *Trichoderma* (e.g. *Trichoderma reesei* QM6a and derivatives thereof) and yeasts like *Saccharomyces*, e.g. *Saccharomyces cerevisiae* or *Pichia*, such as *Pichia pastoris* or *Hansenula*, e.g. *H. polymorpha* (DSMZ 70277). Such micro-organisms can be obtained from recognised depositories, e.g. American Type Culture Collection (ATCC), *Centraalbureau voor Schimmelcultures* (CBS) [Central Office for Mildew Cultures] or *Deutsche Sammlung für Mikroorganismen und Zellkulturen GmbH* (DSMZ) [German Collection of Micro-Organisms and Cell Cultures] or any other depository.

Additionally to the use of a special promoter, other types of elements can influence the expression of cloned genes. It was shown in particular that introns have the potential for enhancing the gene expression.

The expression cassette may also include further elements, such as elements that can be regulated by endogenous or exogenous elements like zinc finger proteins, including naturally occurring zinc finger proteins or chimeric zinc finger proteins.

The expression cassette used according to the present invention can also include enhancer elements or upstream promoter elements.

Vectors used according to the present invention can be constructed in such a way that they include an enhancer element. Thus, the constructs according to the present invention include the gene of interest together with a 3'-DNA sequence, which acts as a signal to terminate the transcription and to allow for the polyadenylation of the thus obtained mRNA. Any signal sequence that allows secretion from the selected host organism possible can be used. The most preferred signal sequences for the secretion from filamentous fungi are the glucoamylase (glaA) or phytase signal sequence from *Aspergillus niger*, the TKA-amylase signal sequence from *A. oryzae*, and the cellobiohydrolase I signal sequence from *T. reesei*, or signal sequences derived from these. Alternatively, the signal sequence of the desired protein could be used.

It is also possible to use a special leader sequence, since the DNA sequence between the transcription start site and the start of the encoding sequence, i.e. the non-translated leader sequence, may influence the gene expression. Preferred leader sequences include sequences that control the optimal expression of the attached gene, i.e. they have a preferred consensus leader sequence that increases or preserves the mRNA stability and avoids an inappropriate translation initiation. The choice of such sequences is well known to the person skilled in the art.

As soon as the expression cassette or DNA sequence according to the present invention is obtained, it can be inserted into vectors by means of known methods to overexpress the encoded polypeptide in appropriate host systems. However, DNA sequences themselves may also be used to transform appropriate host systems of the present invention to attain an overexpression of the encoded polypeptide.

As soon as a DNA sequence according to the present invention is expressed in an appropriate host cell in a suitable medium, the encoded enzyme can be concentrated and/or isolated by known methods either from the medium if the enzyme is secreted into the medium or from the host organism if the enzyme occurs intracel-

lularly, or in periplasmatic space. Known methods for separating the biomass and solids of the culture medium followed by methods for concentrating the enzyme can be used for the production of concentrated enzymatic solutions or as preparation for the dehydration of the enzyme.

5

The invention also relates to preparations that include the polypeptide according to the invention. In general these preparations are liquid or dry. Liquid preparations preferably include the enzyme in a purified or enriched form. However, adjuvants such as a stabiliser with glycerol, sorbitol or propylene glycol, borate, additives such as salts, sugar, preservatives, means for adjusting the pH value, etc. can be added. Typical liquid preparations are aqueous or oily suspensions. As used in the present context, the "enzyme preparation" refers to any enzyme product which contains at least one pectinolytic enzyme of the invention. Thus, such an enzyme preparation may be a spent culture medium or filtrate. Spent culture medium means the culture medium of the host comprising the produced enzymes. Preferably, the host cells are separated from said medium after the production. If desired, such preparations may be spray-dried, granulated or lyophilized or the the preparations may be otherwise concentrated and/or stabilized for storage. If required, a desired enzyme may be further purified in accordance with conventional methods, such as extraction, precipitation, chromatography, electrophoresis, or the like.

20

However, it is an advantage of the invention that the culture medium with or without host cells may be utilized as an enzyme preparation as such without further purification, because the pectinolytic enzyme of the invention can be secreted into the culture medium and displays activity in the ambient conditions of the spent culture medium. Such enzyme preparations are very economical to provide and use, because isolation of a specific enzyme from the culture medium is unnecessary.

25

30

In addition to the pectinolytic enzyme, the enzyme preparations may comprise one or more other enzymes, which may be, for example, other cellulases, amylases,

lipases, proteases, hemicellulases, xylanases, pectinases and/or oxidases such as laccases and peroxidases.

In addition to the pectinolytic enzyme, the enzyme preparation may contain additives such as stabilizers, buffers, preservatives, surfactants and/or culture medium components. Preferred additives are such which are commonly used in enzyme preparations intended for the application where the enzyme preparation is used.

Dry preparations can include freeze-dried, spray-dried, instantized, granulated or extruded preparations, which can solely comprise the enzyme, or have additives like starch, dextrin, sugar, flour, protein or oil.

The enclosed Figures are to illustrate the invention in more detail:

Figure 1. Schematic picture of the expression cassettes used in the transformation of *Trichoderma reesei* protoplasts for overproducing the pectinase proteins. The pectinase genes were under the control of *T. reesei cbh1* (*cel7A*) promoter (*p cbh1*) and the termination of the transcription was ensured by using *T. reesei cbh1* terminator sequence (*t cbh1*). Either the *amdS* gene or *pyr4* gene was included as a transformation selection marker.

Figure 2 A-C) pH dependencies of the state-of-the-art *Aspergillus* PG1 (2A), *Aspergillus* PG2 (2B) and the overproduced crude *Trichoderma* PGA1 preparation of the invention (2C) determined at various pH-values (40 °C, 60 min).

Figure 2 D-F) Temperature dependency of the state-of-the-art *Aspergillus* PG1 (2D), *Aspergillus* PG2 (2E) and the overproduced crude *Trichoderma* PGA1 preparation of the invention (2F) determined at various temperatures (2D and 2E pH 4.5, 2F pH 5.0, 60 min).

Figure 3. SDS-PAGE analysis of the *Trichoderma reesei* PGA1 protein. MW: molecular weight marker, lane 1: culture supernatant of transformant overproducing

Trichoderma PGA1 as described in Example 3. Protein bands were visualised by staining with Coomassie Brilliant Blue. The size of the *Trichoderma* PGA1 is about 38 kDa.

5 **Figure 4.** A) Juice yield after pressing the enzyme treated apple mash preparations. Dosage of 100 ppm of a mixture containing 50 000 PG units/mg of either the *Trichoderma* PGA1s (F050183 and F050200) or the state-of-the-art *Aspergillus* PG1 (REFERENCE), all supplied with 2000 PE units/g of *A. niger* pectin methyl esterase, was used in the experiment. In one of the mash trials no enzyme was
10 added (BLANK). Enzyme incubation time was 60 min at 25 °C.

B) Press diagram showing the juice yield after each pressure step.

Figure 5. Turbidity (measured as NTU) of the juice after enzyme treatment and
15 pressing. Dosage of 100 ppm of a mixture containing 50 000 PG units/mg of either the *Trichoderma* PGA1s (F050183 and F050200) or the state-of-the-art *Aspergillus* PG1 (REFERENCE), all supplied with 2000 PE units/g of *A. niger* pectin methyl esterase, was used in the experiment. In one of the mash trials no enzyme was added (BLANK). Enzyme incubation time was 60 min at 25 °C.

20

Figure 6. A photo of the sample juices after the enzyme treatment and pressing. Samples from left to right: Blank (no enzyme), F050183, F050200 and state of art *Aspergillus* PG1 as a reference.

25 **Figure 7.** Yield (%) of the juice obtained from pressings of mashes of different fruits/vegetables after treatment with *Trichoderma reesei* PGA1.

The *E. coli* strain including the plasmid pALK1958 (RF 6249) was deposited at the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ),
30 Mascheroder Weg 1 b, D-38124 Braunschweig, Germany on 19 July 2006 and assigned accession number DSM18450. The pALK1958 carries the *Trichoderma*

pga1 gene (Table 2) on a 1690 bp *Sac*II-*Xho*I fragment (including 305 bp of the gene 3'-region) cloned into similarly cut pBluescript II SK+ -vector.

5 The following non-limiting Examples are intended to illustrate the subject-matter of the present invention in detail.

EXAMPLE 1: Genome-Wide Screening of *T. reesei* Pectinolytic Enzymes

10 Standard molecular biology methods were used in the isolation and enzyme treatments of DNA (plasmids, DNA fragments), in *E. coli* transformations, etc. The basic methods used are described in the standard molecular biology handbooks, e.g. Sambrook et al. (1989). Molecular cloning, a laboratory manual. Cold Spring Harbor Laboratory, New York, USA and Sambrook and Russell (2001). Molecular cloning, a laboratory manual. Cold Spring Harbor Laboratory, New York,
15 USA.

The *Trichoderma reesei* (the anamorph of *Hypocrea jecorina*) genome database (<http://gsphere.lanl.gov/trire1/trire1.home.html>) was searched with the sequences of various *Aspergillus* pectinases (Table 1) by using the TBLASTN program (Altschul et al., 1990. Basic local alignment search tool. J. Mol. Biol. 215:403-410).
20

Only search with *A. niger* endo-polygalacturonases, *A. tubingensis* exo-polygalacturonases, *A. niger* putative exo-rhamnogalacturonases, and *A. tubingensis* endo-xylogalacturonase produced hits with significant similarity (lower than
25 e-20 over at least 80 % of its length), resulting in the identification of four different open reading frames (Table 2). The complete coding regions of these sequences were obtained from <http://gsphere.lanl.gov/trire1/trire1.home.html>.

Table 1. *Aspergillus* Pectinase Gene Sequences Used in the Mining of the *T. reesei* Genome Database.

Enzyme name	Genes	Accession No.
<i>A. niger</i> endo-polygalacturonases	<i>pgaA</i>	CAB72125
	<i>pgaB</i>	CAB72126
	<i>pgaC</i>	CAA45707
	<i>pgaD</i>	CAB72931
	<i>pgaE</i>	CAA74744
	<i>pgal</i>	CAA41693
	<i>pgall</i>	CAA41694
<i>A. niger</i> pectin lyases	<i>pelA</i>	CAA43130
	<i>pelB</i>	CAA46521
	<i>pelC</i>	AAW03313
	<i>pelD</i>	AAA32701
<i>A. niger</i> pectate lyase	<i>plyA</i>	CAC33162
<i>A. tubingensis</i> exo-polygalacturonase	<i>pgaX</i>	CAA68128
<i>A. tubingensis</i> (endo)-xylogalacturonase	<i>xghA</i>	CAC07733
<i>A. niger</i> rhamnogalacturonases	<i>rhgA</i>	CAA63911
	<i>rhgB</i>	CAA63912
<i>A. niger</i> exo-rhamnogalacturonases	<i>rgxA</i>	ABD61566
	<i>rgxB</i>	ABD61567
	<i>rgxC</i>	ABD61568
<i>A. aculeatus</i> rhamnogalacturonan lyase	<i>rhgB</i>	1NKG_A
<i>A. aculeatus</i> pectin methyl-esterase	<i>pme1</i>	AAB42153
<i>A. oryzae</i> pectin methyl-esterase	<i>pmeA</i>	BAA75474
<i>A. tubingensis</i> pectin methyl-esterase	<i>pmeA</i>	P17872
<i>A. aculeatus</i> rhamnogalacturonan acetyl-esterase	<i>rha1</i>	CAA61858
<i>A. niger</i> rhamnogalacturonan acetyl-esterase	<i>rgaeA</i>	CAC41360

Table 2. Putative pectinase encoding genes identified from the *T. reesei* genome database.

Gene	Gene model / ProtID	Scaffold: Region (bp)	Identity (%)
<i>pga1</i>	fgenes5_pg.C_ scaffold_1000682 / 103049	1 : 2495262-2496642	<i>A. fumigatus</i> polygalacturonase EAL91052; 76% identity <i>A. nidulans</i> endo-polygalacturonase ABF50893; 74% identity
<i>pgx1</i>	fgenes5_pg.C_ scaffold_33000038 / 112140	33 : 88035-89368	<i>F. oxysporium</i> exo-polygalacturonase BAE97149; 56% identity <i>A. nidulans</i> exo-polygalacturonase ABF50895; 54% identity
<i>rgx1</i>	estExt_fgenes5_ pg.C_150014 / 122780	15 : 45898-47405	<i>A. niger</i> exo-rhamnogalacturonase ABD61567; 42% identity <i>A. fumigatus</i> exo-polygalacturonase EAL86831; 40% identity
<i>xga1</i>	e_gw1.33.41.1 / 70186	33 : 90062-91279	unnamed protein from <i>A. oryzae</i> BAE61127; 54% identity <i>A. fumigatus</i> exopolygalacturonase XP_747488; 53% identity

5 Analysis of the derived protein sequences with InterProScan
(<http://www.ebi.ac.uk/InterProScan/>, Apweiler *et al.*, 2000 Bioinformatics
16(12):1145-50) identified them as members of glycoside hydrolase family 28
(GH28; InterPro acc. no. PF00295). The putative pectinase encoding sequences
from *T. reesei* all showed closest homology to enzymes from other fungi involved
10 in pectin degradation (BLASTP search, Altschul *et al.*, 1990. Basic local alignment
search tool. J. Mol. Biol. 215: 403-410, Table 2) and were consequently named
pga1 (endo-polygalacturonase), *pgx1* (exo-polygalacturonase), *xga1* (xylogalactu-
ronase) and *rgx1* (exo-rhamnogalacturonase). The identification was further veri-
fied by phylogenetic approach (Mega 3.1, Kumar *et al.*, 2004 MEGA3: Integrated

software for molecular evolutionary genetics analysis and sequence alignment. Briefings in Bioinformatics. 5:150-163). Three “not-endo-polygalacturonase” sections of the phylogenetic tree thereby formed four branches. PGX1 is found in a branch that also contains the already characterized exo-polygalacturonases from *A. niger*, *A. tubingensis* and *Cochliobolus carbonum*. XGA1 is the most similar to a small branch, in which an *A. tubingensis* xylogalacturonan hydrolase is the only characterized enzyme. XGA1 shares less similarity with the other sequences in that branch than these show towards each other, and it is therefore possible that the *T. reesei* enzyme has developed some unique characteristics. The same applies to RGX1, which shows the highest degree of sequence identity to putative exo-rhamnogalacturonases. In the corresponding branch only an enzyme from *A. niger* has been tested with regard to its functionality without determining the exact reaction mechanism (Martens-Uzunova, E. S., Zandleven, J. S., Benen, J. A., Awad, H., Kools, H. J., Beldman, G., Voragen, A. G., Van den Berg, J. A. & Schaap, P. J. (2006) A new group of exo-acting family 28 glycoside hydrolases of *Aspergillus niger* that are involved in pectin degradation, Biochem J. 400, 43-52).

EXAMPLE 2: Cloning of the identified *T. reesei* pectinase genes

The *pga1*, *pgx1*, *rgx1* and *xga1* genes were amplified from *T. reesei* genomic DNA using the GoTaq[®] system (Promega, USA) with 2 mM MgCl₂ and 0.4 μM of sequence specific primers presented in Table 3. The conditions for the PCR reaction were the following: 2 min initial denaturation step at 95 °C, followed by 28 cycles of 1 min at 95 °C, 45 s annealing at the primer specific temperature (Table 3_TP), 2 min extension at 72 °C and a final elongation at 72 °C for 5 min. The DNA fragments of the expected sizes were isolated, and were then cloned to pBlueScript II SK+ vector (Stratagene, USA). The inserts were characterized by sequencing.

Table 3. The Primers Used to Amplify the *T. reesei* Pectinase Genes. The genomic DNA of *T. reesei* QM9414 was used as a template in the PCR reactions. The name of the plasmid containing the amplified gene fragment is shown.

Gene	Primer name	5' -> 3' sequence	T _m ^(a) [°C]	Plasmid
<i>pga1</i>	Forward: C22000155for SEQ ID NO: 9	GATCCCGCGGCAACATGCTCAA GCTATCAC	50	pALK1958
	Reverse: C22000155rev SEQ ID NO: 10	GATCCTCGAGCATTCTTCACGG CATTCTAC		
<i>pgx1</i>	Forward: C42000032fw SEQ ID NO: 11	CAGTCCGCGGCTAAGCAAAGG AGCACG	49	pALK1961
	Reverse: C42000032rv SEQ ID NO: 12	CGTAGGATCCGTTAGTAGAGTTT CATTGCATC		
<i>xga1</i>	Forward: C42000033fw SEQ ID NO: 13	GACTCCGCGGCGACTTCCATCA TGCTCCTTG	51	pALK1964
	Reverse: C42000033rv SEQ ID NO: 14	GATCACCGCGGATGCTTTATG		
<i>rgx1</i>	Forward: C12000223fw SEQ ID NO: 15	GTACCCGCGGTCGACAGAATG GTGGCGCTATC	58	pALK1970 ^(b) /pALK1971
	Reverse: C12000223rv SEQ ID NO: 16	GTCAGGATCCAGAGCGGTATCA AGCAGTATC		

^(a) Annealing temperature used to amplify the *T. reesei* pectinase gene.

^(b) The encoding region of the full-length *rgx1* gene consisted of two plasmids.

The relevant information on the pectinase genes and the deduced protein sequences are summarized in Table 4 and Table 5, respectively.

5

Table 4. Summary of the *T. reesei* Pectinase Genes.

Pecti-nase gene	Length with introns (bp)^(a)	Coding region (bp)^(b)	No of in-trons	Lengths of introns (bp)
<i>pga1</i>	1381	1137	4	64, 59, 59, 59
<i>pgx1</i>	1421	1311	2	50, 57
<i>xga1</i>	1218	1215	0	
<i>rgx1</i>	1374	1371	0	

^(a) The STOP codon is included.

10 ^(b) The STOP codon is not included.

Table 5. Summary of the deduced *T. reesei* pectinase sequences.

CBH pro-tein	No of aas	Length of ss NN/HMM^(a)	Predicted MW (Da, ss not incl)^(b)	Predicted pI (ss not incl)	Putative glycosylation sites^(c)	N-si-
PGAI	379	21/21	36 187	5.51	3	
PGXI	437	22/22	45 559	5.51	12	
XGAI	405	18/18	40 023	7.10	8	
RGXI	457	17/21	48 700 ^(d) /48 340	4.79	7	

15 ^(a) The prediction on the signal sequence (ss) was made using the program SignalP V3.0 (Nielsen *et al.*, 1997. Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. Protein Engineering 10:1-6;

Bendtsen *et al.*, 2004. Improved prediction of signal peptides: SignalP 3.0. J. Mol. Biol. 340:783-795); the NN value was obtained using neural networks and HMM value using hidden Markov models.

5 ^(b) The predicted signal sequence was not included. The prediction was made using the Compute pI/MW tool at ExPASy server (Gasteiger *et al.*, 2003. ExPASy: the proteomics server for in-depth protein knowledge and analysis. Nucleic Acids Res. 31:3784-3788).

^(c) The number of sequences N-X-S/T.

10 ^(d) The values marked for RGXI are calculated after deleting two possible signal sequences.

The amino acid residues reported to be crucial for catalytic action of the *A. niger* endo-polygalacturonase II (van Santen *et al.*, 1999. 1.68-A crystal structure of endopolygalacturonase II from *Aspergillus niger* and identification of active site residues by site-directed mutagenesis. J. Biol. Chem. 274:30474-30480; Armand *et al.*, 2000. The active site topology of *Aspergillus niger* endopolygalacturonase II as studied by site-directed mutagenesis. J Biol Chem. 275:691-696) are identified also in *T. reesei* PGAI, PGXI and XGAI. This indicates similar catalytic properties of the *T. reesei* pectinase enzymes to those of *A. niger*. In addition to the active site signature typical for GH28 glycoside hydrolases, PGAI, PGXI and XGAI contain several PbH1 (parallel beta-helix repeats) domains, which are also found in several types of pectinolytic enzymes (Jenkins & Pickersgill, 2001. The architecture of parallel beta-helices and related folds. Prog Biophys Mol Biol. 77:111-175.). The findings further confirmed the pectinolytic features of the *T. reesei* genes indicated here.

EXAMPLE 3: Overexpression of the Pectinase Genes in *Trichoderma reesei*

30 Expression plasmids were constructed for overexpression of the *T. reesei* pectinase genes. The expression plasmids constructed are listed in Table 6. The

pga1, *pgx1*, *rgx1* and *xga1* genes, including their own signal sequences, were exactly fused to the *T. reesei cbh1* (*cel7A*) promoter. The transcription termination was ensured by the *T. reesei cel7A* terminator and the *A. nidulans amdS* marker gene was used for selection of the transformants as described in Paloheimo *et al.* (2003) High-yield production of a bacterial xylanase in the filamentous fungus *Trichoderma reesei* requires a carrier polypeptide with an intact domain structure. Appl. Env. Microbiol. 69:7073-7082). The linear expression cassettes (Fig. 1), were isolated from the vector backbones after *NofI* digestion and were transformed into *T. reesei* RF5455 protoplasts (the strain has the genes encoding the two major cellulases CBHI/*Cel7A* and EGII/*Cel5A* deleted).

The expression plasmid including endogenous *pyr4* marker gene was also constructed for the *pga1* gene by ligating 4.7-kb *XbaI-HindIII* genome fragment of *T. reesei pyr4* locus after the *cel7A* terminator in the plasmid. The linear expression cassette was isolated from the vector backbone after *NofI* digestion and was transformed into *T. reesei* RF5514 protoplasts (the strain has the genes encoding the two major cellulases CBHI/*Cel7A* and EGII/*Cel5A* deleted and strain is also pyrimidine auxotroph).

The transformations were performed as in Penttilä *et al.* (1987, A versatile transformation system for the cellulolytic filamentous fungus *Trichoderma reesei*. Gene 61:155-164) with the modifications described in Karhunen *et al.* (1993, High frequency one-step gene replacement in *Trichoderma reesei*. I. Endoglucanase I overproduction. Mol. Gen. Genet. 241:515-522), selecting either acetamide as a sole nitrogen source (*amdS* marker gene) or without uridine supplement (*pyr4* marker gene). The transformants were purified on selection plates through single conidia prior to sporulating them on PD.

Table 6. The expression cassettes constructed to overproduce pectinase proteins in *Trichoderma reesei*. The overall structure of the expression cassettes was as described in Fig. 1. The cloned *pga1*, *pgx1*, *rgx1* and *xga1* genes were exactly fused to the *T. reesei cbh1/cel7A* promoter.

<i>T. reesei</i> pecti- nase	Expression plasmid	Size of the expr. cas- sette ^(a)	<i>cbh1</i> termina- tor ^(b)
PGAI	pALK1967 (<i>amdS</i> ^(c)) pALK1960 (<i>pyr4</i> ^(c))	9.0 kb 10.0 kb	627 bp (AvalI)
PGXI	pALK1968	9.2 kb	627 bp (AvalI)
RGXI	pALK1974	8.8 kb	627 bp (AvalI)
XGAI	pALK1969	8.6 kb	627 bp (AvalI)

^(a) The expression cassette for *T. reesei* transformation was isolated from the vector backbone by using *NotI* digestion.

^(b) The number of the nucleotides from the genomic *cbh1* terminator region after the STOP codon. The restriction site at the 3'-end, used in excising the genomic gene fragment, is included in the parenthesis.

^(c) Two expression plasmids were constructed for *pga1* gene; the pALK1967 plasmid included *amdS* marker gene for transformant selection, and the pALK1960 included *pyr4* marker gene.

The pectinase production of the transformants was analysed from the culture supernatants of the shake flask cultivations (50 ml). The transformants were grown for 7 days in a complex lactose-based cellulase-inducing medium (Joutsjoki *et al.* 1993. Transformation of *Trichoderma reesei* with the *Hormoconis resinae* glucoamylase P (*gamP*) gene: production of a heterologous glucoamylase by *Trichoderma reesei*. Curr. Genet. 24:223-228) buffered with 5% KH₂PO₄. The polygalacturonase activity was assayed by a viscosimetric method using citrus pectin (Copenhagen pectin X-2955, Denmark) as the substrate, as described in

patent EP0388593. One polygacturonase unit (PGU) is defined as the quantity of enzyme which caused 15 nPas^{-1} reduction in viscosity under standard conditions. The genotypes of the chosen transformants were confirmed by using Southern blots in which several genomic digests were included and the respective expression cassette was used as a probe. Overexpression of the PGAI, PGXI, RGXI and XGAI proteins was analyzed by SDS-PAGE with subsequent Coomassie staining. The PGAI protein was noticeable overproduced in *T. reesei* (see Figure 3), whereas no PGU activity or visible protein overproduction in SDS-PAGE could be detected for the PGXI, RGXI and XGAI transformants that were, however, shown to contain integrated expression cassette. This suggests that very low amount of the PGXI, RGXI and XGAI proteins are produced in *T. reesei*.

The chosen PGAI transformants were cultivated in lab bioreactors at 28 °C in the medium indicated above for 3-4 days with pH control 4.4 ± 0.2 ($\text{NH}_3/\text{H}_3\text{PO}_4$) to obtain material for the application tests. The supernatants were recovered by centrifugation and filtering through Seitz-K 150 and EK filters (Pall SeitzSchenk Filter-systems GmbH, Bad Kreuznach, Germany). Two preparations were produced with identical enzyme profiles (PGA1+++, CBHI-, EGII-). F050183 was produced with RF5514 derived transformant and F050200 with RF5455 derived transformant; the former was selected with the *pyr4* marker and the latter with the *amdS* marker. Thus the former strain carries only homologous DNA.

EXAMPLE 4: Characterization of the *T. reesei* PGAI Enzyme

The crude *T. reesei* PGAI enzyme was characterized in terms of pH optimum and thermal stability.

The pH dependency of the overproduced *T. reesei* PGAI protein (sample F 050183) was determined within a pH range of 3.0-8.0 by preparing the sample buffer by mixing 0.1 M citric acid and 0.2 M Na_2HPO_4 (both supplemented with bovine serum albumin (BSA) 100 microgram/ml (Fluka, Cat.# 05470) to the desired pH. The activity was assayed at the desired pH with 60 min incubations. Fig.

2A-C shows the results. The *Aspergillus* enzymes have a pH optimum around 4.5, whereas the *Trichoderma* PGA1 has a slightly more neutral pH optimum at pH 5.0. The *Trichoderma* PGA1 still retains about 70% activity at pH 5.5, at which the *Aspergillus* PG1 has only 30% of the maximal activity left. *Aspergillus* PG2 loses its activity at pH 5.5.

The temperature dependency of the overproduced *Trichoderma* PGA1 protein (sample F050183) was determined at pH 5.0 within the range of 40 °C – 75 °C, and compared to the state-of-the-art *Aspergillus* PG1 and PG2 assayed at their optimal pH 4.5. Surprisingly, the *Trichoderma* PGA1 has a high temperature optimum at about 65 °C, and still about 60% of maximal activity at 70 °C, whereas the *Aspergillus* enzymes have virtually no activity left at 65°C (Fig. 2D-F), and have their optimum around 50 °C (about 15 °C lower than *Trichoderma* PGA1).

Colorimetric Method for PG Activity

For the pH dependency determinations, the assay was carried out at the desired pH of the substrate and the sample buffer at 40 °C for 60 min. For the temperature dependence determinations, the assay was carried out at pH 4.5 for the *Aspergillus* samples ('13 and '22) and pH 5.0 for the *T. reesei* PGA1 sample. Assays were carried out at the desired temperature for 60 min.

Substrate: 0.7 % (w/v) potassium pectate (Fluka, Cat# 51186). 0.7 g substrate was dissolved in 100 ml hot water. After cooling down to room temperature the pH value was adjusted with acetic acid or sodium hydroxide.

Enzyme solution: The enzymes were diluted in the sample buffer.

PAHBAH-Reagent:

Stock solution (5%) 50 g p-hydroxybenzhydrazide 98% (Fluka Cat # 54600) was dissolved in 1000 ml 0.5 M hydrochloride acid.

Working solution: 0.233 g Titriplex III was dissolved in 30 ml 0.5 M sodium hydroxide.

5 ml stock solution was added and filled up to 50 ml with 0.5 M NaOH

5 Assay volumes:

Substrate: 0.25 ml

Enzyme: 0.1 ml

PAHBAH: 0.65 ml

10 Temperature of colour incubation: 80°C

Duration of colour incubation: 15 min

Sample value:

Substrate was pipetted into a test tube. The reaction was started by adding the
15 enzyme solution. The batch was mixed and incubated at 40 °C for 60 min. After
the incubation the reaction was stopped by adding the PAHBAH reagent. For the
colour development the samples were incubated for 15 min at 80 °C. Thereafter
the samples were cooled down in an ice bath for about 5 min and centrifuged
(2 min, 13000 rpm). The supernatants were measured photometrically against the
20 blank sample at 412 nm.

Blank:

Substrate and PAHBAH reagent were mixed. After adding the enzyme solution the
samples were incubated for 60 min at 40 °C and then for 15 min at 80 °C for col-
25 our development. Cooling, centrifugation and photometric measurement were per-
formed as for the sample values.

EXAMPLE 5: Preparation of Apple Juice

30

The cell-free culture supernatants were tested in apple juice preparation. For this
purpose apples (cultivar Golden Delicious) were ground, and 500 g resulting apple

mash were used in the experiment. After enzyme addition the mash was incubated 60 min at room temperature (25 °C), and the mash was then pressed with a laboratory press (Hafico). The pressing routine was 2 minutes at 50, 100, 150 and 200 bar, followed by 1 minute at 300 and 400 bar, respectively.

5

Two *Trichoderma* PGA1 preparations F050183 (*T. reesei* RF5514 / pALK1960/#4) and F050200 (*T. reesei* RF5455 / pALK1967/#4) were compared to the state-of-the-art product containing *Aspergillus* PGI (reference sample). All three samples were supplemented with *Aspergillus* pectin methylesterase. The polygalacturonase activity was assayed by a viscosimetric method using citrus pectin (Copenhagen pectin X-2955, Denmark) as the substrate, as described above and in patent EP0388593. One polygalacturonase unit (PGU) is defined as the quantity of enzyme which caused 15 nPas⁻¹ reduction in viscosity under standard conditions. A dosage of 100 ppm of a mixture having 50 000 PG units/mg of either the *Trichoderma* PGA1s or the reference, supplied with 2000 PE units/g (patent EP0388593) was used in the experiment. In one of the mash trials no enzyme was added (blank sample).

Juice yield results (Fig. 4) show that the *Trichoderma* PGA1 is equal or better than the state-of-the-art pectinase preparation. In particular, the turbidity of the *Trichoderma* PGA1 treated samples was superior, i.e. *Trichoderma* PGA1 treated juices were much more clear and transparent as compared the state-of-the-art *Aspergillus* PG treated juice (Fig. 5). The result is clearly visible (Fig. 6).

Alcohol test for remaining pectin (1+1 volume juice and absolute ethanol) showed after 6 h incubation at 25 °C that the *Trichoderma* PGA1 treated juices contained no residual pectin, whereas the *Aspergillus* PG treated juice contained some residual pectin. In the blank sample considerable amounts of residual pectin were found.

30

Example 6: Test of *Trichoderma* PGA1 in the preparation of juice from different fruits and vegetables.

Trichoderma PGA1 preparation F050183 produced as described in Example 3
5 was tested in preparation of juice from fruits and vegetables without added pectin methyl esterase.

Strawberries and raspberries were mashed manually with a metal device. Plums
and carrots were mechanically crushed with a mincer. Carrots were blanched by
10 micro wave heating at 95 °C. 1000 g of mash was placed in a 2000 ml bottle and
temperature was adjusted for 20 min before adding enzyme solution. Reaction
temperature was 65 °C and reaction time 60 min. Mashing after enzyme reaction
was performed by pressing in a Hafico lab press using textile press clothes. The
received juice was collected in an Imhoff-flask for settling.

15

Enzyme and Dosage:

The dosage was based on general recommendation in the art: 200 ppm at 30.000
PGU/mg corresponds with $6 \cdot 10^6$ PGU/kg carrots, plums, strawberries and rasp-
berries. The activity of enzyme preparation FEA 2005027 was 10300 PGU/mg.
20 The dosage of the enzyme preparation FEA 2005027 per 1000 g carrots was,
thus, 0.58 g. The blank test was without an enzyme dosage.

The press diagram used was the following:

1 min filling - 2 min 0 bar - 2 min 50 bar - 2 min 100 bar - 2 min 150 bar - 2
25 min 200 bar - 1 min 300 bar - 1 min 400 bar.

The turbidity measurement (NTU) was carried out with a Dr Lange LTP5 labora-
tory turbidity photometer at 860 nm. The values are reported as NTU (Neo-
phelometric turbidity Units) on the basis of the DIN 38404 method. The results are
30 shown in the Table 7.

Table 7. Test results with juice preparation from raspberries, strawberries, plums and carrots.

	Juice yield [g]	Juice yield [%]	°Brix	Turbidity NTU	Turbidity 24h NTU	Sediment [%]
Raspberry blank test	795	79,5	9,4	58	58	1,0
Raspberry F050183	789	78,9	9,4	50	52	1,3
Strawberry blank test	803,9	80,39	6,3	170	79	12,2
Strawberry F050183	906	90,6	6,3	183	56	14,5
Plums blank test	543	54,3	15	157	142	20,0
Plums F050183	764	76,4	17,2	143	124	9,0
Carrots blank test	742	74,2	9,5	161	17,8	8,3
Carrots F050183	723	72,3	9,5	150	72	7,7

- 5 The above table 7 shows that treatment of strawberries and plums with *Trichoderma* PGA1 increased the juice yield and °Bx (sugar content) without pectin esterase and other pectinolytic activities.

10 The results are graphically presented in Figure 7. Figure 7 shows the yield of a juice obtained from pressing of mashes of different fruits/vegetables after treatment with *Trichoderma reesei* PGA1. The results are shown in comparison to the respective blank values.

15 Surprisingly, *Trichoderma* PGA1 gave superior result with fruits containing pectin that is low esterified and soluble. Based on the results presented above, it is possible to carry out the treatment of said fruit mash only with the *Trichoderma* PGA1 enzyme at 65 °C in one hour without any additional pectinases. This superior result could not have been expected.

CLAIMS

1. Use of one or more pectinolytic enzymes for the treatment of fruit or vegetable mash, wherein at least one of the pectinolytic enzymes is a polypeptide having pectinolytic activity and comprising an amino acid sequence selected from:
 - a) an amino acid sequence having at least 95% identity to the amino acid sequence of SEQ ID NO:2; and
 - b) a fragment of the amino acid sequence of SEQ ID NO:2, the fragment having pectinolytic activity.
2. The use according to claim 1, wherein the one or more pectinolytic enzymes is a polygalacturonase having the amino acid sequence of amino acids 22-379 of SEQ ID NO: 2.
3. The use according to claim 1 or 2, wherein the fruit or vegetable is selected from apples, pears, grapes, berries, carrots and tomatoes.
4. The use according to claim 1 or 2, wherein the fruit is apple.
5. The use according to claim 1, wherein the one or more pectinolytic enzymes is a polygalacturonase from *Trichoderma reesei* having the amino acid sequence of amino acids 22-379 of SEQ ID NO: 2, and the fruit is apple.
6. A process for enzymatic treatment of fruit or vegetable mash comprising:
 - 1) providing one or more pectinolytic enzymes, wherein each of the one or more pectinolytic enzymes is a polypeptide having pectinolytic activity and comprising an amino acid sequence selected from:

- a) an amino acid sequence having at least 95% identity to the amino acid sequence of SEQ ID NO:2; and
 - b) a fragment of the amino acid sequence of SEQ ID NO:2, the fragment having pectinolytic activity; and
- 2) adding the one or more pectinolytic enzymes to the fruit or vegetable mash.
7. The process according to claim 6, wherein the one or more pectinolytic enzymes is a polygalacturonase having the amino acid sequence of amino acids 22-379 of SEQ ID NO: 2.
8. The process according to claim 6 or 7, wherein the fruit or vegetable is selected from apples, pears, grapes, berries, carrots and tomatoes.
9. The process according to claim 6, wherein the fruit is apple.
10. The process according to claim 6, wherein the one or more pectinolytic enzymes is a polygalacturonase from *Trichoderma reesei* having the amino acid sequence of amino acids 22-379 of SEQ ID NO: 2 and the fruit is apple.
11. A process for the preparation of a fruit or vegetable juice comprising a process for the enzymatic treatment of fruit or vegetable mash according to any one of claims 6 to 10.
12. An isolated polypeptide having endo-polygalacturonase activity and being encoded by a recombinant DNA molecule, which upon expression in a prokaryotic or eukaryotic host cell encodes said polypeptide having endo-polygalacturonase activity, the recombinant DNA molecule comprising a DNA sequence selected from

- a) DNA sequences comprising SEQ ID NO: 1;
 - b) DNA sequences hybridizing to the complement of the DNA sequences of SEQ ID NO:1 under stringent conditions which comprise hybridization of the DNA sequences provided on filters at 65°C, 18 h in dextranulphate solution, washing of the filters for 30 minutes, first with 6x SSC, twice with 2x SSC, three times with 3x SSC, with 0.1% SDS and after that 0.2x SSC at 65°C; and
 - c) DNA sequences which are at least 95% identical to the sequences of SEQ ID NO:1.
13. A preparation comprising the polypeptide according to claim 12 and an excipient.
14. An isolated polypeptide having pectinolytic activity and comprising an amino acid sequence selected from:
- a) an amino acid sequence having at least 95% identity to the amino acid sequence of SEQ ID NO: 2; and
 - b) a fragment of the amino acid sequence of SEQ ID NO:2, the fragment having pectinolytic activity.
15. The isolated polypeptide according to claim 14, comprising an amino acid sequence having at least 95% identity to SEQ ID NO: 2.
16. The isolated polypeptide according to claim 14, comprising an amino acid sequence having at least 98% identity to SEQ ID NO: 2.
17. A transformed host cell selected from a fungal, a yeast, a bacterial and a mammalian cell comprising at least one recombinant DNA molecule, which upon

expression in said host cell encodes a polypeptide having endo-polygalacturonase activity, the recombinant DNA molecule comprising a DNA sequence selected from:

- a) DNA sequences comprising SEQ ID NO: 1;
- b) DNA sequences hybridizing to the complement of the DNA sequences of SEQ ID NO:1 under stringent conditions which comprise hybridization of the DNA sequences provided on filters at 65°C, 18 h in dextran sulphate solution, washing of the filters for 30 minutes, first with 6x SSC, twice with 2x SSC, three times with 3x SSC, with 0.1% SDS and after that 0.2x SSC at 65 °C; and
- c) DNA sequences which are at least 95% identical to the sequences of SEQ ID NO:1.

18. The transformed host cell according to claim 17 belonging to the category of Kluyveromyces, Pichia, Hansenula, Schizosaccharomyces, Aspergillus, Rhizopus, Trichoderma, Hypocrea, Myceliophthora, Chrysosporium, Neurospora, Mucor, Penicillium, Saccharomyces or Fusarium.

19. An isolated polypeptide having endo-polygalacturonase activity selected from the group consisting of:

- a) an isolated polypeptide which is encoded by a nucleic acid sequence comprising the nucleotide sequence of SEQ ID NO: 1;
- b) an isolated polypeptide which is encoded by a nucleic acid sequence having at least 95% identity to the nucleotide sequence of SEQ ID NO: 1;
- c) an isolated polypeptide which is encoded by a nucleic acid sequence having at least 98% identity to the nucleotide sequence of SEQ ID NO: 1; and
- d) an isolated polypeptide which is encoded by a nucleic acid sequence consisting of the nucleotide sequence of SEQ ID NO: 1.

20. An isolated polypeptide having endo-polygalacturonase activity selected from the group consisting of:

a) an isolated polypeptide comprising an amino acid sequence having at least 95% identity to SEQ ID NO: 2; and

b) an isolated polypeptide comprising an amino acid sequence having at least 98% identity to SEQ ID NO: 2.

1/8

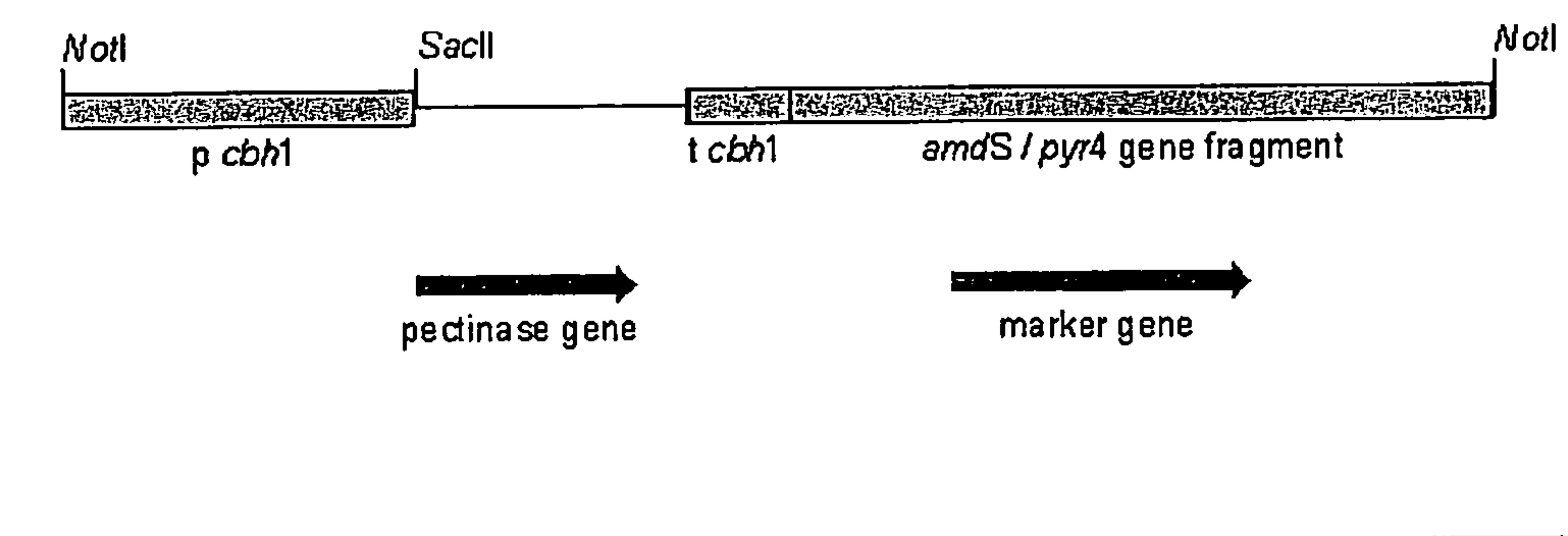
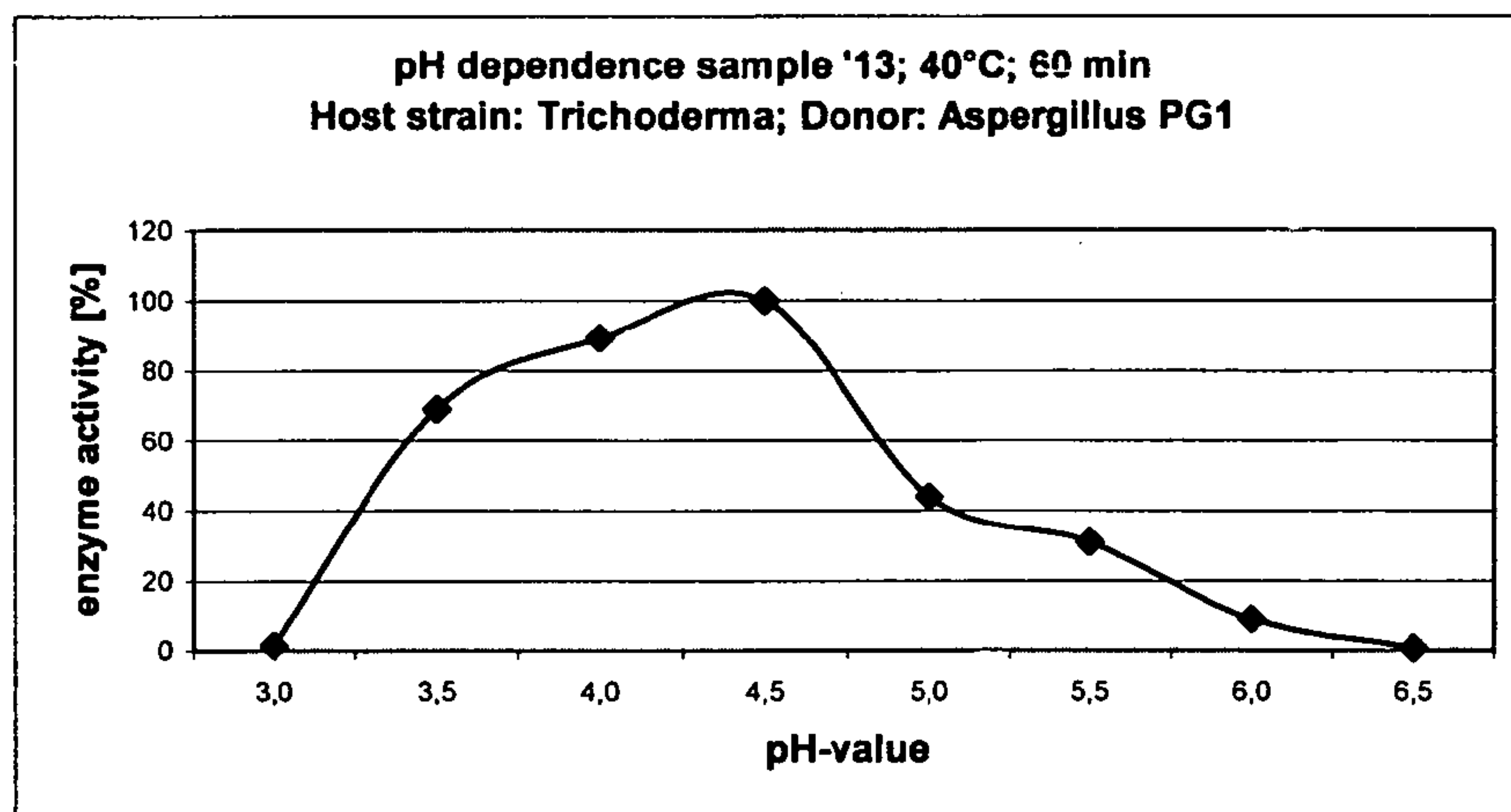


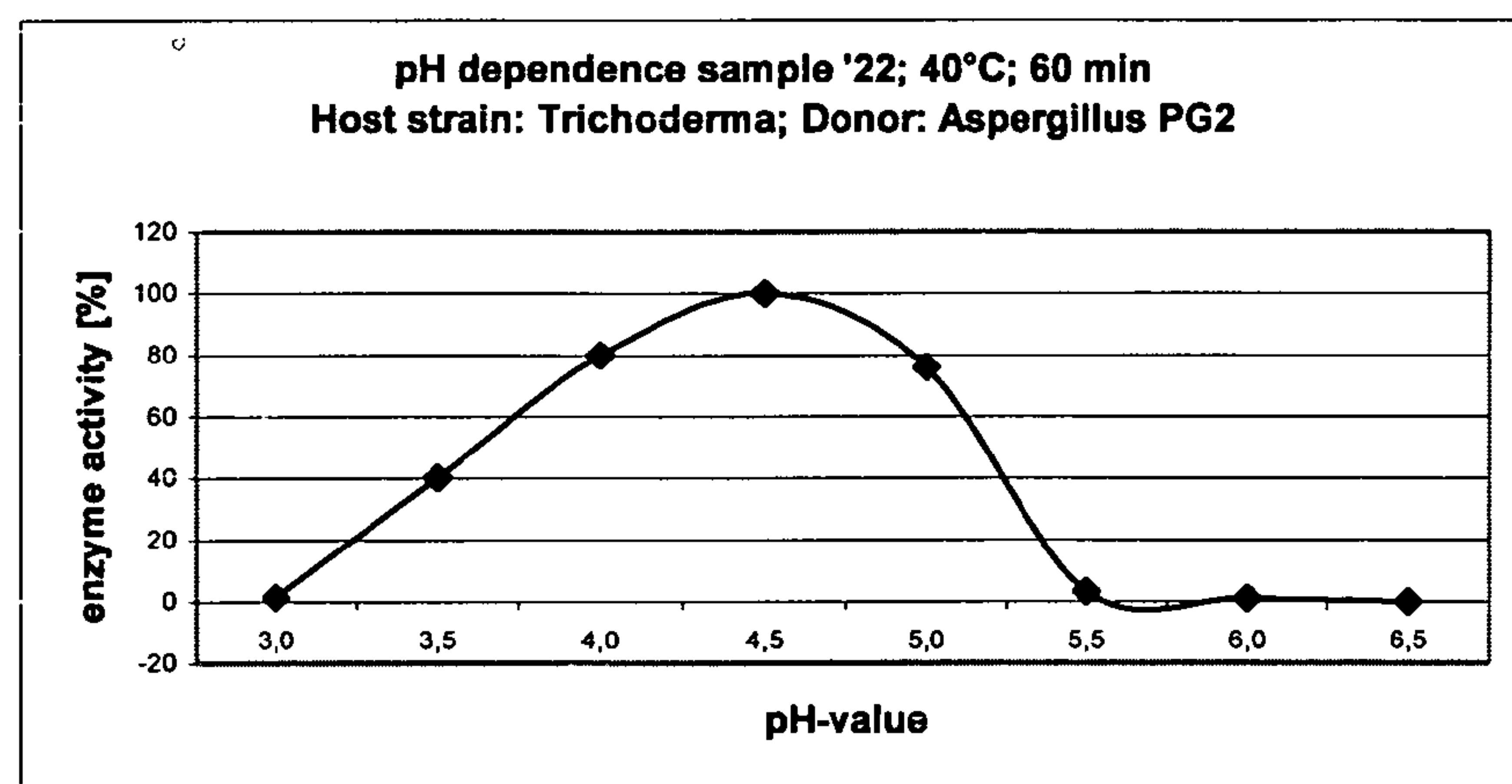
Figure 1

2/8

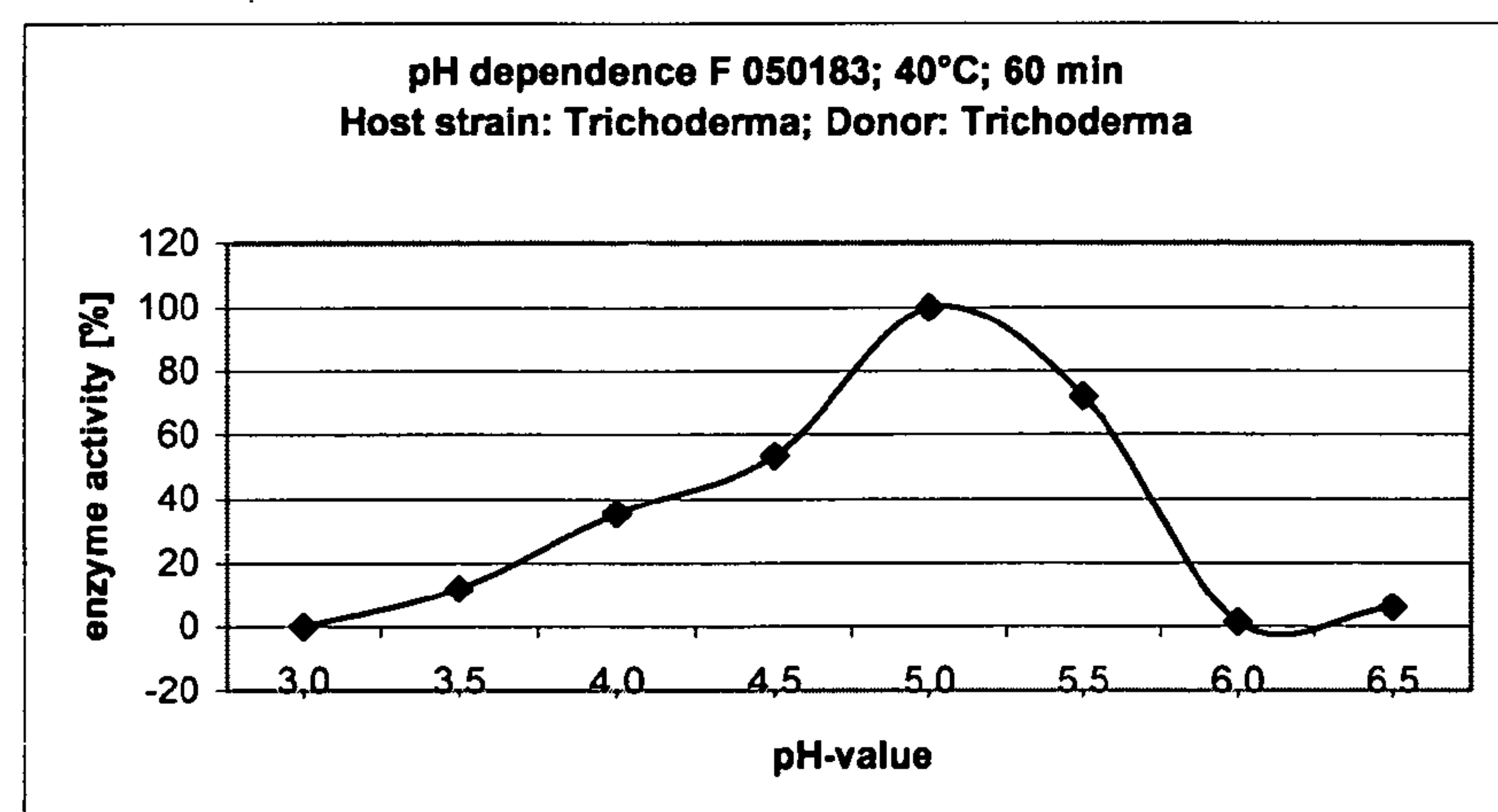
2A



2B



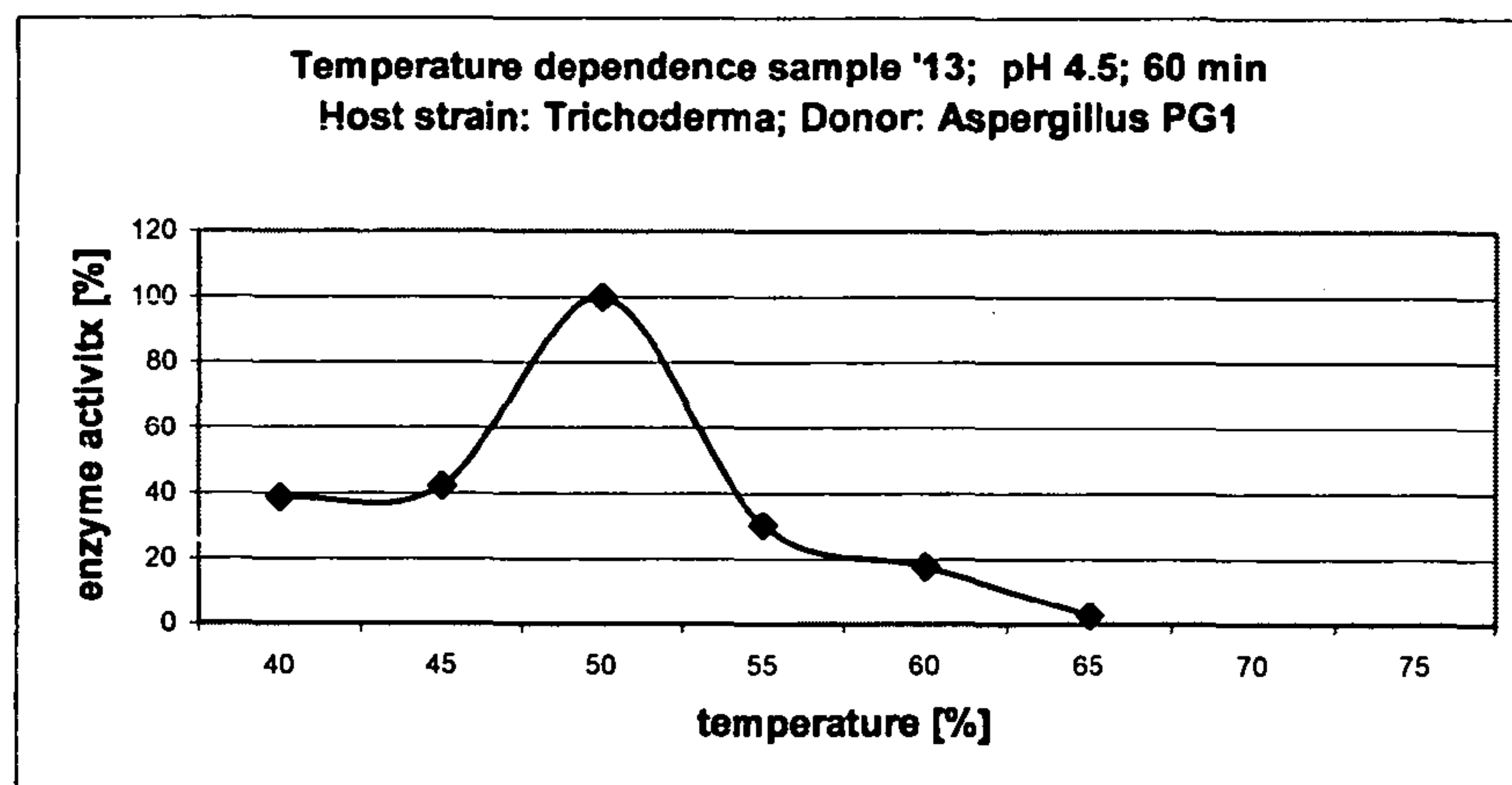
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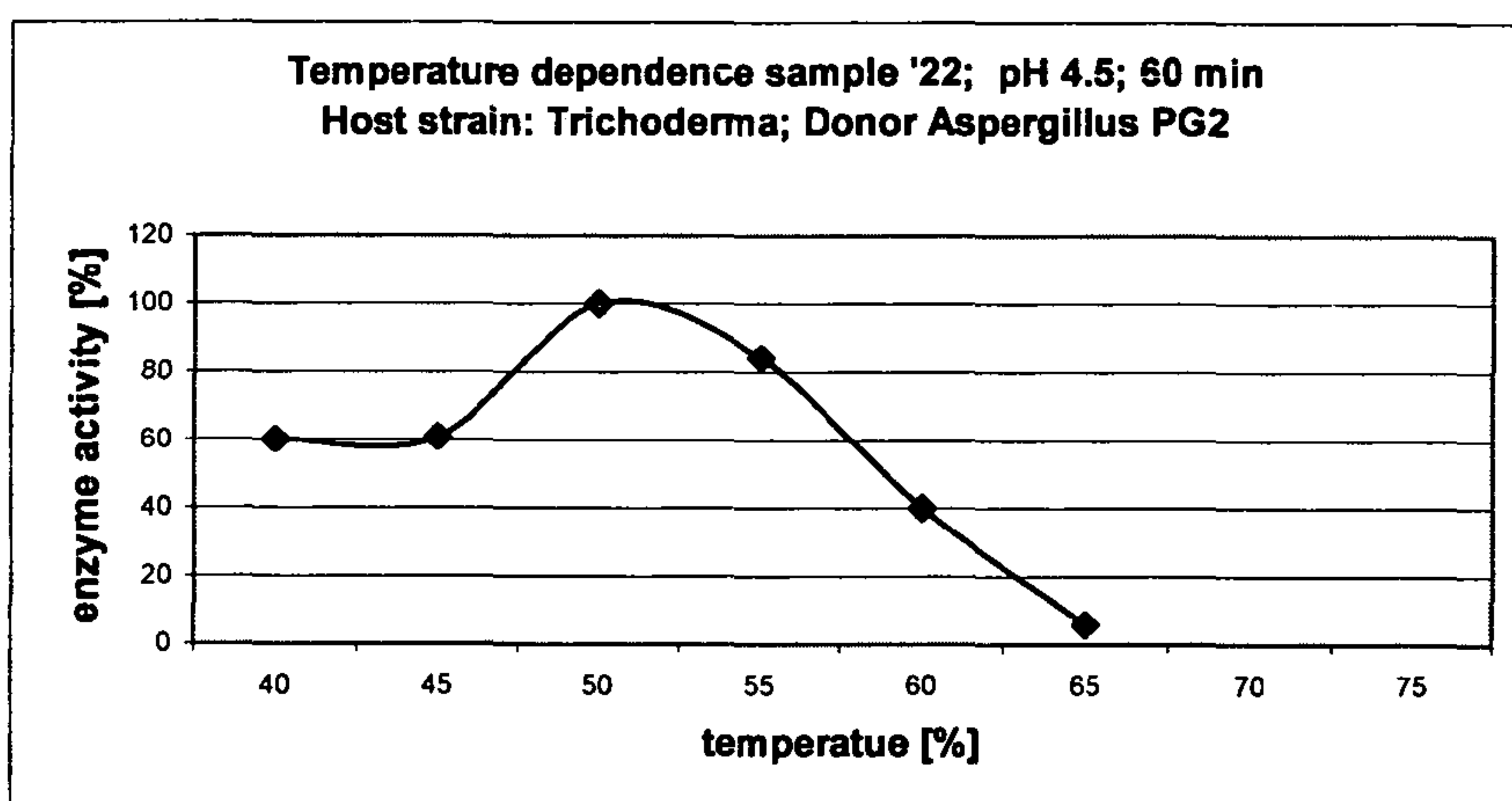
Figures 2A - C

3/8

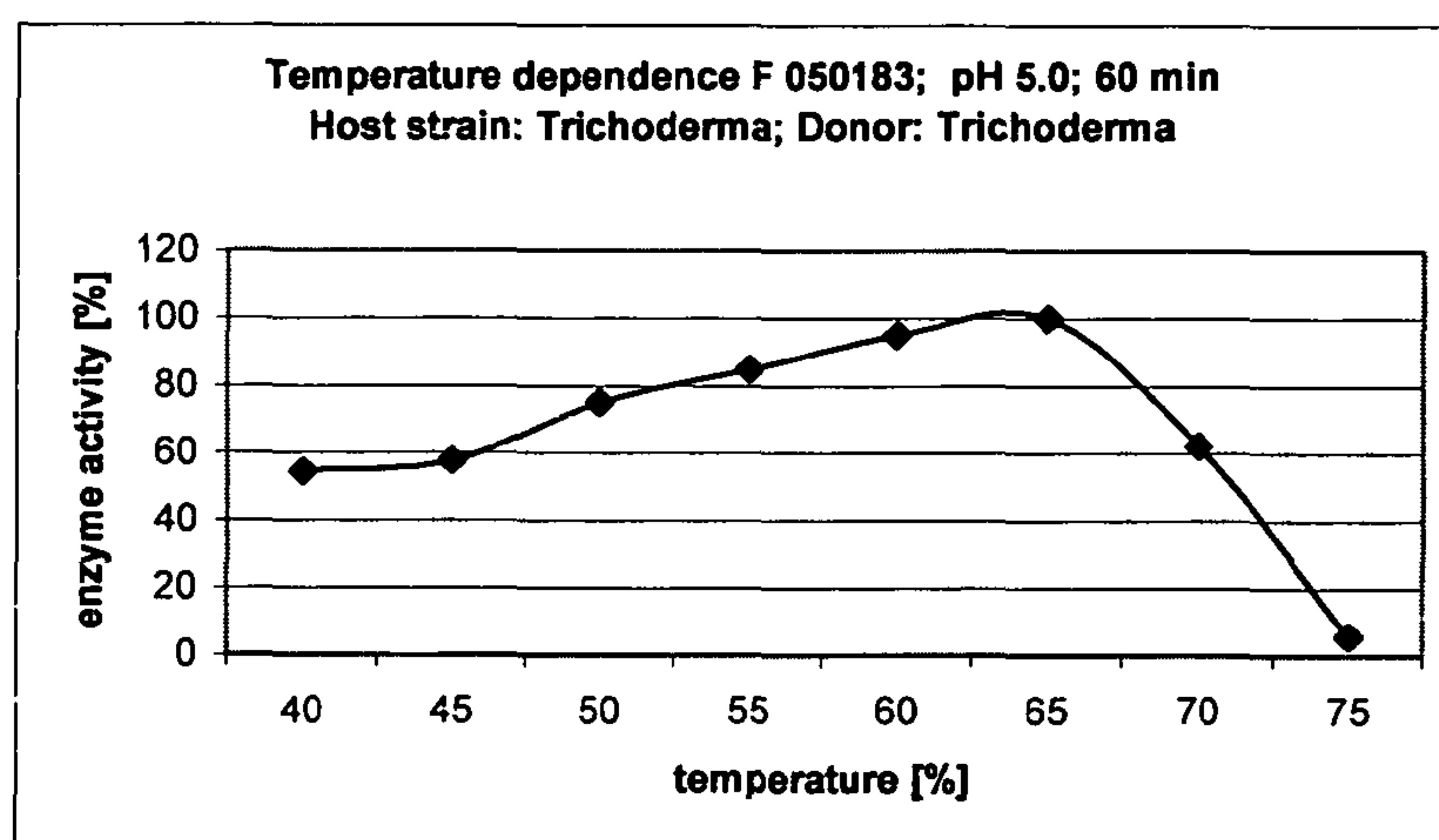
2D



2E



2F



Figures 2D - F

4/8

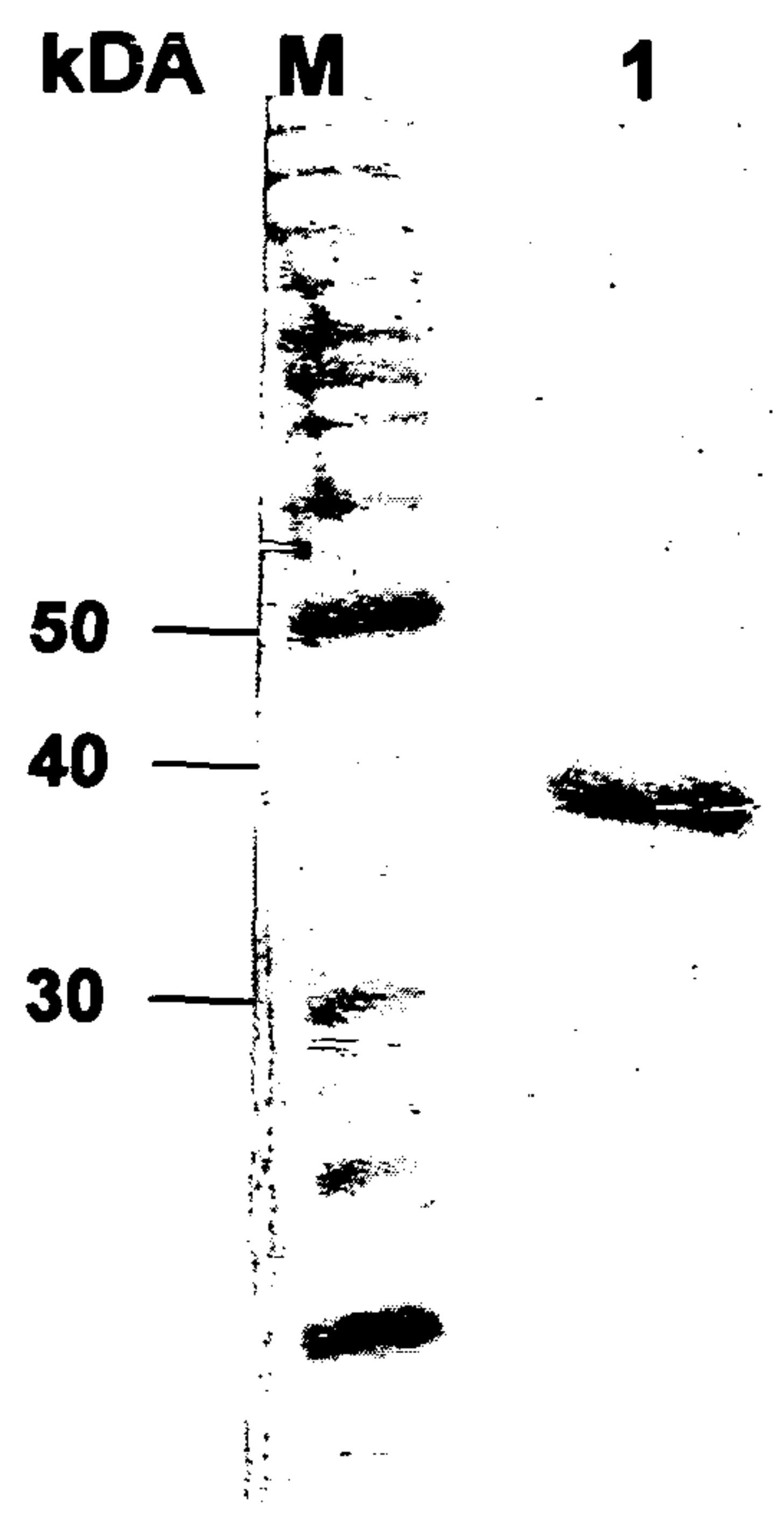


Figure 3

5/8

Fig. 4A

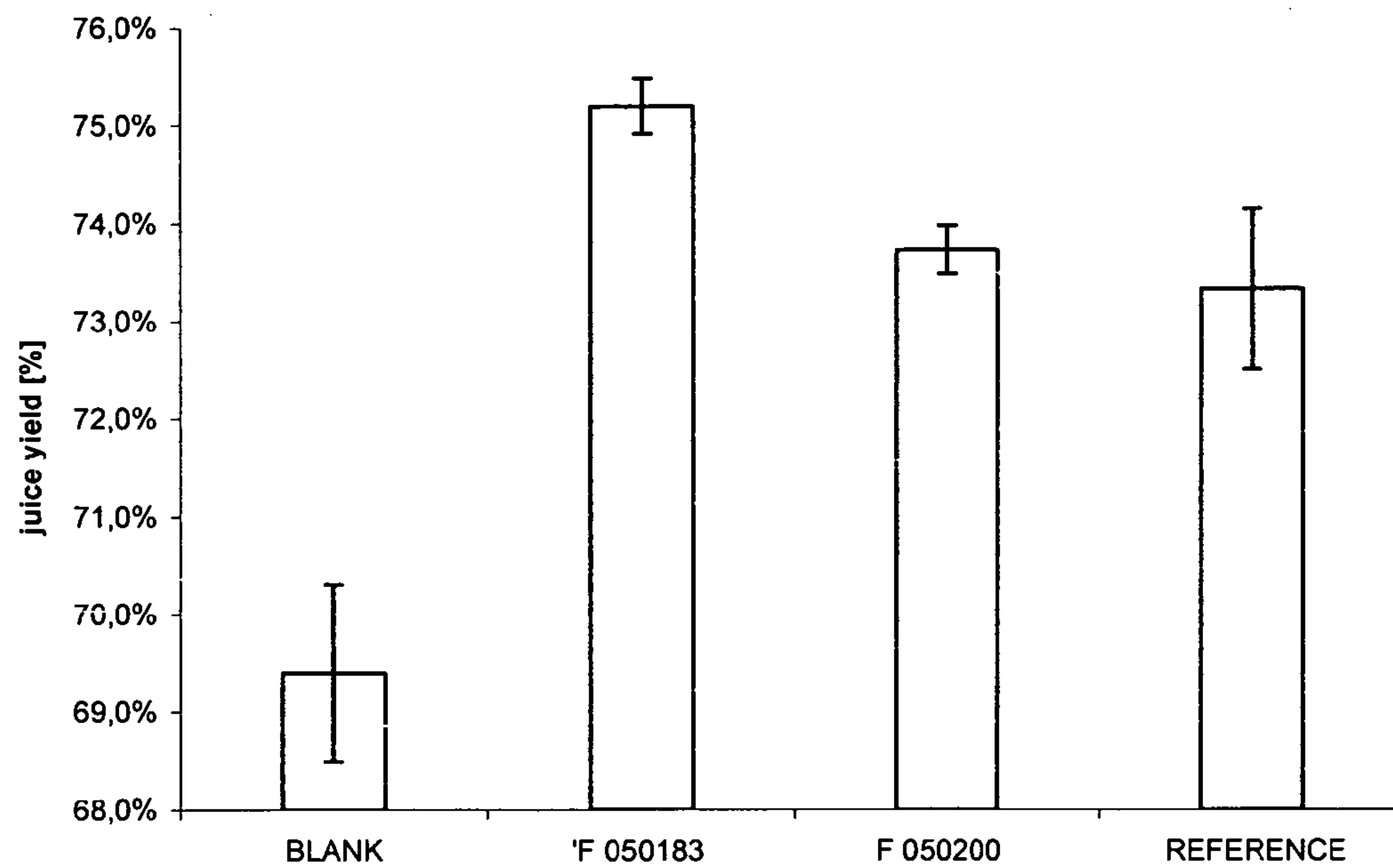
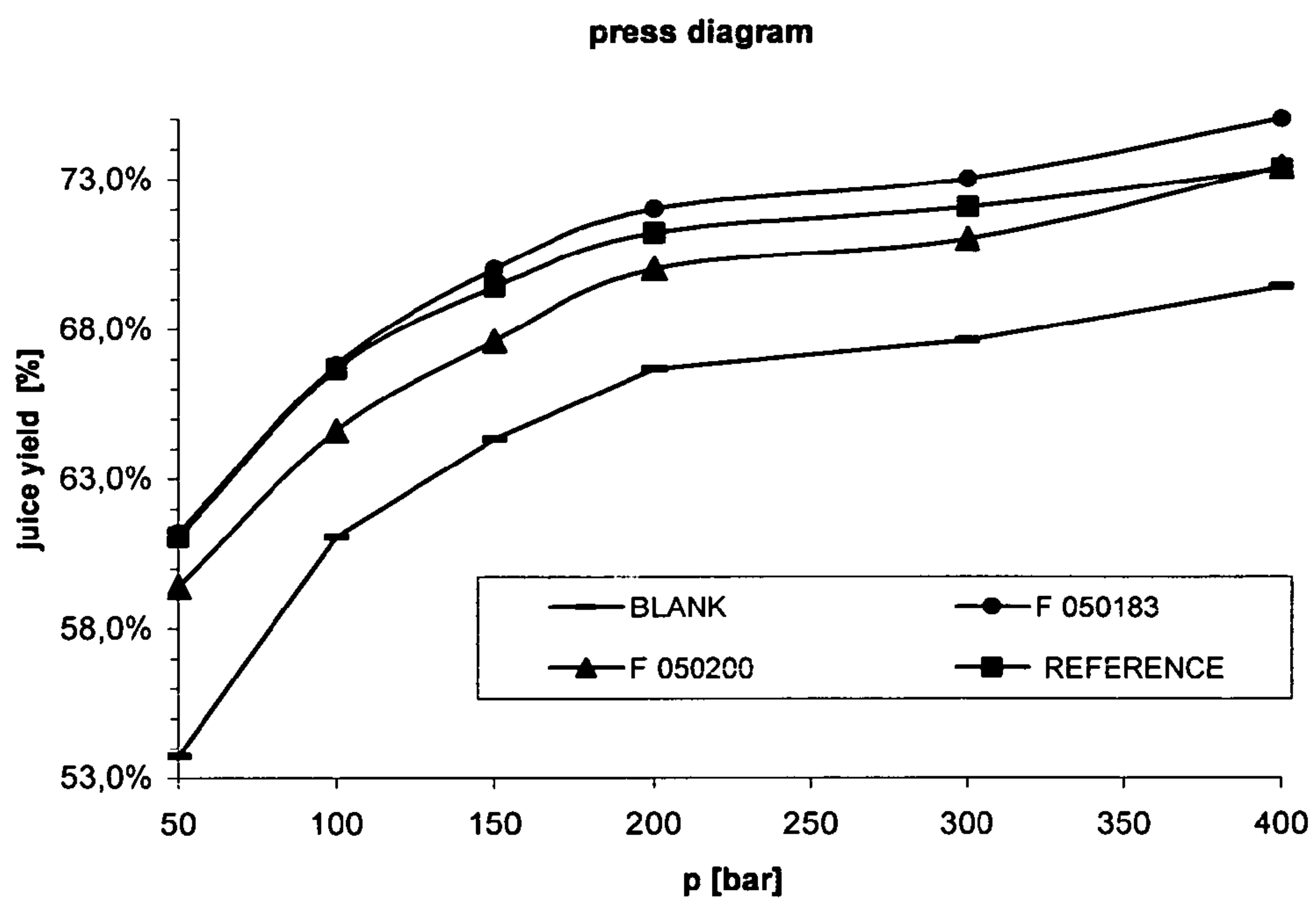
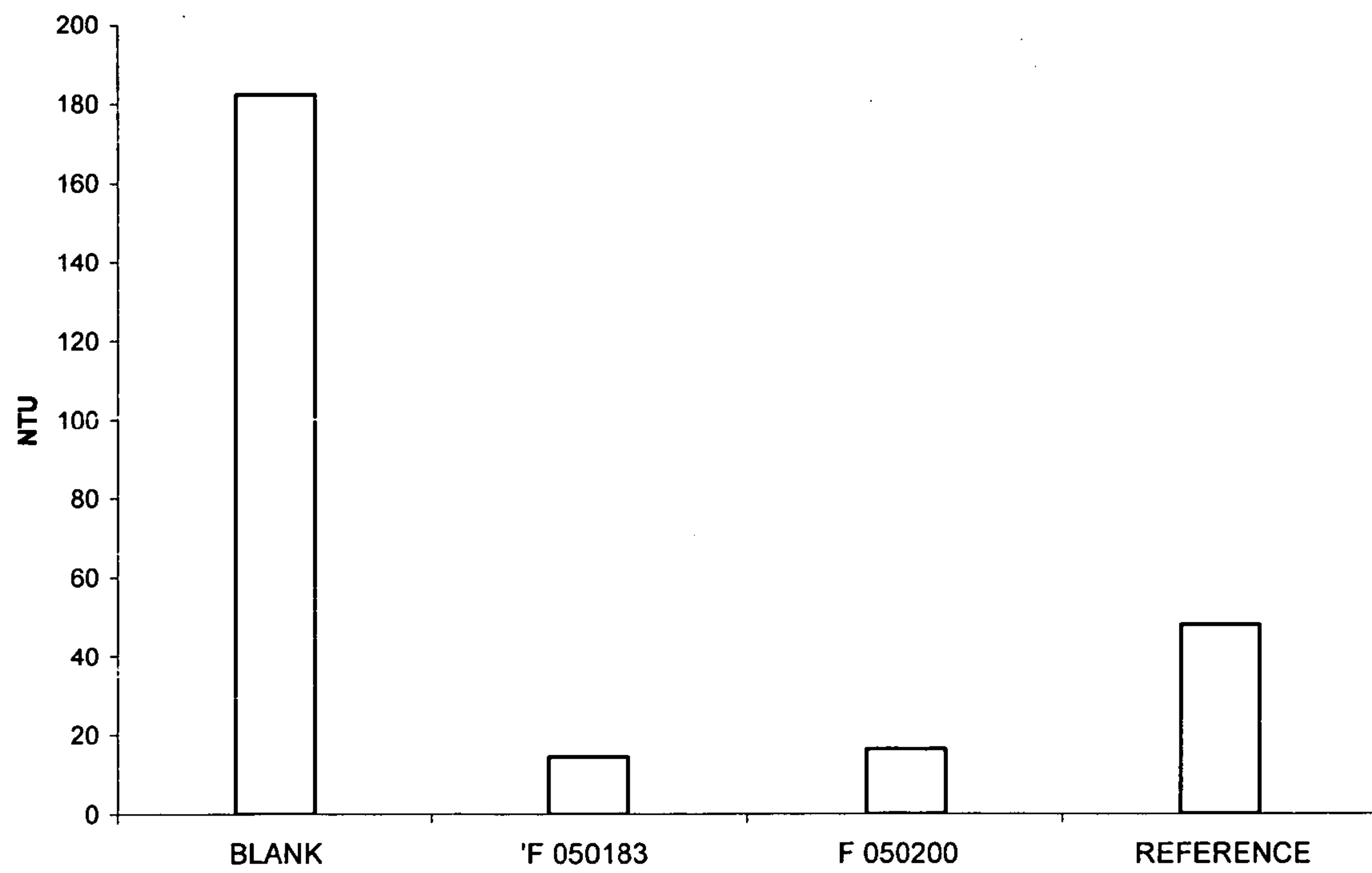


Fig. 4B



Figures 4A + B

6/8**Figure 5**

7/8

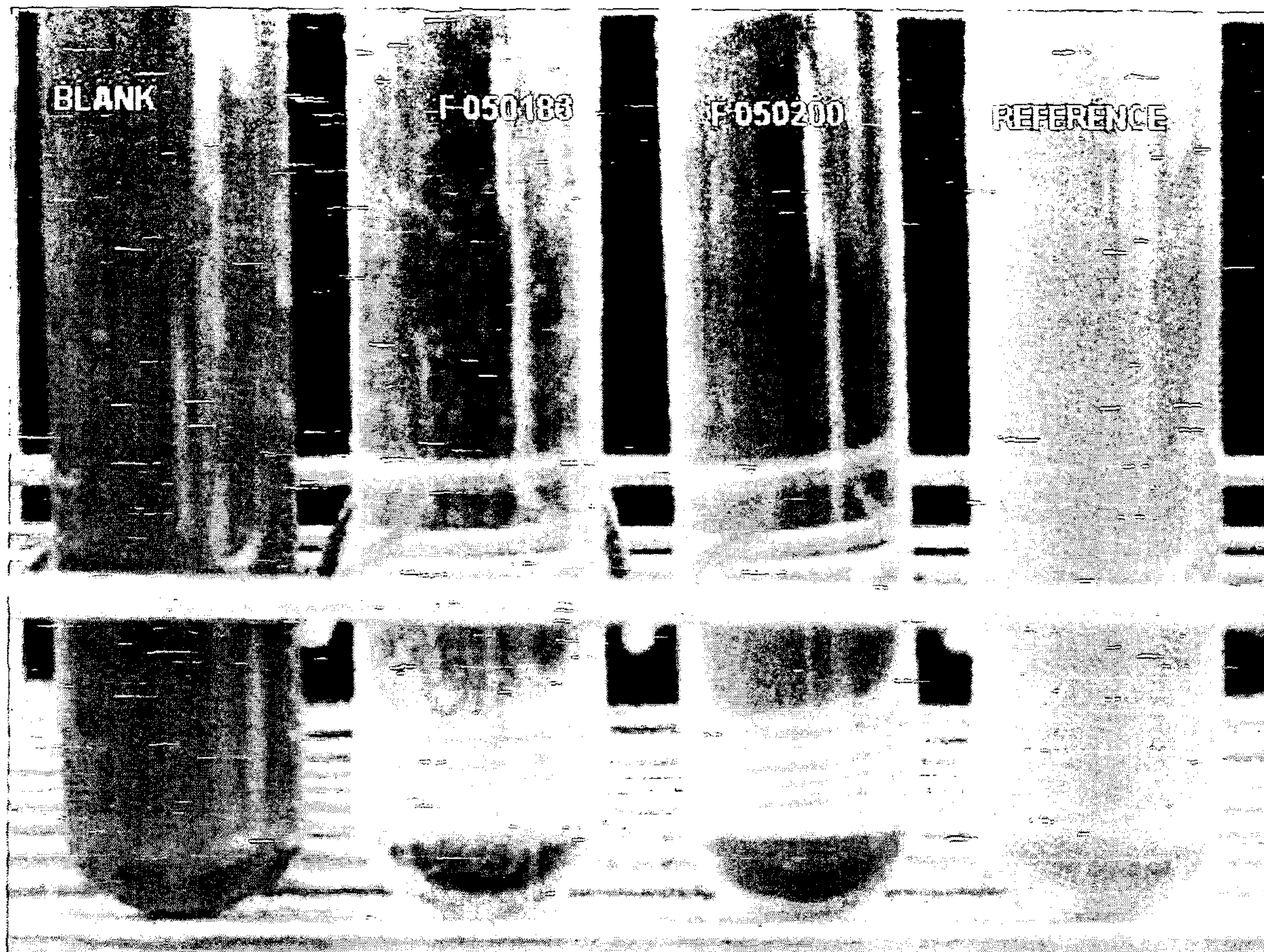


Figure 6

8/8

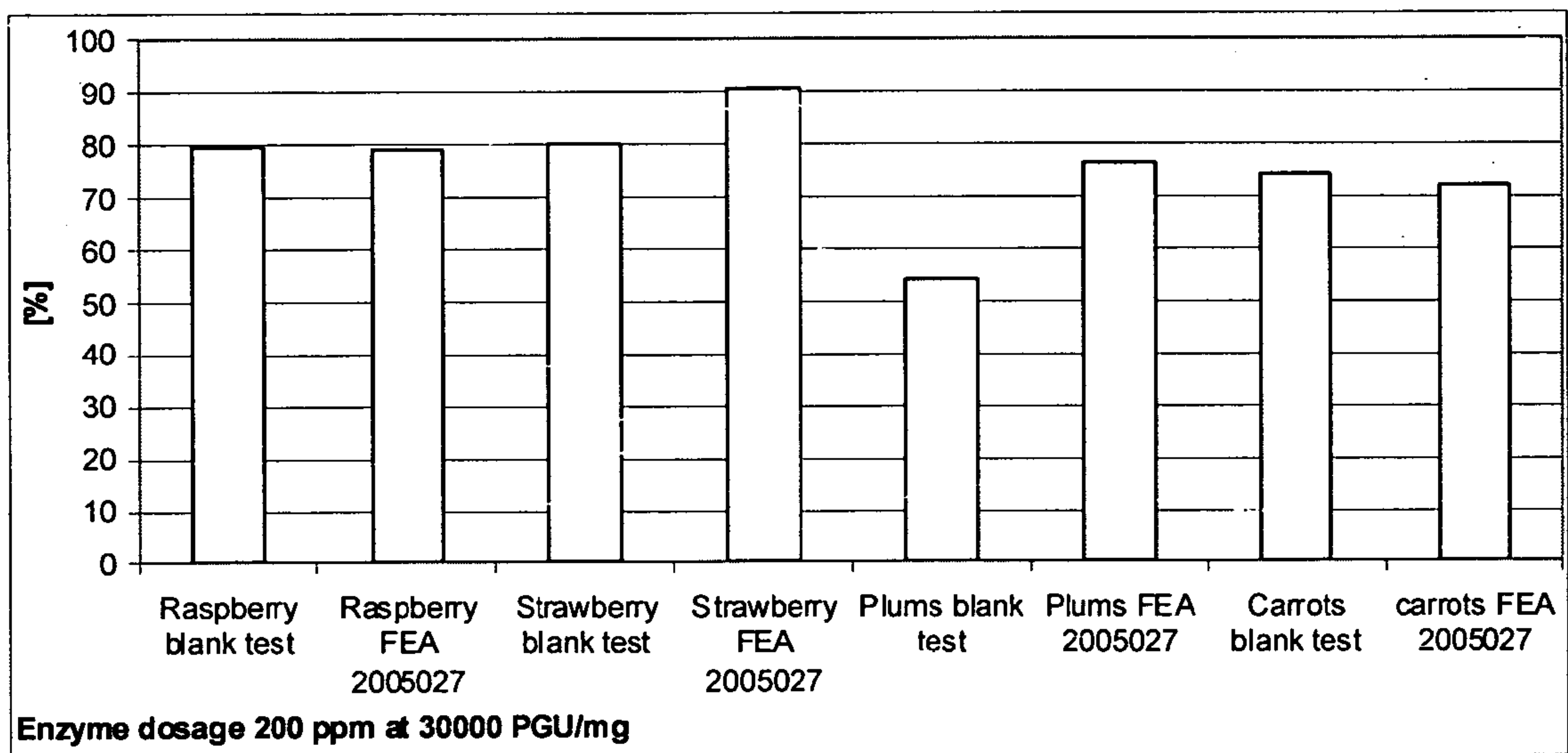


Figure 7