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CA 2198828 C 2011/03/29
(11)(21) **2 198 828**

(12) BREVET CANADIEN
CANADIAN PATENT

(13) **C**

(22) Date de dépôt/Filing Date: 1997/02/28
(41) Mise à la disp. pub./Open to Public Insp.: 1998/08/28
(45) Date de délivrance/Issue Date: 2011/03/29

(51) Cl.Int./Int.Cl. *C12N 15/56* (2006.01), *C12N 15/81* (2006.01), *C12N 9/42* (2006.01)

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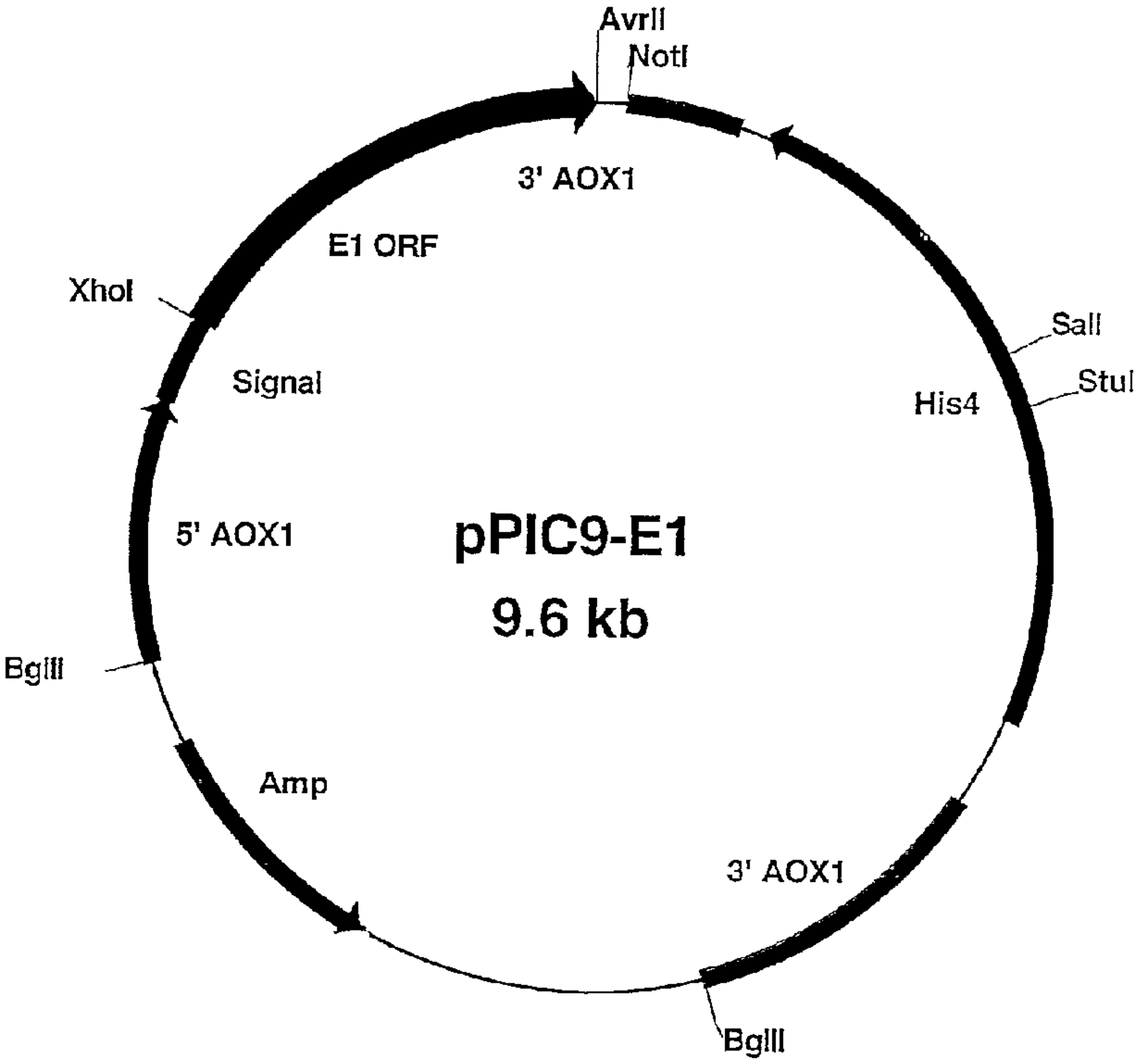
(54) Titre : METHODE POUR ACCROITRE LA THERMOSTABILITE DES ENZYMES CELLULASIQUES PAR ELIMINATION DES DOMAINES DE FIXATION DE LA CELLULOSE
(54) Title: METHOD FOR INCREASING THERMOSTABILITY IN CELLULASE EMZYMES BY REMOVAL OF THE CELLULOSE BINDING DOMAINS



(57) Abrégé/Abstract:

The gene encoding Acidothermus cellulolyticus E1 endoglucanase is cloned and expressed in Pichia pastoris. A new modified E1 endoglucanase enzyme comprising the catalytic domain of the full size E1 enzyme demonstrates enhanced thermostability and is produced by two methods. The first method of producing the new modified E1 is proteolytic cleavage to remove the cellulose binding domain and linker peptide of the full size E1. The second method of producing the new modified E1 is genetic truncation of the gene encoding the full size E1 so that the catalytic domain is expressed in the expression product.

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ABSTRACT

The gene encoding *Acidothermus cellulolyticus* E1 endoglucanase is cloned and expressed in *Pichia pastoris*. A new modified E1 endoglucanase enzyme comprising the catalytic domain of the full size E1 enzyme demonstrates enhanced thermostability and is produced by two methods. The first method of producing the new modified E1 is proteolytic cleavage to remove the cellulose binding domain and linker peptide of the full size E1. The second method of producing the new modified E1 is genetic truncation of the gene encoding the full size E1 so that the catalytic domain is expressed in the expression product.

METHOD FOR INCREASING THERMOSTABILITY IN CELLULASE ENZYMES BY REMOVAL OF THE CELLULOSE BINDING DOMAINS

This application is related-in-part to Canadian Patent Application File No. 2,194,478 filed July 14, 1995.

FIELD OF THE INVENTION

The invention relates to methods for increasing the thermostability of cellulase enzymes.

BACKGROUND OF THE INVENTION

The development of an economic process for the conversion of low-value biomass to useful products via fermentation requires the optimization of several key steps, including cellulase production and performance. Practical utilization of cellulose by hydrolysis with cellulase to produce glucose requires large amounts of cellulase to fully depolymerize cellulose. For example, about one kilogram cellulase preparation may be used for every fifty kilograms of cellulose. Economical production of cellulase is also compounded by the relatively slow growth rates of cellulase-producing fungi and the long times required for cellulase induction. Therefore, improvements in or alternative cellulase production systems capable of great productivities of cellulas activity than may be possible from currently available systems would significantly reduce the cost of cellulose hydrolysis and make the large-scale bioconversion of cellulosic biomass more economical.

Highly thermostable cellulase enzymes are secreted by the cellulolytic thermophile *Acidothermus cellulolyticus gen. nov., sp. nov.* These are discussed in U.S. Patents 5,110,735, 5,275,944, 5,366,884, and 5,432,075. The disclosures of all four of these patents may be referred to for further details. This bacterium was originally isolated from decaying wood in an acidic, thermal pool at Yellowstone National Park and deposited with the American Type Culture Collection (ATCC) under collection number 43068 (Mohagheghi et al. 1986. Int. J. System. Bacteriol. 36:435-443).

The cellulase complex produced by this organism is known to contain several different cellulase enzymes with maximal activities at temperatures of 75°C to 83°C. These cellulases are resistant to inhibition from cellobiose, an end product of the reactions catalyzed by cellulase. Also, the cellulases from *Acidothermus cellulolyticus* are active over a broad pH range centered about pH 6. A high molecular weight cellulase isolated from growth broths of *Acidothermus cellulolyticus* was found to have a molecular weight of approximately 156,600 to 203,400 daltons by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). This enzyme is described in U.S. Patent 5,110,735.

A novel cellulase enzyme, known as the E1 endoglucanase, also secreted by *Acidothermus cellulolyticus* into the growth medium, is described in detail in U.S. Patent 5,275,944. In its native form, this endoglucanase demonstrates a temperature optimum of 83°C and a specific activity of 40μmole glucose release from carboxymethylcellulose/min/mg protein. This E1 endoglucanase was further identified as having an isoelectric pH of 6.7. It is this E1 endoglucanase which has been modified and made the subject of this patent application. The E1 endoglucanase is a multidomain cellulase having a catalytic domain and a cellulose binding domain connected to the catalytic domain by a linker peptide.

SUMMARY OF THE INVENTION

The E1 endoglucanase described above, has been modified to increase its thermostability. The present modification has increased the thermostability of this enzyme by effectively doubling the length of time which this enzyme demonstrates half-maximal activity at elevated temperatures, as well as increasing the temperature at which maximal rates of catalysis are observed. The modification comprises eliminating the cellulose binding domain and linker peptide of the enzyme from the catalytic domain. It is the catalytic domain containing the catalytically active portion of the molecule, which remains after elimination of the cellulose binding domain and linker peptide, which demonstrates these improved thermal properties. This modification has been accomplished by two methods. The first method for eliminating the cellulose binding domain and linker peptide is by subjecting the entire molecule to proteolytic cleavage, which removes the cellulose binding domain and linker peptide from the catalytic domain. The second method for removing the cellulose binding domain and linker peptide from full size E1 involves modification of the gene which encodes full size E1 so that the cellulose binding domain and linker peptide are not present in the expression product. The E1 enzyme of the present invention which demonstrates enhanced thermostability by elimination of the cellulose binding domain and linker peptide is referred to as "modified", "truncated", "catalytic domain", or "E1 CAT". Also, E1 CAT produced by proteolytic cleavage may be referred to as pE1 CAT and E1 CAT produced by genetic transformation may be referred to as gE1 CAT.

In addition to the modified E1 endoglucanase having improved thermostability, this invention teaches the expression of full size E1 in a yeast.

Accordingly, the present invention seeks to transform and express the full size E1 endoglucanase gene in a yeast under the same and/or a different gene regulatory system.

Further, the present invention seeks to modify the gene encoding the E1 endoglucanase from *Acidothermus cellulolyticus* to enhance its thermostabiilty by eliminating expression of the cellulose binding domain and linker peptide in the gene product.

Still further, the present invention seeks to provide the DNA sequence which encodes the modified form of the E1 endoglucanase from *Acidothermus cellulolyticus.*

Further still, the present invention seeks to provide the amino acid sequence of the modified form of the E1 endoglucanase from *Acidothermus cellulolyticus.*

Yet further, the present invention seeks to provide a method for proteolytic cleavage of the cellulose binding domain and linker peptide from the E1 endoglucanase.

Moreover, the present invention seeks to prepare modified E1 endoglucanases which have different properties from the natural enzyme.

The present invention describes the gene for and the nucleotide sequence of the segment of *Acidothermus cellulolyticus* DNA encoding the catalytic domain of the E1 endoglucanase gene. This 2293 base fragment of DNA is unique in nature and discretely defined. The natural gene contains a promoter, a ribosome binding site, a signal peptide, an open reading frame, a termination codon and a putative transcriptional terminator. The modified gene contains a promoter, a ribosome binding site, a signal peptide and one or more termination codons inserted at the C terminus of the catalytic domain.

The cloned gene may also be expressed in other microorganisms under its natural promotor or another promotor recognized by the host microorganism. The cloned gene may be expressed in mammalian systems, higher plants or viral vectors. Alternatively, additional copies of the gene may be introduced into *Acidothermus cellulolyticus* or other heterologous host organism to enhance expression of the enzyme. Additionally, DNA encoding one or more domains of the *Acidothermus cellulolyticus* E1 endoglucanase may be ligated to domains in other compatible endoglucanases to make a recombinant DNA capable of expressing a hybrid endoglucanase enzyme having beneficial properties from both endoglucanases.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a plasmid map of pPIC9-E1.

Figure 2 compares the functional half-life of the full size E1 enzyme to the functional half-life of the catalytic domain of the E1 enzyme at 80°C.

Figure 3 compares the temperature optimum of the full size E1 enzyme to the temperature optimum of the catalytic domain of the E1 enzyme.

Figure 4 is a chromatogram showing purified constituent domains of full size E1 by size exclusion chromatography.

Figure 5 shows the amino acid translation of the coding sequence of E1 CAT.

Figure 6 shows the 2293 base pair nucleotide sequence of the region of *Acidothermus cellulolyticus* genomic DNA which contains the modified E1 endoglucanase gene which expresses only the catalytic domain of the enzyme, without the linker peptide or cellulose binding domain.

Figure 7 is a plasmid map of pYCC101.

Figure 8 is a DSC thermogram comparing the denaturation endotherm peak at 78°C for the full size E1 and E1 CAT showing no peak below at least 88°C.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

According to the present invention the entire E1 coding sequence for *Acidothermus cellulolyticus* E1 endoglucanase is cloned and expressed in a different microbial host than is described in the U.S. Patent 5,536,655 issued July 16, 1996 the disclosure of which may be referred to for further details. The enzyme described in U.S. Patent 5,536,655 is a ß-1,4 endoglucanase which can hydrolyze cellulose or carboxymethylcellulose and is hereafter referred to as E1 endoglucanase or full size E1. The result is a vastly improved rate of E1 enzyme production over native or genetically engineered bacterial systems, thereby lowering the cost of cellulase. The instant application teaches expression of full size E1 in a yeast, namely *Pichia pastoris*.

EXPRESSION OF FULL SIZE E1 AND MODIFIED E1

For expressing the E1 endoglucanase gene, either the full size E1 gene or the modified E1 gene, one may use a variety of hosts including most bacteria, yeast, fungi, algae, viruses, plants and animals. Organisms which naturally produce cellulase enzymes are preferred host cells along with easy to grow host cells and host cells known to express and secrete heterologous genes in large quantities.

If the host cell is a bacterium, generally a bacterial promoter and regulatory system will be used. For a typical bacterium such as *E. coli*, representative examples of well known promoters include, for example, trc, lac, tac, trp, bacteriophage lambda P_L, T7 RNA polymerase promoter, etc. When the expression system is yeast, examples of well known promoters include, but are not limited to GAL 1/GAL 10, alcohol dehydrogenase

(ADH), alcohol oxidase (AOX), his3, cycI, etc. For eukaryotic hosts, enhancers such as the yeast Ty enhancer, may be used.

Alternatively, if one wished for the full size or modified E1 endoglucanase gene to be expressed at only a particular time, such as after the culture or host organism has reached maturity, an externally regulated, environmentally-responsive promoter is particularly useful. Examples include those based upon the nutritional or chemical composition of the medium (e.g. methanol, lac, trp, his), temperature regulation (e.g. temperature sensitive regulatory elements), heat shock promoters (e.g. HSP80A, U.S. Patent 5,187,267), stress response (e.g. plant EF1A promoter, U.S. Patent 5,177,011) and chemically inducible promoters (e.g. tetracycline inducible promoter or salicylate inducible promoter U.S. Patent 5,057,422).

Other suitable hosts for expressing full size or modified E1 endoglucanase include members of the genera: *Trichoderma, Fusarium, Penicillium, Bacillus, Xanthomonas, Streptomyces, Aspergillus* and *Pichia*, for example. Some of these microorganisms also serve as sources of endoglucanase genes for the formation of mixed domain genes for the production of hybrid enzymes.

Expression of the full size E1 endoglucanase gene has been demonstrated in *E. coli, Pichia pastoris* and in *Streptomyces lividans.*

Expressing full size or modified E1 endoglucanase in *E. coli* may be performed under control of a T7 bacteriophage promoter or other promotor recognizable by *E. coli.* Expression of full size E1 in *E. coli* has been enhanced considerably relative to the native gene with the constructs of the present invention. Expression of the full size E1 endoglucanase coding sequence in *S. lividans* has been achieved twice more with several different constructs employing two different promoters. These are the *tipA* promoter (thiostrepton-inducible) and the ST I-II promoter isolated from a trypsin inhibitor gene from *Streptomyces longisporus.* Expression levels of active, full size, secreted E1 endoglucanase up to 20 mg/L have been achieved with the ST I-II promoter.

Expression of the full size or modified E1 endoglucanase coding sequence in the filamentous fungi, *Aspergillus niger* , *A. awamori, A. oryzae, A. terreus* and/or *A.*

nidulans and *Trichoderma reesei* is achievable using various promoters derived from *Aspergillus* or *Trichoderma*. These promoters include, but are not limited to, G3PDH (glyceraldehyde-3-phosphate dehydrogenase), glucoamylase, β-tubulin and IPNS (isopenicillin N synthase) from *Aspergillus* and CBH I (cellobiohydrolase I), alcohol dehydrogenase, triosephosphate isomerase and α-amylase from *T. reesei*.

The *Acidothermus cellulolyticus* full size E1 endoglucanase gene was cloned, as described in U.S. Patent No. 5,536,655 granted July 16, 1996, and expressed in *Pichia pastoris* using the AOX1 promoter and 3' sequences. This expression is taught in Example 1.

Example 1

Expression of the entire Full Size E1 gene in *Pichia pastoris*

Expression of active E1 endoglucanase in the range of 0.75-1.5 g/L has been accomplished in the yeast, *Pichia pastoris*, by splicing the methanol-inducible alcohol oxidase (AOX1) promoter, including the signal sequence from the *P. pastoris* alcohol oxidase polypeptide to the mature coding sequence of the E1 endoglucanase gene.

P. pastoris has been shown to be a useful host organism for expression of large quantities of diverse heterologous proteins. *P. pastoris* was used to express large quantities of active full size E1.

Plasmid 4-5, a pGEM-7 (Promega Corp.) derivative carrying a 3.7 kb genomic fragment of *Acidothermus cellulolyticus* DNA and harboring the entire E1 gene, was used as a template in PCR reactions to amplify the E1 coding sequence for subsequent cloning into the *Pichia* secretion vector, pPIC9. The primer annealing to the non-coding strand of the template, "E1-f", is a 30-mer with 18 bases of homology to the template, beginning at the mature N-terminus of the E1 polypeptide. The segment of E1-f which is not homologous to the template molecules incorporates 4 codons which encode the C-terminal 4 amino acids of the αF signal peptide which is present in pPIC9 and also includes an XhoI site at the 5' end for use in subsequent cloning into pPIC9. The primer annealing to the coding strand and priming synthesis of the non-coding strand, "E1-r", is a 24-mer with 18 bases of homology to the template. The sequence of E1-r corresponds to the last 5 codons of the E1 coding sequence and the stop codon. The non-homologous 5' tail of E1-r adds an AvrII restriction site for use in subsequent cloning into pPIC9.

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                    signal cleavage site
         XhoI       |
E1-f:5'-CTC GAG AAA AGA GCG GGC GGC GGC TAT TGG-3'(SEQ ID NO:1)
AA seq   L   E   K   R   A   G   G   G   Y   W (SEQ ID NO:2)

          AvrII
E1r: 5'- CCT AGG TTA ACT TGC TGC GCA GGC -3' (SEQ ID NO:3)
AA seq       stop S   A   A   C   A    (SEQ ID NO:4)
```

Cloning of the E1 expression construct in pPIC9 was accomplished using the *Pichia* Expression Kit supplied by Invitrogen (San Diego, CA). All procedures are those recommended by Invitrogen. The PCR reaction produces a 1584 bp fragment containing the entire open reading frame for the full size, mature E1 polypeptide. The PCR product was cloned directly into the TA vector, PCRII (Invitrogen, San Diego, CA). A single clone containing the PCR product was digested with XhoI and AvrII and the resulting 1.6 kb fragment was cloned into the same sites of pPIC9 to produce pPIC9-E1, which is diagrammatically represented in Figure 1. Several independent pPIC9-E1 isolates were screened for the existence of XhoI and AvrII sites and subsequently subjected to DNA sequencing across the signal peptide/E1 fusion junction to verify the correct sequence context in this region.

The pPIC9-E1 plasmid was linearized at the unique StuI site and transformed into spheroplasts of *P. pastoris* strain GS115. His$^+$ transformants were selected for the Mut$^-$ phenotype. Twenty independent His$^+$Mut$^-$ isolates were screened by PCR using the AOX1 primers. Clones which displayed a 1.8 kb PCR product were screened for expression of E1 endoglucanase activity after growth on methanol.

The media and intracellular contents of cells from cultures grown in the presence of methanol for two days were screened by Western blot analysis using a monoclonal antibody specific for the E1 endoglucanase. All *P. pastoris* clones containing the foreign

DNA were shown to express E1 on Western blots. Some of the reactive material on these blots runs as two diffuse high molecular weight bands. This material may be heavily glycosylated relative to the main band on the blots, which runs at a molecular weight only slightly higher than native E1 (75-80 kDa vs. 72 kDa). Most of the E1 produced in these cultures was secreted into the medium, as intended. The activity of the E1 secreted into the medium was demonstrated by the ability of these crude culture filtrates to hydrolyze 4-methylumbelliferyl-ß-D-cellobioside (MUC), whereas control culture supernatants did not hydrolyze MUC.

Fed-batch fermentations of one of the *Pichia* transformants were conducted over a period of 4 days. Cultures were grown to a high optical density on 4% glycerol (w/v). When glycerol was exhausted (approx. 30 hours), methanol was fed as the sole carbon source over a period of 66 additional hours. After purification of the E1 from a portion of this culture, the yield was estimated at 1.5 g/L.

The present invention also comprises a further improvement of the E1 enzyme by modification of the physical structure of the enzyme to enhance its thermostable properties. At 80°C, the modified enzyme of the present invention demonstrates increased stability relative to the parent, full size E1, as well as an approximately 10°C increase in its optimal temperature for activity.

This enhancement is brought about by the cleavage of the cellulose binding domain from the catalytic domain of the full size E1 enzyme. The cleavage of the cellulose binding domain from the catalytic domain can be accomplished by more than one method. The first method of cleaving the cellulose binding domain from the catalytic domain is the enzymatic cleavage of the cellulose binding domain from the full-sized E1. This is taught in Example 2.

Example 2

Method for the production, purification and papain cleavage of full size E1 endoglucanase to produce E1 CAT.

Full size E1 enzyme was recombinantly produced using *S. lividans* strain TK24 expressing E1-pIJ702 grown in 30 g/L Tryptic Soy Broth (Difco) with 5 ug/mL thiostrepton using a New Brunswick Microferm™ fermenter. The fermentation broth (10L) was harvested using a CEPA continuous flow centrifuge, the supernatant concentrated and diafiltered against 20 mM Bis-Tris™, pH 5.8 to a final volume of 300 mL using an Amicon CH2 concentrator and 10,000 MW cutoff hollow-fiber cartridges. Yields of native 72 kDa molecular weight E1 from these fermentations ranged from 1.2 to 2 mg/L, as estimated from purification yields.

The recombinant full size enzyme was purified with essentially a three step purification process consisting of hydrophobic interaction chromatography (HIC) followed by anion exchange and finally, size exclusion chromatography. The HIC step employed a column packed with 250 mL of Pharmacia Fast Flow Phenyl Sepharose™. This was followed by anion-exchange chromatography using a 6 mL Pharmacia Resource Q™ anion exchange column.

For the hydrophobic interaction step, ammonium sulfate was added to the concentrated culture supernatant of one 10 L fermentation to a final concentration 0.5 M. A total volume of 300 mL of this concentrate was loaded onto the phenyl sepharose column and washed extensively with 20 mM Tris, 0.5 M $(NH_4)_2SO_4$ pH 8.0. The column was developed with a linear decreasing gradient (0.5 M - zero) of $(NH_4)_2SO_4$. Recombinant full size E1 eluted at zero salt concentration. Fractions containing E1 activity were identified using 4-methylumbelliferyl β-D-cellobioside (MUC) assays. Active fractions were combined, concentrated and diafiltered against 20 mM Tris pH 8.0, after which they were loaded directly onto the anion-exchange column and eluted with a increasing NaCl gradient (0-300 mM). A final buffer exchange and purification step was done using size exclusion chromatography with a 2.6 cm x 10 cm Pharmacia Superdex™ 200 column and using one of two buffer systems; either 20 mM acetate, 100 mM NaCl, pH 5.0 buffer or 50 mM ammonium acetate, pH 6.2 buffer depending upon the eventual use of the enzyme.

The E1 catalytic domain was produced by proteolytic cleavage with papain. The proteolytic digestions were done in 50 mM ammonium acetate, pH 6.2 buffer. Molar ratios of 72kDa MW E1/23 kDa MW papain cleaved E1 of 6/1 were used. Papain digestions were incubated at 28°C for 24 h. The catalytic domain was separated from full size recombinant E1, E1-CBD (cellulose binding domain), linker peptides and papain by SEC using a 2.6 cm x 10 cm Pharmacia Superdex 200 column.

A chromatogram demonstrating the purification of modified catalytic domain from papain cleaved *S. lividans* recombinant E1 by size exclusion chromatography is shown in Figure 4. Peak A is unmodified full size E1 enzyme, peak B is the catalytic domain, and peak C is the cellulose binding domain of the cleaved product. Also, peak B reacts with a monoclonal antibody (MAB) specific for full size E1, thereby confirming that this fragment contains epitopes for which this MAB shows specificity.

In order to confirm the identity of the peptide isolated from the papain digestion, the peak B peptide was subjected to analytical ultracentrifugation using a Beckman Optima™ XLa centrifuge. Sedimentation equilibrium analysis yielded an estimated molecular weight of 41,600 daltons for this peptide. SDS-PAGE analysis rendered a molecular weight estimate of 42,000 daltons. These values compare well with the calculated molecular weight predicted from amino acid sequence for the catalytic domain (i.e., 40,192 da.) Due to the limits of detection of the techniques utilized herein, these molecular weight values are well within the range of experimental error and therefore the molecular weight of the E1 CAT is in the range of about 40,000 to 42,000 Daltons.

Comparison of the thermal stability at 80°C of pE1 CAT and full size E1 endoglucanase, both produced from a *Streptomyces* host, can be seen in Figure 2. The enzyme was incubated at 80°C in 20 mM acetate, 100 mM NaCl, pH 5.0 buffer with timed aliquots removed, and each assayed for activity using 1 mg/mL p-nitrophenyl-ß-D-cellobioside substrate in 20 mM acetate, 100 mM NaCl, pH 5.0, at 65°C for 30 minutes. Activity values are expressed as a percentage of the activity detected at time zero (e.g., 100%).

Comparison of the temperature optima of modified E1 and full size E1 was performed using 1 mg/mL p-nitrophenyl-ß-D cellobioside substrate in 20 mM acetate, 100 mM NaCl, pH 5.0, at various temperatures with a 30 minute incubation time. See Figure 3. The temperature optimum of the E1 CAT produced by papain cleavage is increased by

10°C relative to the full size E1. Activity values in Fig. 3 are expressed as a percentage of the maximum activity detected.

It is further contemplated that one may include more than one catalytic domain in the hybrid enzyme. This may allow for a further increase in specific activity. Also, a catalytic domain containing cellulase activity other than endoglucanase activity may be included as well to reduce the number of cellulase enzymes one needs to add to a cellulosic substrate for polymer degradation.

Example 3

Production of E1 CAT by genetic truncation of the full size E1 coding sequence

The amino acid sequence of E1 CAT is shown in Figure 5. The entire amino acid sequence of pE1 CAT, including the C-terminus, is confirmed by the x-ray crystal structure derived from this molecule. An alternative to production of E1 CAT by papain cleavage of full size E1 is taught. A molecular genetic approach was also developed to produce E1 CAT.

A strategy was desired to generate a genetically truncated E1 gene which would produce E1 CAT without requiring any downstream processing to achieve E1 CAT from a precursor molecule, as for papain cleavage. One way to accomplish this is to introduce a translational stop codon at or near the C-terminal residue of the catalytic domain.

The 2.3 kb Bam H1 fragment containing most of the E1 gene was subcloned into pAlter-1 in preparation for site-directed mutagenesis (pYCC100). The nucleotide sequence for this fragment is shown in Figure 6. A mutagenic 36-mer oligonucleotide (underlined in Figure 6) was synthesized (5'- ATTTTCGATC CTGTCTAATG ATCTGCATCG CCTAGC -3')(SEQ ID NO:5). Using the Altered Sites® II kit (Promega Corp.) two consecutive codons immediately downstream of the C-terminal residue of E1-CAT (as determined by x-ray crystallography) were changed to different stop codons (TAA, TGA). The six mutagenic nucleotides are double-underlined in Figure 6. The DNA sequence of this clone (pYCC101) has been confirmed by dideoxy DNA sequencing in the region of the site-directed mutations using the T7 Sequenase kit supplied by US Biochemical. (Cleveland, OH). A plasmid map of pYCC101 is shown in Figure 7.

Native AA seq S S I F D P V G A S A S P S S Q (SEQ ID NO:6)

Native DNA seq TCGTCGATTTTCGATCCTGTCGGCGCGTCTGCATCGCCTAGCAGTCAA (SEQ ID NO:7)

Mutagenic oligo ATTTTCGATCCTGTCTAATGATCTGCATCGCCTAGC (SEQ ID NO:8)

Mutated DNA seq TCGTCGATTTTCGATCCTGTCTAATGATCTGCATCGCCTAGCAGTCAA (SEQ ID NO:9)

Mutated AA seq S S I F D P V . . S A S P S S Q (SEQ ID NO:10)

Mutagenized DNA was transformed into *E. coli* strain ES1301. Transformants were screened for resistance to ampicillin and sensitivity to tetracycline in order to identify clones carrying the putatively mutagenized E1 gene. Many ampicillin-resistant candidate clones were subsequently screened on plates containing 1 mM 4-methylumbelliferyl-ß-D-cellobioside (MUC) to verify expression of active E1. Plasmid DNA was prepared from several clones and employed as templates in dideoxy DNA sequencing reactions using the Sequenase® kit (U.S. Biochemical, Cleveland, OH) to verify the sequence of E1 DNA in the region of the intended mutation. The mutated sequence was detected in every clone which was sequenced. One of these clones was selected and designated pYCC101. Each of the successfully mutated clones expresses a protein not present in control cells and which migrates at a molecular weight of approximately 42 kDa in SDS-PAGE gels. This 42 kDa protein also reacts with a monoclonal antibody specific for the E1 endoglucanase on Western blots, thus confirming its identity as E1 CAT.

Example 4

Differential Scanning calorimetry (DSC)

Calorimetric studies of the denaturation of the full size E1 enzyme and the proteolytically cleaved E1 CAT were carried at pH 5.0 in 50 mM sodium acetate, using a Microcal™ MC-2 differential scanning microcalorimeter over a temperature range of 25-95°C and using a scan rate of 20°C/h. For the examples shown in Figure 2, the protein concentrations were 0.24 mg/mL for the native E1 enzyme and 0.14 mg/mL for E1 CAT.

Figure 8 shows the DSC thermogram for full size E1 enzyme displaying a prominent denaturation endotherm peak at approximately 78°C. This compares to the thermogram of E1 CAT, which shows no peak below at least 88°C. Since the thermograms are uninterpretable above 88°C (limitation of the instrumentation), a reasonable conclusion is that the denaturation endotherm for the catalytic domain lies somewhere above 88°C.

Example 5

Method for the determination of the functional half-life of E1 and E1 CAT

The time at which half of the original endoglucanase activity remains following pre-incubation at 80°C is referred to as its functional half-life. Enzymes were pre-incubated at 80°C in 20 mM acetate, 100 mM NaCl, pH 5.0, in concentrations of 13.14 μg/mL for the full size enzyme E1 and 19.53 μg/mL for E1 CAT. Small aliquots (100μL) from single tubes were removed at various times and assayed for activity by adding the 100 μL enzyme aliquot to 700 μL 20 mM acetate, 100 mM NaCl, pH 5.0, and 200 μL of 5 mg/mL p-nitrophenyl β-D cellobioside. The assay mixture was incubated at 65°C for 30 minutes and the reaction stopped by adjusting the pH by addition of 2 mL of 1 M Na_2CO_3. The activity was then measured by determining the concentration of the nitrophenolate anion, released as a result of catalytic activity of the enzyme, by measuring the absorbance at 410 nm of the quenched samples. Results shown in Figure 2 demonstrate that E1 CAT has a functional half-life at 80°C which nearly doubles that of full size E1 (16 h vs. 9 h).

Other options for generating genetically truncated E1 contemplated by the present inventors to be part of this invention include PCR of the coding sequence incorporating a non-homologous stop codon into the downstream synthetic primer; or using available restriction sites downstream of the DNA encoding the catalytic domain to delete the DNA sequences encoding the linker peptide and cellulose binding domain.

Unless specifically defined otherwise, all technical or scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials have been described.

The foregoing description of the specific embodiments reveal the general nature of the invention so that others can, by applying current knowledge, readily modify and/or adapt for various applications such specific embodiments without departing from the generic concept, and, therefore, such adaptations and modifications should and are intended to be comprehended within the meaning and range of equivalents of the disclosed embodiments. It is to be understood that the phraseology or terminology employed herein is for the purpose of description and not of limitation.

08/604,913

SEQUENCE LISTING

(1) GENERAL INFORMATION:
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  (ii) TITLE OF INVENTION: METHOD FOR INCREASING THERMOSTABILITY IN CELLULASE ENZYMES

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    (A) ADDRESSEE: National Renewable Energy Laboratory
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    (C) CITY: Golden
    (D) STATE: CO
    (E) COUNTRY: U.S.A.
    (F) ZIP: 80401-3393

  (v) COMPUTER READABLE FORM:
    (A) MEDIUM TYPE: Floppy disk
    (B) COMPUTER: IBC PC compatible
    (C) OPERATING SYSTEM: PC-DOS/MS-DOS
    (D) SOFTWARE: ASC II (DOS) text

  (vi) CURRENT APPLICATION DATA:
    (A) APPLICATION NUMBER: U.S./not yet assigned
    (B) FILING DATE:
    (C) CLASSIFICATION:

  (vii) PRIOR APPLICATION DATA:
    (A) APPLICATION NUMBER: 08/276,213
    (B) FILING DATE: 15-070-1994

  (viii) ATTORNEY/AGENT INFORMATION
    (A) NAME: Edna M. O'Connor
    (B) REGISTRATION NUMBER: 29,252
    (C) REFERENCE/DOCKET NUMBER: 95-56

  (ix) TELECOMMUNICATION INFORMATION:
    (A) TELEPHONE: 303/384-7573
    (B) TELEFAX: 303/384-7499

(2) INFORMATION FOR SEQ ID NO:1:

  (i) SEQUENCE CHARACTERISTICS:
    (A) LENGTH: 30 base pairs
    (B) TYPE: nucleic acid
    (C) STRANDEDNESS: double
    (D) TOPOLOGY: linear

  (ii) MOLECULE TYPE: DNA

(ix) FEATURE:
(A) NAME/KEY: E1-f primer

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

CTC GAG AAA AGA GCG GGC GGC GGC TAT TGG 30

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Protein

(ix) FEATURE:
(A) NAME/KEY: E1-f primer

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

```
Leu Glu Lys Arg Ala Gly Gly Gly Tyr Trp
                 5                   10
```

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic Acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(ix) FEATURE:
(A) NAME/KEY: E1r

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

CCT AGG TTA ACT TGC TGC GCA GGC 24

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Protein

(ix) FEATURE:
(A) NAME/KEY: E1r

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

Ser Ala Ala Cys Ala
5

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic Acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

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

ATTTTCGATC CTGTCTAATG ATCTGCATCG CCTAGC 36

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

Ser Ser Ile Phe Asp Pro Val Gly Ala Ser Ala Ser Pro Ser Ser Gln
5 10 15

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 48 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

TCGTCGATTT TCGATCCTGT CGGCGCGTCT GCATCGCCTA GCAGTCAA 48

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 35 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(ix) FEATURE:
(A) NAME/KEY: mutagenic olige

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

ATTTTCGATC TGTCTAATGA TCTGCATCGC CTAGC 35

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 47 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(ix) FEATURE:
(A) NAME/KEY: mutated DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

TCGTCGATTT TCGATCCTGT TAATGATCTG CATCGCCTAG CAGTCAA 47

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Protein

(ix) FEATURE:
(A) NAME/KEY: MOD_RES
(B) LOCATION: 8 and 9
(D) OTHER INFORMATION: Xaa is any amino acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

```
Ser Ser Ile Phe Asp Pro Val Xaa Xaa Ser Ala Ser Pro Ser Ser Gln
                 5                  10                  15
```

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 358 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Protein

(ix) FEATURE:
(A) NAME/KEY: E1-CAT

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

```
Ala Gly Gly Gly Tyr Trp His Thr Ser Gly
 1               5                  10

Arg Glu Ile Leu Asp Ala Asn Asn Val Pro
                 15                  20

Val Arg Ile Ala Gly Ile Asn Trp Phe Gly
                 25                  30

Phe Glu Thr Cys Asn Tyr Val Val His Gly
                 35                  40

Leu Trp Ser Arg Asp Tyr Arg Ser Met Leu
                 45                  50

Asp Gln Ile Lys Ser Leu Gly Tyr Asn Thr
                 55                  60

Ile Arg Leu Pro Tyr Ser Asp Asp Ile Leu
                 65                  70

Lys Pro Gly Thr Met Pro Asn Ser Ile Asn
                 75                  80

Phe Tyr Gln Met Asn Gln Asp Leu Gln Gly
                 85                  90

Leu Thr Ser Leu Gln Val Met Asp Lys Ile
                 95                 100

Val Ala Tyr Ala Gly Gln Ile Gly Leu Arg
                105                 110

Ile Ile Leu Asp Arg His Arg Pro Asp Cys
                115                 120

Ser Gly Gln Ser Ala Leu Trp Tyr Thr Ser
                125                 130

Ser Val Ser Glu Ala Thr Trp Ile Ser Asp
                135                 140

Leu Gln Ala Leu Ala Gln Arg Tyr Lys Gly
                145                 150

Asn Pro Thr Val Val Gly Phe Asp Leu His
                155                 160

Asn Glu Pro His Asp Pro Ala Cys Trp Gly
                165                 170

Cys Gly Asp Pro Ser Ile Asp Trp Arg Leu
                175                 180

Ala Ala Glu Arg Ala Gly Asn Ala Val Leu
                185                 190
```

Ser Val Asn Pro Asn Leu Leu Ile Phe Val
195 200

Glu Gly Val Gln Ser Tyr Asn Gly Asp Ser
205 210

Tyr Trp Trp Gly Gly Asn Leu Gln Gly Ala
215 220

Gly Gln Tyr Pro Val Val Leu Asn Val Pro
225 230

Asn Arg Leu Val Tyr Ser Ala His Asp Tyr
235 240

Ala Thr Ser Val Tyr Pro Gln Thr Trp Phe
245 250

Ser Asp Pro Thr Phe Pro Asn Asn Met Pro
255 260

Gly Ile Trp Asn Lys Asn Trp Gly Tyr Leu
265 270

Phe Asn Gln Asn Ile Ala Pro Val Trp Leu
275 280

Gly Glu Phe Gly Thr Thr Leu Gln Ser Thr
285 290

Thr Asp Gln Thr Trp Leu Lys Thr Leu Val
295 300

Gln Tyr Leu Arg Pro Thr Ala Gln Tyr Gly
305 310

Ala Asp Ser Phe Gln Trp Thr Phe Trp Ser
315 320

Trp Asn Pro Asp Ser Gly Asp Thr Gly Gly
325 330

Ile Leu Lys Asp Asp Trp Gln Thr Val Asp
335 340

Thr Val Lys Asp Gly Tyr Leu Ala Pro Ile
345 350

Lys Ser Ser Ile Phe Asp Pro Val
355

(2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2293 base pairs
(B) TYPE: nucleic acid

(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(ix) FEATURE:
(A) NAME/KEY: E1-CAT

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

GGATCCACGT TGTACAAGGT CACCTGTCCG TCGTTCTGGT AGAGCGGCGG GATGGTCACC 60

CGCACGATCT CTCCTTTGTT GATGTCGACG GTCACGTGGT TACGGTTTGC CTCGGCCGCG 120

ATTTTCGCGC TCGGGCTTGC TCCGGCTGTC GGGTTCGGTT TGGCGTGGTG TGCGGAGCAC 180

GCCGAGGCGA TCCCAATGAG GGCAAGGGCA AGAGCGGAGC CGATGGCACG TCGGGTGGCC 240

GATGGGGTAC GCCGATGGGG CGTGGCGTCC CCGCCGCGGA CAGAACCGGA TGCGGAATAG 300

GTCACGGTGC GACATGTTGC CGTACCGCGG ACCCGGATGA CAAGGGTGGG TGCGCGGGTC 360

GCCTGTGAGC TGCCGGCTGG CGTCTGGATC ATGGGAACGA TCCCACCATT CCCCGCAATC 420

GACGCGATCG GGAGCAGGGC GGCGCGAGCC GGACCGTGTG GTCGAGCCGG ACGATTCGCC 480

CATACGGTGC TGCAATGCCC AGCGCCATGT TGTCAATCCG CCAAATGCAG CAATGCACAC 540

ATGGACAGGG ATTGTGACTC TGAGTAATGA TTGGATTGCC TTCTTGCCGC CTACGCGTTA 600

CGCAGAGTAG GCGACTGTAT GCGGTAGGTT GGCGCTCCAG CCGTGGGCTG GACATGCCTG 660

CTGCGAACTC TTGACACGTC TGGTTGAACG CGCAATACTC CCAACACCGA TGGGATCGTT 720

CCCATAAGTT TCCGTCTCAC AACAGAATCG GTGCGCCCTC ATGATCAACG TGAAAGGAGT 780

ACGGGGGAGA ACAGACGGGG GAGAAACCAA CGGGGGATTG GCGGTGCCGC GCGCATTGCG 840

GCGAGTGCCT GGCTCGCGGG TGATGCTGCG GGTCGGCGTC GTCGTCGCGG TGCTGGCATT 900

GGTTGCCGCA CTCGCCAACC TAGCCGTGCC GCGGCCGGCT CGCGCCGCGG GCGGCGGCTA 960

TTGGCACACG AGCGGCCGGG AGATCCTGGA CGCGAACAAC GTGCCGGTAC GGATCGCCGG 1020

CATCAACTGG TTTGGGTTCG AAACCTGCAA TTACGTCGTG CACGGTCTCT GGTCACGCGA 1080

CTACCGCAGC ATGCTCGACC AGATAAAGTC GCTCGGCTAC AACACAATCC GGCTGCCGTA 1140

CTCTGACGAC ATTCTCAAGC CGGGCACCAT GCCGAACAGC ATCAATTTTT ACCAGATGAA 1200

TCAGGACCTG CAGGGTCTGA CGTCCTTGCA GGTCATGGAC AAAATCGTCG CGTACGCCGG 1260

TCAGATCGGC CTGCGCATCA TTCTTGACCG CCACCGACCG GATTGCAGCG GGCAGTCGGC 1320

GCTGTGGTAC ACGAGCAGCG TCTCGGAGGC TACGTGGATT TCCGACCTGC AAGCGCTGGC 1380

GCAGCGCTAC AAGGGAAACC CGACGGTCGT CGGCTTTGAC TTGCACAACG AGCCGCATGA 1440

CCCGGCCTGC TGGGGCTGCG GCGATCCGAG CATCGACTGG CGATTGGCCG CCGAGCGGGC 1500

CGGAAACGCC GTGCTCTCGG TGAATCCGAA CCTGCTCATT TTCGTCGAAG GTGTGCAGAG 1560

CTACAACGGA GACTCCTACT GGTGGGGCGG CAACCTGCAA GGAGCCGGCC AGTACCCGGT 1620

CGTGCTGAAC GTGCCGAACC GCCTGGTGTA CTCGGCGCAC GACTACGCGA CGAGCGTCTA 1680

CCCGCAGACG TGGTTCAGCG ATCCGACCTT CCCCAACAAC ATGCCCGGCA TCTGGAACAA 1740

GAACTGGGGA TACCTCTTCA ATCAGAACAT TGCACCGGTA TGGCTGGGCG AATTCGGTAC 1800

GACACTGCAA TCCACGACCG ACCAGACGTG GCTGAAGACG CTCGTCCAGT ACCTACGGCC 1860

GACCGCGCAA TACGGTGCGG ACAGCTTCCA GTGGACCTTC TGGTCCTGGA ACCCCGATTC 1920

CGGCGACACA GGAGGAATTC TCAAGGATGA CTGGCAGACG GTCGACACAG TAAAAGACGG 1980

CTATCTCGCG CCGATCAAGT CGTCGATTTT CGATCCTGTC TAATGATCTG CATCGCCTAG 2040

CAGTCAACCG TCCCCGTCGG TGTCGCCGTC TCCGTCGCCG AGCCCGTCGG CGAGTCGGAC 2100

GCCGACGCCT ACTCCGACGC CGACAGCCAG CCCGACGCCA ACGCTGACCC CTACTGCTAC 2160

GCCCACGCCC ACGGCAAGCC CGACGCCGTC ACCGACGGCA GCCTCCGGAG CCCGCTGCAC 2220

CGCGAGTTAC CAGGTCAACA GCGATTGGGG CAATGGCTTC ACGGTAACGG TGGCCGTGAC 2280

AAATTCCGGA TCC 2293

WHAT IS CLAIMED IS:

1. A DNA comprising the nucleotide sequence of SEQ ID NO: 12, wherein the DNA encodes a truncated *Acidothermus cellulolyticus* E1 endoglucanase that has endoglucanase activity and exhibits an increased thermostability compared to full-size *Acidothermus cellulolyticus* E1 endoglucanase.

2. The DNA according to claim 1, encoding the amino acid sequence of SEQ ID NO. 11.

3. A vector carrying the DNA according to claim 1 and a vector sequence encoding either an origin of replication or an integration site for a host genome.

4. The vector according to claim 3 further comprising DNA encoding a signal sequence operably linked thereto.

5. The vector according to claim 3 further comprising exogenous regulatory sequences capable of causing expression of said DNA in *Pichia pastoris*; wherein *Pichia pastoris* contains the vector.

6. A DNA according to claim 1 encoding a catalytic domain of *Acidothermus cellulolyticus* E1 endoglucanase.

7. A DNA according to claim 1 encoding a catalytic domain and a linker peptide of *Acidothermus cellulolyticus* E1 endoglucanase.

8. The DNA according to claim 6 further comprising at least one domain from a cellulase gene other than *Acidothermus cellulolyticus* E1 endoglucanase.

9. The DNA according to claim 7 wherein the DNA encodes a protein having an endoglucanase activity.

10. A microorganism containing the vector of claim 4.

11. A method for producing an endoglucanase having the amino acid sequence of SEQ ID NO: 11, comprising enzymatic cleavage of a full size *Acidothermus cellulolyticus* E1 endoglucanase, wherein a cellulose binding domain is removed from the endoglucanase.

12. A method for producing an endoglucanase having the amino acid sequence of SEQ ID NO: 11, comprising inserting at least one stop codon after a nucleotide sequence encoding a catalytic domain of full size *Acidothermus cellulolyticus* E1 endoglucanase so that a linker peptide and cellulose binding domain of E1 endoglucanase are not expressed.

13. An endoglucanase comprising the amino acid sequence of SEQ ID NO: 11, wherein the endoglucanase has a molecular weight of approximately 40,000 to 42,000 daltons.

14. The DNA according to claim 2 wherein the DNA consists of the nucleotide sequence of SEQ ID NO: 12.

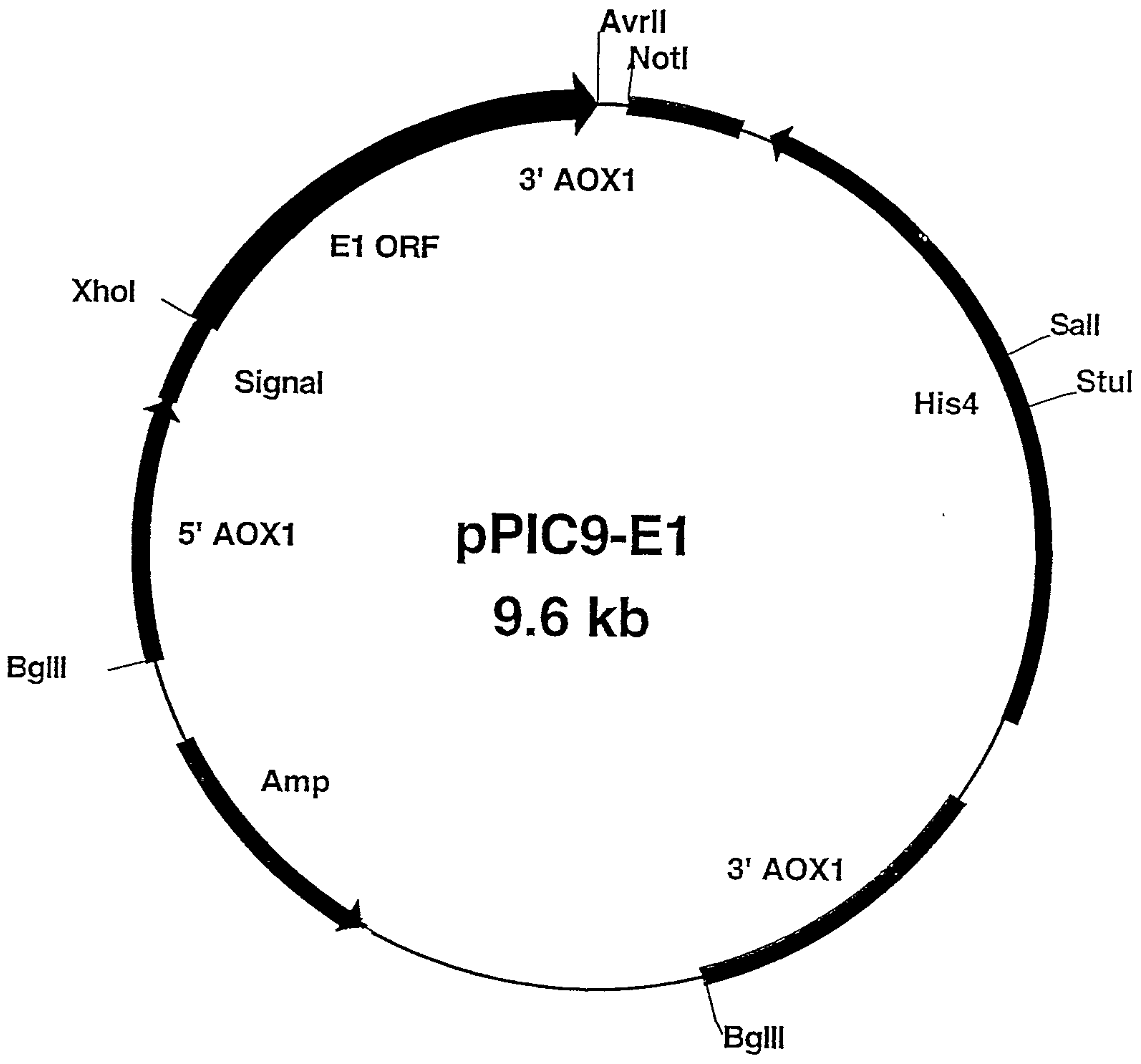

Fig. 1

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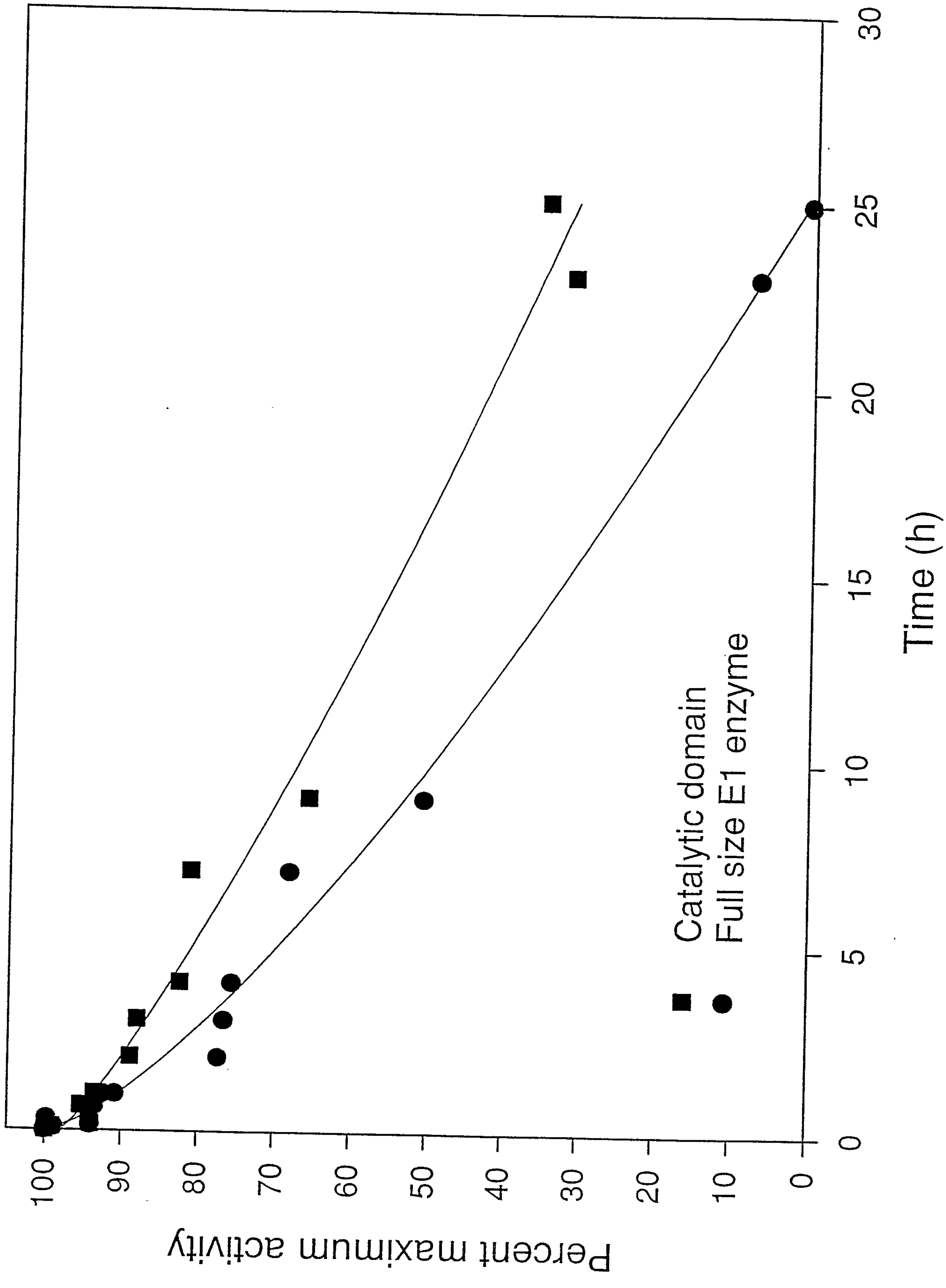

Fig. 2

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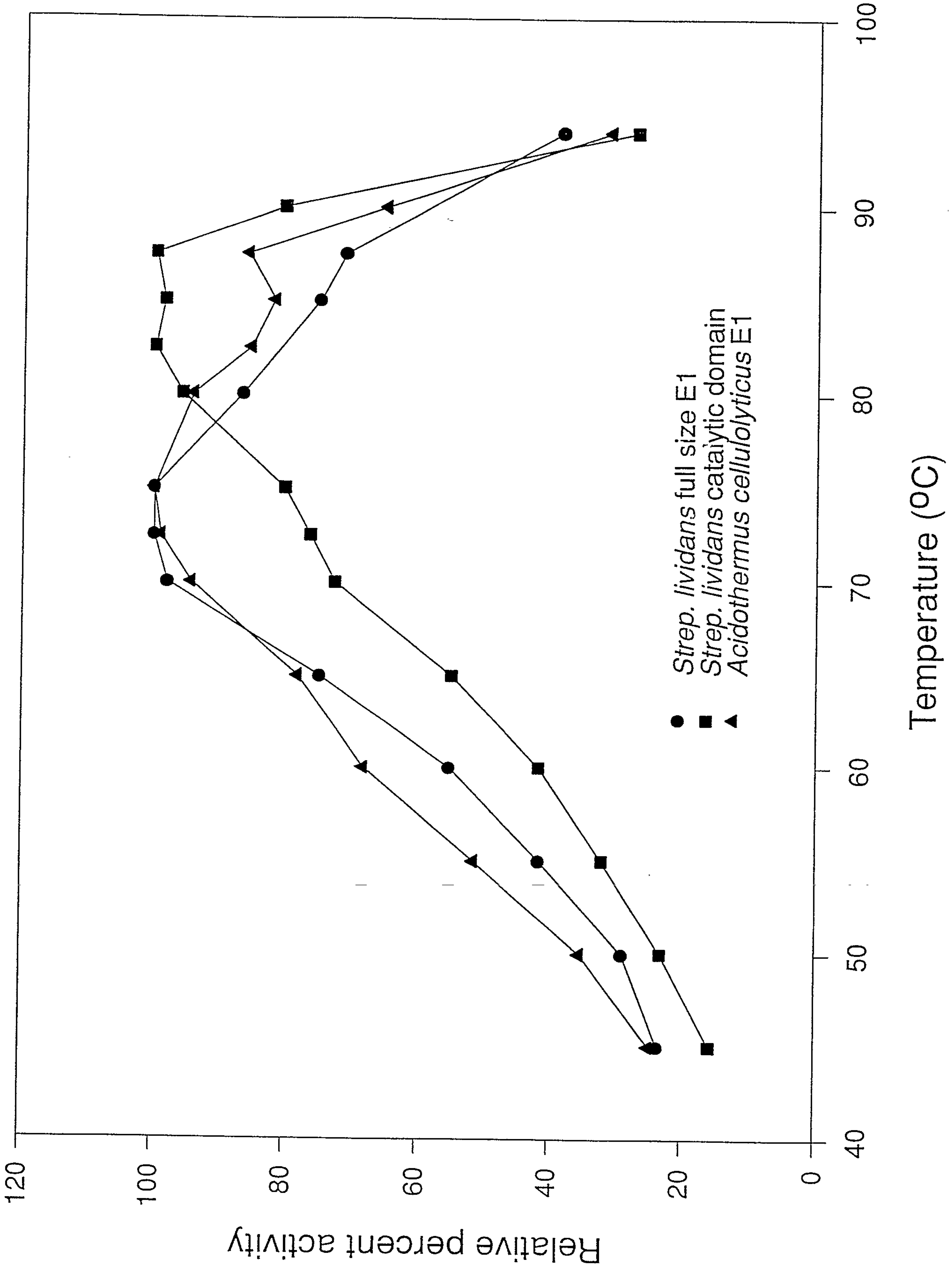

Fig. 3

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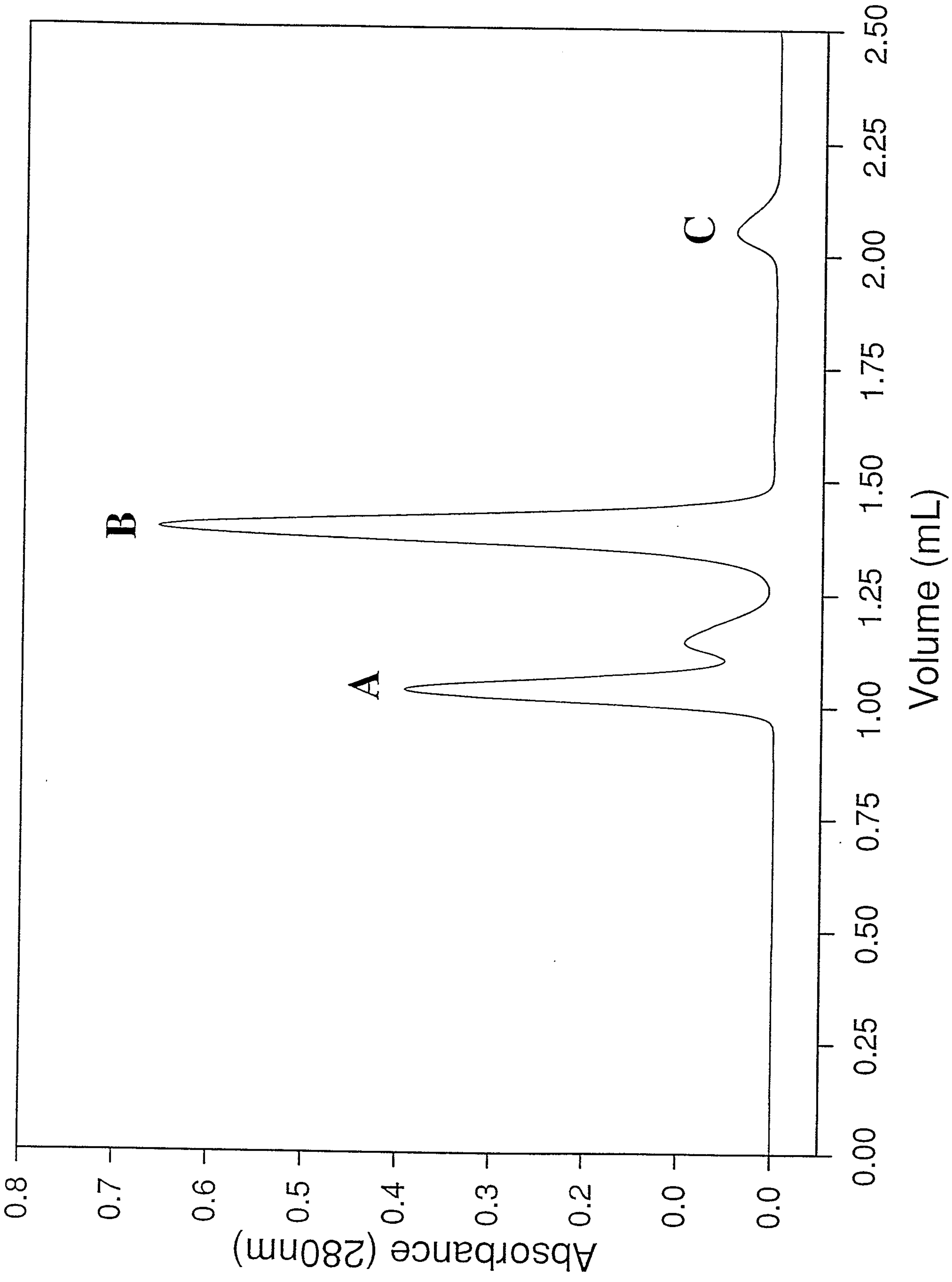

Fig. 4

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```
AGGGYWHTSG REILDANNVP VRIAGINWFG FETCNYVVHG LWSRDYRSML     50
DQIKSLGYNT IRLPYSDDIL KPGTMPNSIN FYQMNQDLQG LTSLQVMDKI    100
VAYAGQIGLR IILDRHRPDC SGQSALWYTS SVSEATWISD LQALAQRYKG    150
NPTVVGFDLH NEPHDPACWG CGDPSIDWRL AAERAGNAVL SVNPNLLIFV    200
EGVQSYNGDS YWWGGNLQGA GQYPVVLNVP NRLVYSAHDY ATSVYPQTWF    250
SDPTFPNNMP GIWNKNWGYL FNQNIAPVWL GEFGTTLQST TDQTWLKTLV    300
QYLRPTAQYG ADSFQWTFWS WNPDSGDTGG ILKDDWQTVD TVKDGYLAPI    350
KSSIFDPV                                                  358
```

Fig. 5

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```
GGATCCACGT TGTACAAGGT CACCTGTCCG TCGTTCTGGT AGAGCGGCGG GATGGTCACC     60
CGCACGATCT CTCCTTTGTT GATGTCGACG GTCACGTGGT TACGGTTTGC CTCGGCCGCG    120
ATTTTCGCGC TCGGGCTTGC TCCGGCTGTC GGGTTCGGTT TGGCGTGGTG TGCGGAGCAC    180
GCCGAGGCGA TCCCAATGAG GGCAAGGGCA AGAGCGGAGC CGATGGCACG TCGGGTGGCC    240
GATGGGGTAC GCCGATGGGG CGTGGCGTCC CCGCCGCGGA CAGAACCGGA TGCGGAATAG    300
GTCACGGTGC GACATGTTGC CGTACCGCGG ACCCGGATGA CAAGGGTGGG TGCGCGGGTC    360
GCCTGTGAGC TGCCGGCTGG CGTCTGGATC ATGGGAACGA TCCCACCATT CCCCGCAATC    420
GACGCGATCG GGAGCAGGGC GGCGCGAGCC GGACCGTGTG GTCGAGCCGG ACGATTCGCC    480
CATACGGTGC TGCAATGCCC AGCGCCATGT TGTCAATCCG CCAAATGCAG CAATGCACAC    540
ATGGACAGGG ATTGTGACTC TGAGTAATGA TTGGATTGCC TTCTTGCCGC CTACGCGTTA    600
CGCAGAGTAG GCGACTGTAT GCGGTAGGTT GGCGCTCCAG CCGTGGGCTG GACATGCCTG    660
CTGCGAACTC TTGACACGTC TGGTTGAACG CGCAATACTC CCAACACCGA TGGGATCGTT    720
CCCATAAGTT TCCGTCTCAC AACAGAATCG GTGCGCCCTC ATGATCAACG TGAAAGGAGT    780
ACGGGGGAGA ACAGACGGGG GAGAAACCAA CGGGGGATTG GCGGTGCCGC GCGCATTGCG    840
GCGAGTGCCT GGCTCGCGGG TGATGCTGCG GGTCGGCGTC GTCGTCGCGG TGCTGGCATT    900
GGTTGCCGCA CTCGCCAACC TAGCCGTGCC GCGGCCGGCT CGCGCCGCGG GCGGCGGCTA    960
TTGGCACACG AGCGGCCGGG AGATCCTGGA CGCGAACAAC GTGCCGGTAC GGATCGCCGG   1020
CATCAACTGG TTTGGGTTCG AAACCTGCAA TTACGTCGTG CACGGTCTCT GGTCACGCGA   1080
CTACCGCAGC ATGCTCGACC AGATAAAGTC GCTCGGCTAC AACACAATCC GGCTGCCGTA   1140
CTCTGACGAC ATTCTCAAGC CGGGCACCAT GCCGAACAGC ATCAATTTTT ACCAGATGAA   1200
TCAGGACCTG CAGGGTCTGA CGTCCTTGCA GGTCATGGAC AAAATCGTCG CGTACGCCGG   1260
TCAGATCGGC CTGCGCATCA TTCTTGACCG CCACCGACCG GATTGCAGCG GGCAGTCGGC   1320
GCTGTGGTAC ACGAGCAGCG TCTCGGAGGC TACGTGGATT TCCGACCTGC AAGCGCTGGC   1380
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CGTGCTGAAC GTGCCGAACC GCCTGGTGTA CTCGGCGCAC GACTACGCGA CGAGCGTCTA   1680
CCCGCAGACG TGGTTCAGCG ATCCGACCTT CCCCAACAAC ATGCCCGGCA TCTGGAACAA   1740
GAACTGGGGA TACCTCTTCA ATCAGAACAT TGCACCGGTA TGGCTGGGCG AATTCGGTAC   1800
GACACTGCAA TCCACGACCG ACCAGACGTG GCTGAAGACG CTCGTCCAGT ACCTACGGCC   1860
GACCGCGCAA TACGGTGCGG ACAGCTTCCA GTGGACCTTC TGGTCCTGGA ACCCCGATTC   1920
CGGCGACACA GGAGGAATTC TCAAGGATGA CTGGCAGACG GTCGACACAG TAAAAGACGG   1980
CTATCTCGCG CCGATCAAGT CGTCGATTTT CGATCCTGTC TAATGATCTG CATCGCCTAG   2040
CAGTCAACCG TCCCCGTCGG TGTCGCCGTC TCCGTCGCCG AGCCCGTCGG CGAGTCGGAC   2100
GCCGACGCCT ACTCCGACGC CGACAGCCAG CCCGACGCCA ACGCTGACCC CTACTGCTAC   2160
GCCCACGCCC ACGGCAAGCC CGACGCCGTC ACCGACGGCA GCCTCCGGAG CCCGCTGCAC   2220
CGCGAGTTAC CAGGTCAACA GCGATTGGGG CAATGGCTTC ACGGTAACGG TGGCCGTGAC   2280
AAATTCCGGA TCC                                                      2293
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Fig. 6

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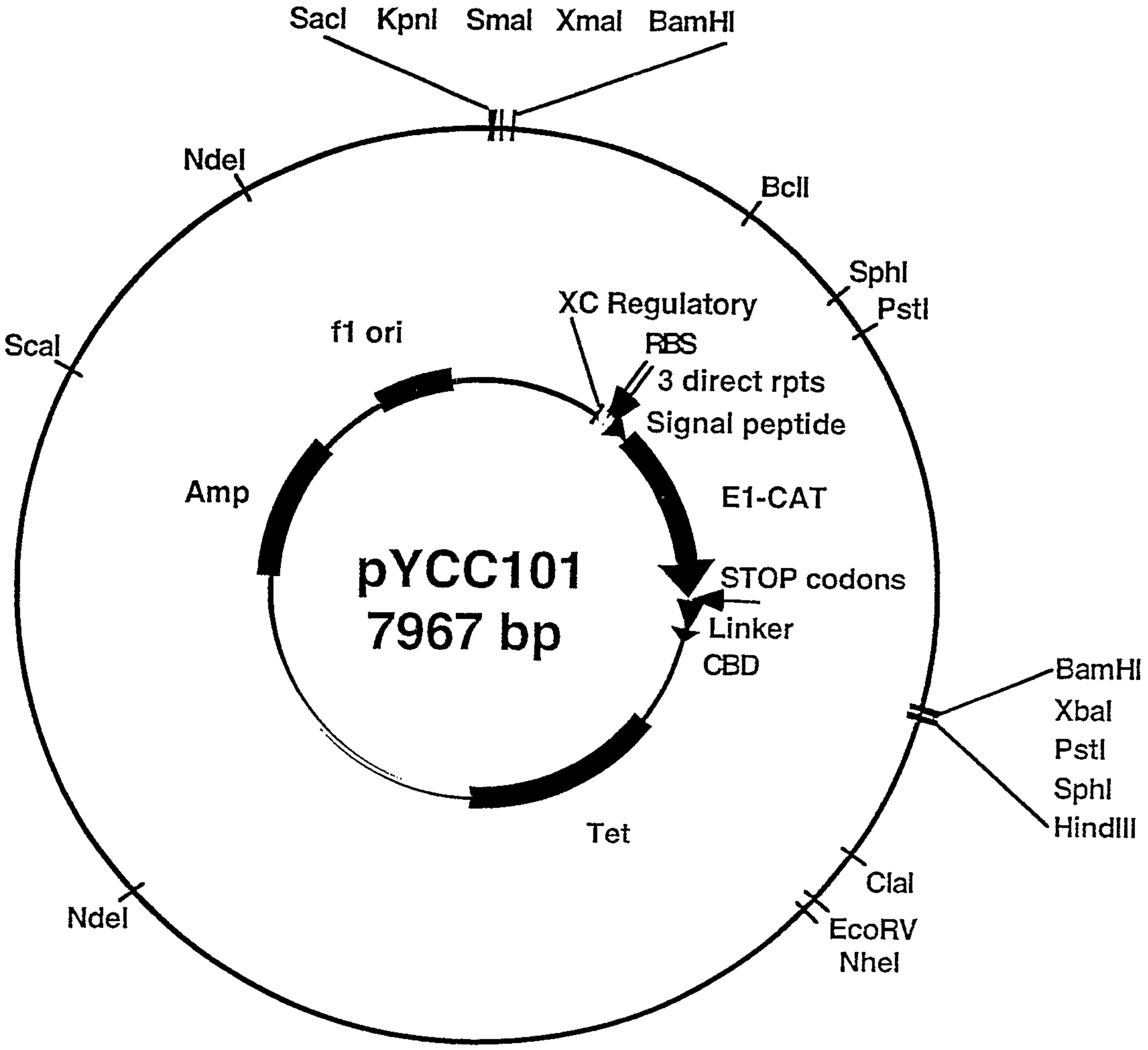

Fig. 7

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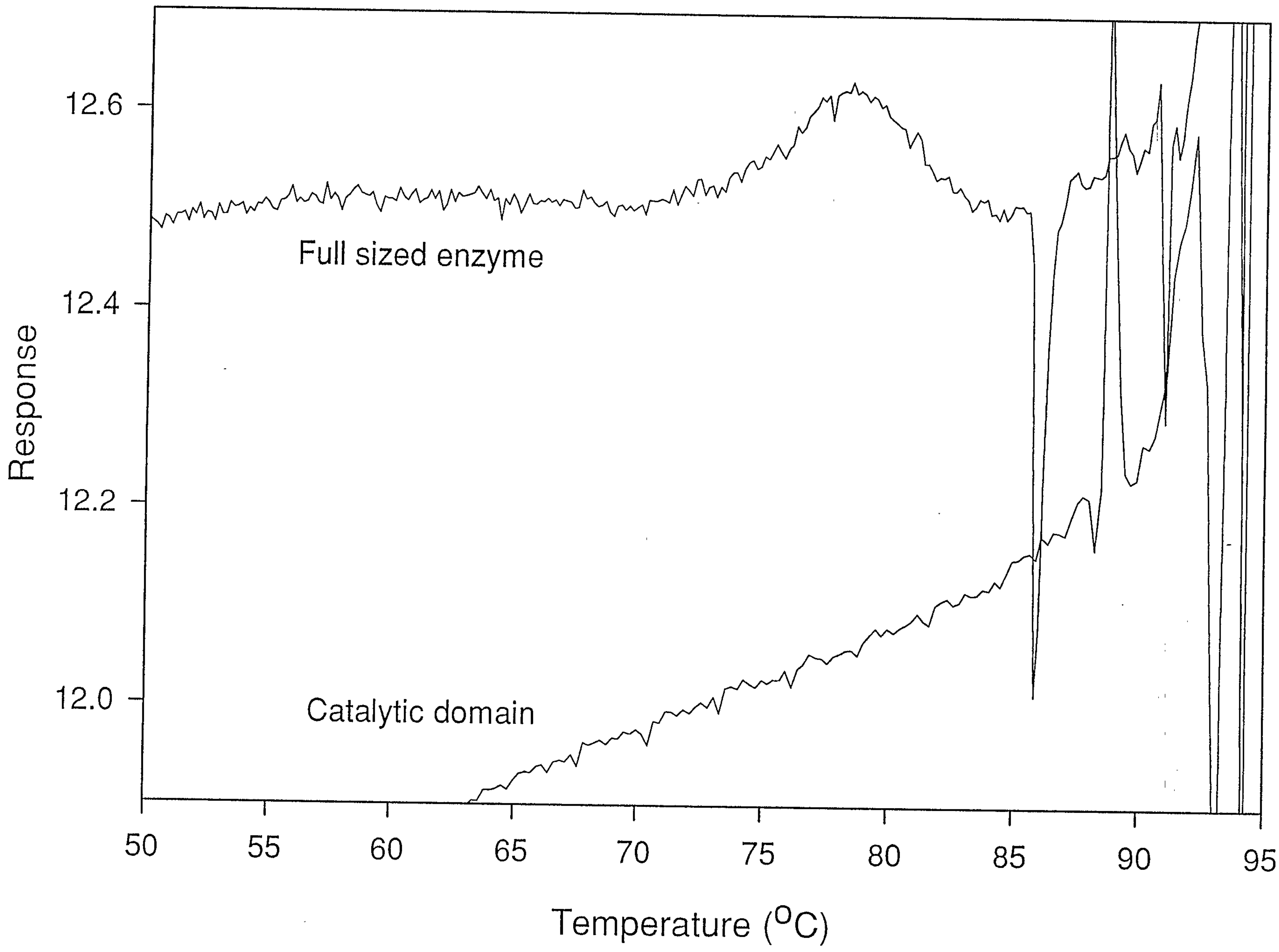

Fig. 8

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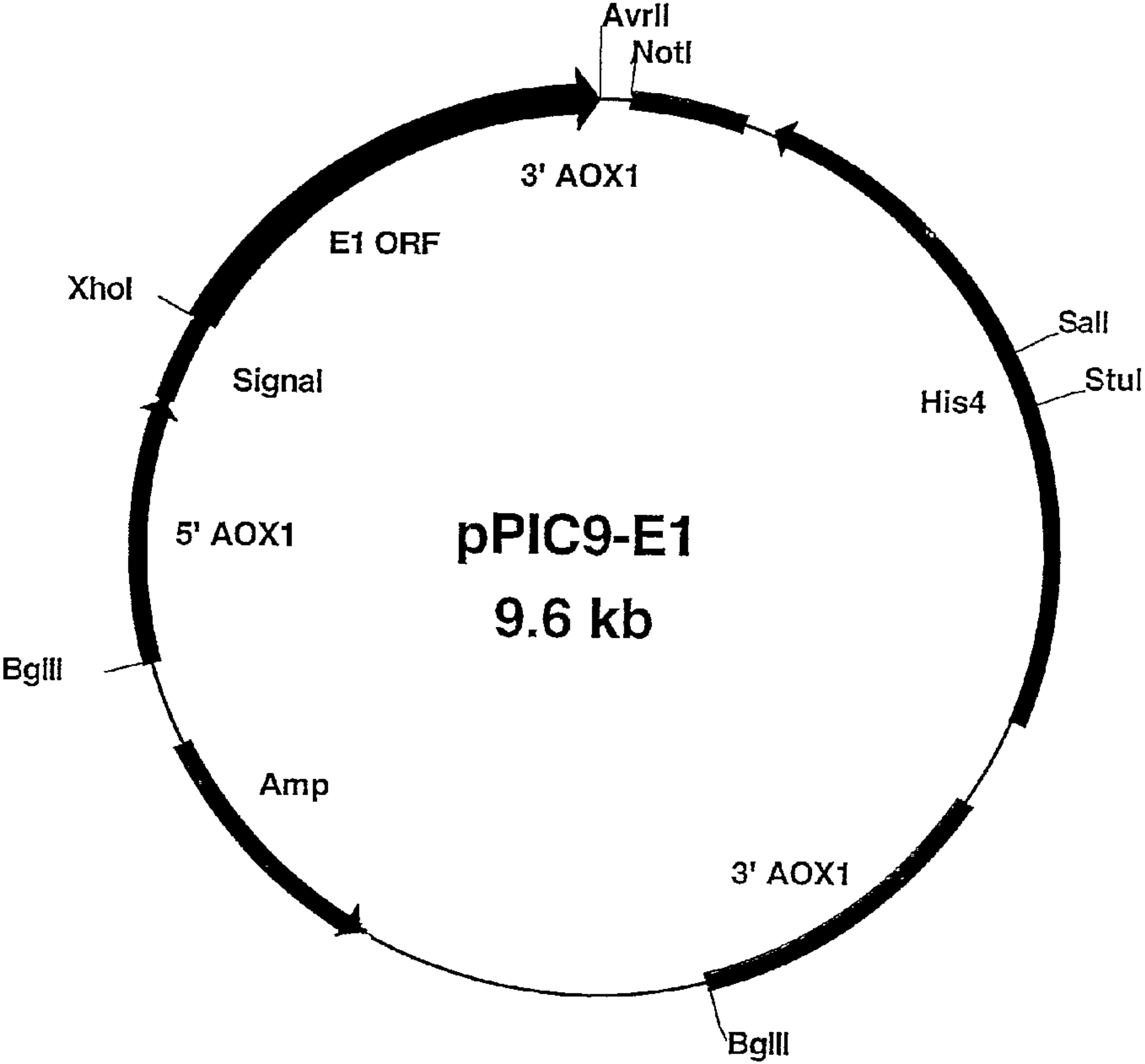
AvrII
NotI
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E1 ORF
XhoI
Signal
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9.6 kb
BglII
Amp
SalI
StuI
His4
3' AOX1
BglII