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(54) Title: NOVEL LACCASE ENZYMES AND THEIR USES

(57) Abstract: The present invention relates to novel laccase enzymes obtainable from the strains of the genus *Thielavia* or from the strains of the genus *Chaetomium*. The invention relates also to nucleic acid sequences encoding the enzymes, recombinant hosts into which the nucleic acid sequences have been introduced and to methods for the production of the enzymes in recombinant hosts. The enzymes of the invention are suitable for several applications, for example for treating denim and for strain removal.

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NOVEL LACCASE ENZYMES AND THEIR USES

FIELD OF THE INVENTION

This invention relates to novel laccase enzymes useful in many applications. This invention relates also to nucleic acids encoding the enzymes, vectors, host cells and methods for producing the enzymes as well as enzyme preparations comprising the enzymes. Furthermore, this invention relates to methods for treating denim, methods for stain removal, methods for treating natural or man-made fibre or lignocellulosic fibre, methods for treating wool, methods for treating hair and methods for bleaching pulp and dye house effluents and methods for decolorizing dyes. This invention relates also to various uses and compositions, which can be used in the mentioned applications.

BACKGROUND OF THE INVENTION

Laccases (EC. 1.10.3.2 *p*-benzenediol:oxygen oxidoreductase) belong to a family of multi-copper oxidases. Laccases are widely distributed enzymes in higher plants, fungi, some insects and bacteria. They are characterized by low substrate specificity, oxidizing various substrate, including diphenols, polyphenols, different substituted phenols, diamines, aromatic amines, and even inorganic compounds like iodine. Laccases oxidize their substrates by a one-electron oxidation mechanism, and they use molecular oxygen as an electron acceptor. Among laccases the primary sequence, induction mechanism, physico-chemical (e.g. isoelectric point and carbohydrate content) and biochemical characteristics are variable. The copper binding sites of laccases are, however, strictly conserved.

Several laccase proteins and genes encoding these laccases have been previously isolated. For example WO 01/92498 describes a fungal laccase enzyme isolated from *Melanocarpus albomyces* strain, the patent publication EP 0765394 B1 (corresponding U.S. Patent No. 5,981,243) describes the cloning of a laccase gene from *Myceliophthora thermophila* and its expression in *Aspergillus* and U.S. Patent No. 5,750,388 describes the cloning of a laccase gene from *Scytalidium thermophilum* and its expression in *Aspergillus*.

Chefetz et al. (1998a) describe isolation and preliminary characterization of a laccase from composted municipal soil waste. The microbe producing this laccase was later identified as *Chaetomium thermophilum*, and the enzyme was further purified and characterized (Chefetz et al., 1998b). The reported enzyme had pI 5.1. The laccase was stable for 1 h at 70°C and had half-lives of 24 and 12 h at

40 and 50°C, respectively. The enzyme was stable at pH 5 to 10 and the pH optimum was 6. Saito et al. (2003) describe purification and characterization of an extracellular laccase of a fungus from family *Chaetomiaceae*. The molecular mass of the enzyme was approximately 73 to 80 kDa and pI of 3.5. The optimum pH for the oxidation of syringaldazine was 7.0 and the optimum temperature was 42°C.
5 The laccase was stable for up to 288 h at 4°C and its respective half-life times at 25 and 40°C were estimated to be 150 and 20 h.

Laccases have many industrially potential applications, such as delignification of wood pulps, methods for treating lignin containing fibers, methods for treating wood fibers in order to functionalize them or glue the fibers, improval of the production of fuel ethanol from renewable raw materials, food
10 applications (for example in baking or clarification of beer or wine), various bioremediative processes and textile applications, such as denim treatment, stain removal, treatment of various fibers for textile industry, methods for decolorizing dyes and methods for treating dye house effluents, or use in hair dyeing composition, in hard-surface cleaning or in detergent formulations.

"Stone washed" look or an abraded look has been denim producers' interest in recent years. Traditional
15 stone washing with pumice stones reduces the strength of fabric and burdens the laundering apparatuses. Past years the trend has been towards enzymatic denim finishing processes. "Bleached look" of denim is normally obtained by means of bleaching chemicals, e.g. sodium hypochlorite. So far bleaching with hypochlorite has been the most efficient bleaching method for denim dyed with Indigo, since almost all shades can be obtained. However, hypochlorite process is environmentally very harmful, it is difficult to
20 control and it damages the fabric easily. It is also very inconvenient or even harmful method for the user, it cannot be used for Lycra containing products and antichlor treatment with several rinsing/washing steps is required. There is thus a need for development of ecologically less harmful alternative for sodium hypochlorite, in particular laccases have been studied for that purpose.

WO 97/25468 describes the use of laccase in a method for providing to dyed denim an abraded look. The
25 method comprises a cellulase treatment and simultaneous or subsequent treatment with a phenol oxidizing enzyme, such as laccase, and an enhancing agent, such as methylsyringate. *Myceliophthora thermophila* laccase is the example of laccases in the patent publication.

In textile industry new materials, finishes and dyes have been developed in recent years. Although the new developments have many advantageous properties, such as easy drying, stain and water resistance,
30 or bright colours of the textiles, their disadvantage quite often is that they must be washed at low temperatures. Low temperatures are preferred also for economical reasons, since the use of low temperatures saves energy. There is thus a need for laccases which function at low temperatures.

Even though numerous publications describing laccases from various microorganisms are available, there is still a need for novel laccases, which would function more effectively and be more suitable for the various conditions in different applications.

5 SUMMARY OF THE INVENTION

It is an aim of the present invention to eliminate at least some of the problems associated with the prior art. In particular, it is an aim of this invention to provide novel laccase enzymes having varying properties suitable for different applications.

10 This invention is based on the surprising finding that laccase enzymes having novel and diverse properties can be isolated from the same genus, species and even from the same strain. Some of the laccases are in particular suitable for use at relatively low temperatures.

One object of this invention is a laccase enzyme, which comprises the amino acid sequence SEQ ID 41 (TaLcc2) or a sequence showing at least 60 % identity to the sequence SEQ ID NO: 41.

15 More specifically the laccase enzyme of this invention is characterized by what is stated in the characterizing part of claim 1.

The enzyme is preferably obtainable from a microorganism, more preferably from a filamentous fungus, in particular from the genus *Thielavia*, more specifically from the species *Thielavia arenaria*. Advantageously, the enzyme is obtainable from the strain CBS 116071 deposited on 2 September 2004 at Centraalbureau voor Schimmelcultures, Uppsalalaan 8, 3584 CT, Utrecht, the Netherlands.

20 The enzyme functions at broad pH range from pH 3 to 9, preferably at pH 4 to 8, most preferably at pH 4.5 to 6.5. The enzyme functions also at broad temperature range. For example, in denim treatment the enzyme is effective at 30 to 80 °C, preferably at temperatures 40 to 60°C. The enzyme is most active at temperatures 40 to 50°C and is thus very useful in applications, where lower temperatures are more advantageous.

25 In particular, in denim treatment laccases that can be used at low temperatures have advantages over laccases which function better in conventional temperatures, such as about 60°C. Lower temperatures save energy and are more economical.

The laccase enzymes of the present invention are suitable also for other applications, where low temperatures are more advantageous. Such applications are for example other textile applications, such as stain removal, or for example hair dyeing.

One object of this invention is also a laccase enzyme, which comprises the amino acid sequence SEQ ID NO: 43 (TaLcc3) or a sequence showing at least 58 % identity to the sequence SEQ ID NO: 43.

More specifically the laccase enzyme of this invention is characterized by what is stated in the characterizing part of claim 17.

The enzyme is preferably obtainable from a microorganism, more preferably from a filamentous fungus, in particular from the genus *Thielavia*, more specifically from the species *Thielavia arenaria*. Advantageously, the enzyme is obtainable from the strain CBS 116071 deposited on 2 September 2004 at Centraalbureau voor Schimmelcultures, Uppsalalaan 8, 3584 CT, Utrecht, the Netherlands.

The enzyme functions at pH 3.5 to 7.5, preferably at pH 4 to 6.5.

One object of this invention is also a laccase enzyme, which comprises the amino acid sequence SEQ ID NO: 45 (TaLcc4) or a sequence showing at least 78 % identity to the sequence SEQ ID NO: 45.

More specifically the laccase enzyme of this invention is characterized by what is stated in the characterizing part of claim 23.

The enzyme is preferably obtainable from a microorganism, more preferably from a filamentous fungus, in particular from the genus *Thielavia*, more specifically from the species *Thielavia arenaria*. Advantageously, the enzyme is obtainable from the strain CBS 116071 deposited on 2 September 2004 at Centraalbureau voor Schimmelcultures, Uppsalalaan 8, 3584 CT, Utrecht, the Netherlands.

The enzyme functions at pH 3.5 to 7.5, more preferably at pH 4 to 7, most preferably at pH 5 to 6.5.

One object of this invention is also a laccase enzyme, which comprises the amino acid sequence SEQ ID NO: 47 (CtLcc1) or a sequence showing at least 74 % identity to the sequence SEQ ID NO: 47.

More specifically the laccase enzyme of this invention is characterized by what is stated in the characterizing part of claim 29.

The enzyme is preferably obtainable from a microorganism, more preferably from a filamentous fungus, in particular from the genus *Chaetomium*, preferably from the species *Chaetomium thermophilum*. Advantageously, the enzyme is obtainable from the strain CBS 730.95 deposited on November 8, 1995 at the Centraalbureau Voor Schimmelcultures at Oosterstraat 1, 3742 SK BAARN, The Netherlands.

The enzyme functions at pH 3.5 to 8, preferably at pH 4 to 7, most preferably at pH 4.5 to 6. For example, in denim treatment the enzyme is effective at temperatures 30 to 80 °C, preferably at 40 to 70 °C, most preferably at 50 to 60 °C.

5 One object of this invention is a laccase enzyme, which comprises the amino acid sequence SEQ ID 49 (CtLcc2) or a sequence showing at least 55 % identity to the sequence SEQ ID NO: 49.

More specifically the laccase enzyme of this invention is characterized by what is stated in the characterizing part of claim 42.

10 The enzyme is preferably obtainable from a microorganism, more preferably from a filamentous fungus, in particular from the genus *Chaetomium* preferably from the species *Chaetomium thermophilum*. Advantageously, the enzyme is obtainable from the strain CBS 730.95 deposited on November 8, 1995 at the Centralbureau Voor Schimmelcultures at Oosterstraat 1, 3742 SK BAARN, The Netherlands.

One object of this invention a laccase enzyme, which comprises the amino acid sequence SEQ ID 51 (CtLcc3) or a sequence showing at least 53 % identity to the sequence SEQ ID NO: 51.

15 More specifically the laccase enzyme of this invention is characterized by what is stated in the characterizing part of claim 46.

The enzyme is preferably obtainable from a microorganism, more preferably from a filamentous fungus, in particular from the genus *Chaetomium* preferably from the species *Chaetomium thermophilum*. Advantageously, the enzyme is obtainable from the strain CBS 730.95 deposited on November 8, 1995 at the Centralbureau Voor Schimmelcultures at Oosterstraat 1, 3742 SK BAARN, The Netherlands.

20 The present invention relates in particular to laccase enzymes, which show at least 60 % identity to the amino acid sequence SEQ ID NO:41 (TaLcc2), laccase enzymes, which show at least 58 % identity to the amino acid sequence SEQ ID NO:43 (TaLcc3), laccase enzymes, which show at least 78 % identity to the amino acid sequence SEQ ID NO:45 (TaLcc4) and laccase enzymes, which show at least 74 % identity to the amino acid sequence SEQ ID NO:47 (CtLcc1) and which are most effective at temperature
25 40 to 60 °C.

One object of this invention is also a nucleic acid sequence, which encodes at least one of the enzymes of the invention.

The nucleic acid sequence is characterized by what is stated in the characterizing part of claim 56.

30 Further objects of this invention are a vector comprising the nucleic acid sequence and a host comprising the nucleic acid sequence or the vector, and a process for the production of a polypeptide having laccase activity.

One further object of the invention is a process for obtaining an enzyme preparation comprising the polypeptide or enzyme, which comprises the steps of culturing a host cell comprising the nucleic acid sequence encoding the enzyme or a vector comprising the nucleic acid sequence and either recovering the polypeptide from the cells or separating the cells from the culture medium and obtaining the supernatant. Furthermore, an object of the invention is the enzyme preparation comprising the laccase enzyme of the invention.

One object of this invention is a method for treating denim, which comprises contacting denim in an aqueous medium with the laccase enzyme or enzyme preparation of the invention under suitable conditions for the function of the enzyme.

One object of this invention is a method for removing stains, which comprises that material to be treated with the method is contacted with a laccase enzyme of the present invention under suitable conditions for the function of the enzyme.

This invention provides also a method of bleaching pulp, for treating natural or man-made fibers, a method for treating wool, a method for treating hair, a method for treating dye house effluents and a method for decolorizing dyes by using the laccase enzyme of the present invention.

Still further objects of this invention are uses of laccase enzyme of the present invention in various applications and compositions.

By using the laccase enzymes of this invention in denim bleaching it is possible to obtain many advantages. By using the laccase enzymes of this invention it is possible to decrease or even avoid the environmentally harmful effects of sodium hypochlorite. If sodium hypochlorite not used, no antichlor treatment is required. By the laccases of the present invention it is also possible to obtain various shades as by sodium hypochlorite bleaching. One advantage of the laccase enzymes of the invention is that the treatment does not damage the fabric. The laccases can also be used for treating Lycra containing products. In addition, the laccase treatment is also convenient for the user.

Furthermore, the enzymes can function on a broad temperature and pH range. Some of the enzymes are in particular suitable for use at low temperatures, in particular at temperatures 40 to 50°C.

Other features, aspects and advantages of the present invention will become apparent from the following description and appended claims.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1A. Production of the *Thielavia* laccase in 50 ml shake flask cultivation.

Figure 1B. Production of the *Chaetomium* laccase in 50 ml shake flask cultivation.

Fig. 2A. SDS-PAGE (15 %) showing the purification of *Thielavia* laccase. 1 MW marker (175, 83, 62, 47, 32.5, 25, 16.5, and 6.5 kDa), 2 culture supernatant, 3 after DEAE Sepharose, 4-7 fractions after gel filtration, about 3-6 µg protein loaded on each lane. Proteins are stained with Coomassie Brilliant Blue.

Fig. 2B. SDS-PAGE (12%) from the purification of *Chaetomium* laccase. 1 culture supernatant (60 µg), 2 after ion exchange (14.5 µg), 3 MW standard (175, 83, 62, 47.5, 32.5, 25, 16.5, 6.5 kDa), 4-9 fractions from HIC (3.5, 3.3, 3.1, 2.4, 2.0, and 1.5 µg), 10 culture supernatant (30 µg).

Fig. 3A. pH optima of the purified *Thielavia* laccase (P TL) and the crude enzyme (CE) determined on guaiacol.

Fig. 3B. pH optima of the purified *Chaetomium* laccase (P TL) and the crude enzyme (CE) determined on guaiacol.

Fig. 4A. Thermal stability of *Thielavia* laccase at 50 and 60°C.

Fig. 4B. Thermal stability of *Chaetomium* laccase at 50 and 60°C.

Fig. 5. The peptide sequences used in the planning of the PCR primers for cloning the *Thielavia arenaria* ALKO4197 and *Chaetomium thermophilum* ALKO4265 laccase genes. All possible codons to encode the sequences are shown. A. The homologous peptide sequences chosen from alignment of several fungal laccase sequences. The first methionine in Peptides II and III (in parenthesis) was not present in all the laccase sequences. B. The tryptic peptide sequences obtained from the purified *Thielavia arenaria* ALKO4197 TaLcc1. C. The N-terminal sequence and the tryptic peptide sequences obtained from the purified *Chaetomium thermophilum* ALKO4265 CtLcc1.

Fig. 6 A-G. The nucleotide and the deduced amino acid sequences of the *Thielavia arenaria* ALKO4197 and *Chaetomium thermophilum* ALKO4265 laccase genes. The stop codon is shown by an asterisk below the sequence. The location of the putative introns and the consensus intron splicing signals (5' GTPuNGPy, 3' PyAG, internal NNCTPuAPy) are marked in the sequence by using lowercase letters and by bolding, respectively. The putative signal peptides, analyzed by SignalP V2.0 program, and the mature C-terminal amino acid sequences, determined from the purified recombinant TaLcc1 and TaLcc2 proteins, are underlined. A double underlining is used for the other potential signal sequence encoded by

the longer *Talcc2* gene. The location of the N-terminal peptide from CtLcc1 and the tryptic peptide sequences obtained from purified TaLcc1 and CtLcc1 are marked by dotted lines below the sequences. The conserved residues involved in copper binding are highlighted. The sites for putative N-glycosylation (N-X-S/T) in are bolded. The two putative translation start sites of TaLcc2 and CtLcc3 are boxed. A. *Talcc1*, B. *Talcc2*, C. *Talcc3*, D. *Talcc4*, E. *Ctlcc1*, F. *Ctlcc2*, G. *Ctlcc3*.

Fig.7 A-C. The expression cassettes used in the transformation of *Trichoderma reesei* protoplasts for producing the recombinant fungal laccases. The laccase genes were under the control of the *cbh1* (*cel7A*) promoter (p *cbh1*) and the termination of the transcription was ensured by using the *cbh1* terminator sequence (t *cbh1*). The *amdS* gene was included as a transformation marker and the *cbh1* 3'-flanking region, together with the *cbh1* promoter, was used to enable targeting of the expression cassette into the *cbh1* locus by homologous recombination.

Fig.8. The performance of laccase preparations in denim bleaching at different pH values at conditions described in Example 7.

Fig.9. The performance of laccase preparations in denim bleaching at different temperatures at conditions described in Example 8.

Fig.10 A and B. Effect of CtLcc1 laccase on grass soiling at 60°C and TaLcc2 and TaLcc4 laccases at 50°C at conditions described in Example 9. Mediator control without the enzyme for both temperatures. A. Lightness values, B. a*-values (-a is the green direction, +a is the red direction)

Fig. 11 A and B. Effect of CtLcc1 laccase on tea soiling at 60°C and TaLcc2 and TaLcc4 laccases at 50°C at conditions described in Example 9. Mediator control without the enzyme for both temperatures. A. Lightness values, B. a*-values (-a is the green direction, +a is the red direction)

Fig.12 A and B. Effect of laccase preparations on grass soiling at 40°C with different dosages at conditions described in Example 10. A. Lightness values, B. a*-values (-a is the green direction, +a is the red direction)

Fig.13 A and B. Effect of laccase preparations on tea soiling at 40°C with different dosages at conditions described in Example 10. A. Lightness values, B. a*-values (-a is the green direction, +a is the red direction)

Sequences

- SEQ ID NO: 1 Sequence of Peptide 1, a tryptic peptide from *Thielavia arenaria* ALKO4197 TaLcc1 protein.
- 5 SEQ ID NO: 2 Sequence of Peptide 2, a tryptic peptide from *Thielavia arenaria* ALKO4197 TaLcc1 protein.
- SEQ ID NO: 3 Sequence of Peptide 3, a tryptic peptide from *Thielavia arenaria* ALKO4197 TaLcc1 protein.
- 10 SEQ ID NO: 4 N-terminal sequence from *Chaetomium thermophilum* ALKO4265 CtLcc1 protein.
- SEQ ID NO: 5 Sequence of Peptide 18.9, a tryptic peptide from *Chaetomium thermophilum* ALKO4265 CtLcc1 protein.
- SEQ ID NO: 6 Sequence of Peptide 22.4, a tryptic peptide from *Chaetomium thermophilum* ALKO4265 CtLcc1 protein.
- 15 SEQ ID NO: 7 Sequence of Peptide 22.7, a tryptic peptide from *Chaetomium thermophilum* ALKO4265 CtLcc1 protein.
- SEQ ID NO: 8 Sequence of the oligonucleotide primer POX1
- SEQ ID NO 9: Sequence of the oligonucleotide primer POX2
- SEQ ID NO 10: Sequence of the oligonucleotide primer POX22
- 20 SEQ ID NO 11: Sequence of the oligonucleotide primer POX3
- SEQ ID NO 12: Sequence of the oligonucleotide primer POX16
- SEQ ID NO 13: Sequence of the oligonucleotide primer POX23
- SEQ ID NO: 14 Sequence of the oligonucleotide primer POX26.
- SEQ ID NO: 15 Sequence of the oligonucleotide primer POX27.
- SEQ ID NO: 16 Sequence of the oligonucleotide primer POX28.
- 25 SEQ ID NO: 17 Sequence of the oligonucleotide primer POX29.
- SEQ ID NO: 18 Sequence of the oligonucleotide primer POX30.
- SEQ ID NO: 19 Sequence of the oligonucleotide primer POX31.
- SEQ ID NO: 20 Sequence of the oligonucleotide primer POX4
- SEQ ID NO: 21 Sequence of the oligonucleotide primer POX5
- 30 SEQ ID NO: 22 Sequence of the oligonucleotide primer POX6
- SEQ ID NO: 23 Sequence of the oligonucleotide primer POX7
- SEQ ID NO: 24 Sequence of the oligonucleotide primer POX8
- SEQ ID NO: 25 Sequence of the oligonucleotide primer POX9
- SEQ ID NO: 26 Sequence of the oligonucleotide primer POX10
- 35 SEQ ID NO: 27 Sequence of the oligonucleotide primer POX11
- SEQ ID NO: 28 Sequence of the oligonucleotide primer POX12
- SEQ ID NO: 29 Sequence of the oligonucleotide primer POX13
- SEQ ID NO: 30 Sequence of the oligonucleotide primer POX14
- SEQ ID NO: 31 Sequence of the oligonucleotide primer POX15
- 40 SEQ ID NO: 32 Sequence of the PCR fragment obtained from *Thielavia arenaria* ALKO4197 using the primers POX27 and POX31.
- SEQ ID NO: 33
- Sequence of the PCR fragment obtained from *Thielavia arenaria* ALKO4197 using the primers POX4 and POX11.
- 45 SEQ ID NO: 34
- Sequence of the PCR fragment obtained from *Thielavia arenaria* ALKO4197 using the primers POX27 and POX9.
- SEQ ID NO: 35
- Sequence of a PCR fragment obtained from *Chaetomium thermophilum* ALKO4265 using the primers
- 50 POX8 and POX11.

SEQ ID NO: 36

Sequence of the PCR fragment obtained from *Chaetomium thermophilum* ALKO4265 using the primers POX4 and POX9.

SEQ ID NO: 37

- 5 Sequence of a PCR fragment obtained from *Chaetomium thermophilum* ALKO4265 using the primers POX8 and POX11.

SEQ ID NO: 38

The nucleotide sequence of the *Thielavia arenaria* ALKO4197 *Talcc1* gene.

SEQ ID NO: 39

- 10 The deduced amino acid sequence of the *Thielavia arenaria* ALKO4197 *TaLcc1*.

SEQ ID NO: 40

The nucleotide sequence of the *Thielavia arenaria* ALKO4197 *Talcc2* gene.

SEQ ID NO: 41

The deduced amino acid sequence of the *Thielavia arenaria* ALKO4197 *TaLcc2*.

- 15 SEQ ID NO: 42

The nucleotide sequence of the *Thielavia arenaria* ALKO4197 *Talcc3* gene.

SEQ ID NO: 43

The deduced amino acid sequence of the *Thielavia arenaria* ALKO4197 *TaLcc3*.

SEQ ID NO: 44

- 20 The nucleotide sequence of the *Thielavia arenaria* ALKO4197 *Talcc4* gene.

SEQ ID NO: 45

The deduced amino acid sequence of the *Thielavia arenaria* ALKO4197 *TaLcc4*.

SEQ ID NO: 46

The nucleotide sequence of the *Chaetomium thermophilum* ALKO4265 *Ctlcc1* gene.

- 25 SEQ ID NO: 47

The deduced amino acid sequence of the *Chaetomium thermophilum* ALKO4265 *CtLcc1*.

SEQ ID NO: 48

The nucleotide sequence of the *Chaetomium thermophilum* ALKO4265 *Ctlcc2* gene.

SEQ ID NO: 49

- 30 The deduced amino acid sequence of the *Chaetomium thermophilum* ALKO4265 *CtLcc2*.

SEQ ID NO: 50

The nucleotide sequence of the *Chaetomium thermophilum* ALKO4265 *Ctlcc3* gene.

SEQ ID NO: 51

The deduced amino acid sequence of the *Chaetomium thermophilum* ALKO4265 *CtLcc3*.

35

Depositions

Thielavia arenaria ALKO4197 was deposited at the Centraalbureau Voor Schimmelcultures at Upsalalaan 8, 3584 CT, Utrecht, the Netherlands on 2 September 2004 and assigned accession number CBS 116071.

- 40 *Chaetomium thermophilum* ALKO4265 was deposited at the Centraalbureau Voor Schimmelcultures at Oosterstraat 1, 3742 SK BAARN, The Netherlands on November 8, 1995 and assigned accession number CBS 730.95. After termination of the current deposit period, samples will be stored under agreements as to make the strain available beyond the enforceable time of the patent.

The *E.coli* strain including the plasmid pALK1342 was deposited at the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ), Mascheroder Weg 1 b, D-38124 Braunschweig, Germany on 7 March 2003 and assigned accession number DSM 15484.

5 The *E.coli* strain including the plasmid pALK1347 was deposited at the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ), Mascheroder Weg 1 b, D-38124 Braunschweig, Germany on 7 March 2003 and assigned accession number DSM 15486.

The *E.coli* strain including the plasmid pALK1345 was deposited at the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ), Mascheroder Weg 1 b, D-38124 Braunschweig, Germany on 7 March 2003 and assigned accession number DSM 15485.

10 The *E.coli* strain including the plasmid pALK1664 was deposited at the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ), Mascheroder Weg 1 b, D-38124 Braunschweig, Germany on 7 March 2003 and assigned accession number DSM 15487.

15 The *E.coli* strain including the plasmid pALK1304 was deposited at the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ), Mascheroder Weg 1 b, D-38124 Braunschweig, Germany on 27 June 2002 and assigned accession number DSM 15075.

The *E.coli* strain including the plasmid pALK1305 was deposited at the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ), Mascheroder Weg 1 b, D-38124 Braunschweig, Germany on 27 June 2002 and assigned accession number DSM 15076.

20 The *E.coli* strain including the plasmid pALK1685 was deposited at the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ), Mascheroder Weg 1 b, D-38124 Braunschweig, Germany on 20 November 2003 and assigned accession number DSM 16040.

DETAILED DESCRIPTION

25 The present invention provides several laccase enzymes, which have diverse properties and which are suitable for different applications.

By "the laccase of the present invention" or "the laccases of the present invention" is here meant the group of laccases as defined in the claims and described herein.

By "laccase enzyme" is in connection of this invention meant an enzyme classified as EC 1.10.3.2 by the enzyme nomenclature. The laccase enzyme may originate from any organism including plants,

preferably it may originate from microorganisms. It may originate from bacteria, for example from a genus selected from the group comprising *Bacillus*, *Azospirillum* and *Streptomyces*. Preferably the enzyme originates from fungi (including filamentous fungi and yeasts), for example from a genus selected from the group comprising *Thielavia*, *Chaetomium*, *Achaetomium*, *Aspergillus*, *Botrytis*,
5 *Collybia*, *Fomes*, *Humicola*, *Hypocrea*, *Lentinus*, *Melanocarpus*, *Myceliophthora*, *Neurospora*, *Phlebia*, *Pleurotus*, *Podospora*, *Polyporus*, *Rhizoctonia*, *Scytalidium*, *Pycnoporus*, *Trametes* and *Trichoderma*.

According to a preferred embodiment of the invention the laccases of the present invention are obtainable from genus *Thielavia*, more preferably from *Thielavia arenaria*. According to a most preferred embodiment of the invention the enzyme is obtainable from a strain deposited at
10 Centraalbureau voor Schimmelcultures under number CBS 116071.

According to another preferred embodiment of the invention the laccases of the present invention are obtainable from genus *Chaetomium*, more preferably from *Chaetomium thermophilum*. According to a most preferred embodiment of the invention the enzyme is obtainable from a strain deposited at Centraalbureau voor Schimmelcultures under number CBS 730.95.

15 The origin of the laccases of the present invention is not restricted to genus *Thielavia* or to the species *T. arenaria* or to *Chaetomium* or to species *C. thermophilum*. By using the description provided herein, a person skilled in the art can find and isolate laccases of the present invention from other genera of fungi, from other microorganisms and also from higher organisms, such as plants.

Laccase of the present invention can be isolated from any organism producing laccase. Preferably the
20 laccase enzyme of the present invention is isolated from a microbial source. Organisms capable of producing laccase can be screened, the activity on various substrates can be determined, and the enzyme characterized. For example, the pH and temperature ranges, where the enzyme functions, pH and temperature optima, and enzyme stability in various temperatures, can be determined. Alternatively, genes encoding laccases in various organisms can be isolated and the amino acid sequences encoded by
25 the genes can be compared with the amino acid sequence of the laccases isolated and characterized in the Examples here. This includes direct cloning from environmental samples.

Microorganisms that produce the laccase of the present invention can be isolated from nature or they can be screened from already isolated and identified strains of culture collections by using screening methods that are well known to a person skilled in the art. Screening can be carried out by studying the
30 production of the enzyme either on a solid culture on plate cultivations or in a liquid culture medium by measuring the enzyme activity. Suitable substrates for measuring the activity include ABTS, dimethoxyphenol (DMP), guaiacol, and syringaldazine. Fungi can be screened for their ability to produce

laccases for example by the methods referred in Example 1 with indicators, such as Remazol Brilliant Blue R-478 and guaiacol or ABTS. Suitable laccases can be isolated and the genes encoding them can be cloned also from higher organisms, such as plants.

5 Microorganism strains, which are found as a result of screening can be cultivated on a suitable medium, and the formation of laccase in the culture solution or plate can be observed. After a sufficient amount of laccase of interest has been produced, the enzyme can be purified and its properties can be more thoroughly characterized.

10 The produced laccase enzymes can be isolated and purified by using conventional methods of protein chemistry, such as salt precipitation, ultrafiltration, ion exchange chromatography, and hydrophobic interaction chromatography. Purification can be monitored by protein determination, enzyme activity assays and by SDS polyacrylamide gel electrophoresis. The enzyme activity of the purified enzyme at various temperatures and pH values can be determined; similarly, the molecular weight and the isoelectric point can be determined.

15 The purified enzyme refers to an enzyme preparation, which has no other proteins or very low amount of other proteins in addition to the laccase protein. The purity of the obtained laccase that is essentially free from other proteins is $\geq 90\%$.

20 The purification of the preferred laccases of the present invention has been exemplified in Example 1. Concentrated *Thielavia* culture filtrate was loaded on Q Sepharose FF column, proteins were eluted with an increasing salt gradient and laccase active fractions were loaded on Sephacryl S100 gel filtration resin. Purification was followed by activity assays and by SDS-PAGE and subsequent staining with Coomassie Brilliant Blue. In order to obtain high purity samples an additional Resource Q anion exchange step was included. The culture supernatant of *Chaetomium* laccase was concentrated and buffer changed to binding buffer by ultrafiltration. Proteins were bound to DEAE Sepharose FF, eluted with a sodium sulphate gradient and laccase positive fractions were pooled and further purified with 25 hydrophobic interaction chromatography. Finally the purity of active fractions were analysed by SDS-PAGE and subsequent Coomassie staining. Naturally, it is possible to separate the enzymes of the present invention by using other known purification methods instead, or in addition to the methods described here.

30 Molecular weight of the laccase can be determined on SDS-PAGE according to Laemmli (1970) and the isoelectric point of the laccase can be determined with isoelectric focusing and bands containing laccase activity can be visualized by staining the gel with ABTS, for example, as described in Example 2.

Determination of laccase activity at various temperatures can be carried out by using ABTS as a substrate, as described in Example 1 in accordance with the method developed by Niku-Paavola *et al.* (1988) or by other methods described in literature.

5 The pH optimum of the laccase can be determined on a suitable substrate in a suitable buffer at different pH values by measuring activity.

The thermal stability can be determined by incubating an enzyme sample for different time periods at various temperatures in a suitable buffer at a certain pH. The residual activity of the enzyme at each temperature can be defined pH values by measuring activity.

10 Specific activities of the purified laccase can be determined towards different laccase substrates, such as ABTS, di-methoxy-phenol (DMP), syringaldazine, and guaiacol.

The effect of various inhibitors on laccase activity can be determined by measuring the oxygen consumption during the enzyme reaction with ABTS, for example, in sealed and fully filled containers with oxygen electrode or following the enzyme activity by spectroscopic means in the presence of an inhibitor.

15 The N-terminus of the protein as well as the internal peptides can be sequenced according to Edman degradation chemistry [Edman and Begg (1967)] as described in Example 2 or by other methods described in the literature.

The molecular weight of the purified major laccase enzymes isolated from *Thielavia arenaria* and *Chaetomium thermophilum* culture supernatants were both approximately 80 kDa. The purified
20 *Thielavia* laccase showed multiple bands in isoelectric focusing at pIs 5.5, 5.9, 6.4, 6.8, and 6.9. The purified *Chaetomium* laccase showed 3 – 4 bands in isoelectric focusing at pIs 4.1 to 4.3.

The pH optimum for the purified *Thielavia* laccase was 5.5 determined on guaiacol, and the enzyme showed substantially high activity still at pH 7. The pH optimum for the purified *Chaetomium* laccase was at pH 5.0. The accuracy of the measurement is ± 0.5 .

25 The specific activity of the *Thielavia* laccase enzyme was the highest on ABTS, 1020 nkat/mg of protein at pH 4.5. The specific activity on DMS was 260, on syringaldazin 490 and on guaiacol 63 nkat/mg at pH 5.5. The specific activity of the *Chaetomium* laccase enzyme was the highest on ABTS, 750 nkat/mg of protein at pH 4.5. The specific activity on DMP was 290, on syringaldazin 400 and on guaiacol 85 nkat/mg at pH 5.5.

The laccase which shows advantageous properties may be either produced by the original or recombinant host by a method comprising cultivating under suitable conditions a host into which a DNA sequence encoding said laccase and sequences needed for expressing said enzyme, have been introduced, and optionally isolating the enzyme. The production host can be any organism capable of expressing the laccase. Preferably the host is a microbial cell, more preferably a fungus. Most preferably the host is a filamentous fungus. Preferably the recombinant host is modified to express and secrete laccase as its main activity or one of its main activities. The spent culture medium of the production host can be used as such, or it may be concentrated, filtrated or fractionated. It may also be dried.

Suitable expression and production host systems are for example the production system developed for the fungus host *Trichoderma* (EP 244 234), or *Aspergillus* production system, such as *A. oryzae* or *A. niger* (WO 9708325 and WO 9533386, US 5,843,745, US 5,770,418), or the production system developed for fungal species of *Fusarium*, such as *F. oxysporum* (Malardier et al., 1989). Suitable production systems developed for bacteria are a production system developed for *Bacillus*, for example *B. subtilis* or for *E. coli*, or for actinomycete *Streptomyces*. Suitable production systems developed for yeasts are systems developed for *Saccharomyces*, *Shizosaccharomyces* or *Pichia pastoris*. Production systems in some other microbes or in mammalian cell can also be used.

Preferred hosts for producing laccase enzyme of the present invention are in particular strains from genus *Trichoderma* or *Aspergillus*.

Within the scope of protection of the present invention are also vectors which can be used when the nucleic acid sequence encoding the chosen laccase are introduced into a host. Within the scope of protection are also sequences facilitating the expression and secretion of the laccase encoding sequence, such as promoters and signal sequences.

Standard molecular biology methods can be used in the cloning of the laccase enzyme i.e. in the isolation and enzyme treatments of DNA, in *E. coli* transformations, etc. The basic methods used are described in the standard molecular biology handbooks, e.g. Sambrook et al. (1989) and Sambrook and Russell (2001).

Genomic library prepared from the chosen host organism was screened with probes prepared by PCR. The sequences of the oligonucleotide primers used in the PCR reactions based on the amino acid sequences of the peptides obtained from the purified laccase enzyme produced by the natural host and on the consensus sequences of fungal laccases. The DNA products obtained were characterized by sequencing and by performing Southern blot hybridizations to the genomic *Thielavia* and *Chaetomium* DNA digested with several restriction enzymes.

Four laccase genes were isolated from *Thielavia* and three from *Chaetomium*. All these genes were included into plasmid vectors and deposited in an *E.coli* strain to the DSMZ collection. The full-length *Thielavia* laccase gene *Talcc1* was included in the plasmid pALK1342 and deposited under number DSM 15484. Accordingly, *Thielavia* laccase gene *Talcc2* was included in the plasmid pALK1347 and deposited under number DSM 15486, *Talcc3* gene was included in the plasmid pALK1345 and deposited under number DSM 15485 and *Talcc4* gene was included in the plasmid pALK1664 under number DSM 15487. *Chaetomium* laccase gene *Ctlcc1* was included in the plasmid pALK1304 and deposited under number DSM 15075. *Ctlcc2* was included in the plasmid pALK1305 and deposited under number DSM 15076. *Ctlcc3* was included in the plasmid pALK1685 and deposited under number DSM 16040. The deduced amino acid sequences of the laccases were analyzed from the DNA sequence.

The sequences of the laccase genes and deduced laccase proteins are shown Figure 6. The relevant information on the genes and the deduced amino acid sequences are summarized in Tables 8 and 9, respectively.

For example, the length of the *Talcc2* gene was 1957 bp (or 1737 bp depending on the translation start site) including the stop codon and the gene had two introns. The deduced protein sequence consisted of 589 amino acids (for the shorter deduced amino acid sequence 579 amino acids) including a predicted signal sequence of 29/24 amino acids and no "tail" after the consensus sequence DSGI. The predicted molecular mass was 61811/62274 Da for the mature polypeptide and the predicted pI was 4.65/4.65 (signal sequence removed). The deduced amino acid sequence included 12 putative N-glycosylation sites.

The length of the *Ctlcc1* gene was 2127 bp (including the stop codon) and the gene had five introns. The deduced protein sequence consisted of 607 amino acids including a predicted signal sequence of 20 amino acids and a "tail" of 13 amino acids after the consensus sequence DSGI. The predicted molecular mass was 63905Da for the mature polypeptide (signal sequence and tail not included) and the predicted pI was 6.09 (signal sequence removed). The deduced amino acid sequence included 9 putative N-glycosylation sites.

The deduced amino acid sequences of TaLcc1 and CtLcc1 were found to be the most homologous to each other, as were also the TaLcc3 and CtLcc2 (also at the gene level, e.g. in the organization of introns of the respective genes). The identity value obtained for TaLcc1 and CtLcc1 using Needleman-Wunsch global alignment (EMBLOSUM62, Gap penalty 10.0, Extend penalty 0.5; European Molecular Biology Open Software Suite program package, version 2.9.0; Rice et al., 2000) was 69.5% and that for TaLcc3 and CtLcc2 was 67.3% (Table 10). The identity values of the other laccase proteins were lower, when aligned with each other and with TaLcc1, CtLcc1, TaLcc3 and CtLcc2 as can be seen in Table 10.

By the term "identity" is here meant the 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) program package, version 2.9.0, with the following parameters: EMBL0SUM62, Gap penalty 10.0, Extend penalty 0.5.

Within the scope of the present invention are enzymes or polypeptides which comprise amino acid sequences which have laccase activity and which show at least 60 % identity to the amino acid sequence SEQ ID NO:41 (TaLcc2). Preferred enzymes comprise amino acid sequences which show at least 65 %, more preferably at least 70 %, even more preferably at least 75 % identity. Still more preferable the amino acid sequences show at least 80 %, more preferably at least 85 %, more and more preferably at least 90 %, most preferably at least 95 % identity to the amino acid sequence SEQ ID NO:41.

Within the scope of the present invention are also enzymes or polypeptides which comprise amino acid sequences which have laccase activity and which show at least 58 % identity to the amino acid sequence SEQ ID NO:43 (TaLcc3). Preferred enzymes comprise amino acid sequences which show at least 65 %, more preferably at least 68 %, even more preferably at least 75 % identity. Still more preferably the amino acid sequences show at least 80 %, more preferably at least 85 %, more and more preferably at least 90 %, most preferably at least 95 % identity to the amino acid sequence SEQ ID NO:43.

Within the scope of the present invention are also enzymes or polypeptides which comprise amino acid sequences which have laccase activity and which show at least 78 % identity to the amino acid sequence SEQ ID NO:45 (TaLcc4). Preferred enzymes comprise amino acid sequences which show at least 80 %, more preferably at least 85 %, even more preferably at least 90 % identity. Most preferable the amino shows at least 95 % identity to the amino acid sequence SEQ ID NO:45.

Within the scope of the present invention are enzymes or polypeptides which comprise amino acid sequences which have laccase activity and which show at least 74 % identity to the amino acid sequence SEQ ID NO:47 (CtLcc1). Preferred enzymes comprise amino acid sequences which show at least 76 %, more preferably at least 80 %, even more preferably at least 85 % identity. Still more preferable the amino acid sequences show at least 90 %, most preferably at least 95 % identity to the amino acid sequence SEQ ID NO:47.

Within the scope of the present invention are enzymes or polypeptides which comprise amino acid sequences which have laccase activity and which show at least 55 % identity to the amino acid sequence SEQ ID NO:49 (CtLcc2). Preferred enzymes comprise amino acid sequences which show at least 60 %, more preferably at least 68 % identity. Still more preferable the amino acid sequences show at least 75 %,

more preferably at least 80 %, still more preferably at least 85 %, more and more preferably at least 90 %, most preferably at least 95 % identity to the amino acid sequence SEQ ID NO:49.

Within the scope of the present invention are enzymes or polypeptides which comprise amino acid sequences which have laccase activity and which show at least 53 % identity to the amino acid sequence
5 SEQ ID NO:51 (CtLcc3). Preferred enzymes comprise amino acid sequences which show at least 60 %, more preferably at least 65 %, even more preferably at least 70 % identity. Still more preferable the amino acid sequences show at least 75%, more preferably at least 80 %, Still more preferable the amino acid sequences show at least 85%, more and more preferably at least 90 %, most preferably at least 95 % identity to the amino acid sequence SEQ ID NO:51.

10 Within the scope of the present invention are also enzymes and truncated polypeptides as defined above, but which lack signal sequence or tail or both. The signal sequence or the tail or both may be cut for example during posttranslational phases of the production or in the spent culture medium or during the storage of the culture medium or enzyme preparation. In addition, a propeptide from the protein may be
15 cleaved by the host. The truncation can also be achieved e.g. by shortening the gene encoding the polypeptide prior to transforming it to the production host.

The laccase according to the invention can be produced to the culture medium of its natural host or a recombinant host, from where it can be isolated and purified by using known methods of protein chemistry. If the culture medium contains a sufficiently high amount of laccase but no other detrimental proteins, it is possible to use the culture medium as such by simply separating the cells. When so desired,
20 the culture solution can be concentrated, filtrated, fractionated and/or purified. It may also be dried. It is preferable to use, in various applications, an enzyme preparation containing an increased amount of laccase. Such an enzyme preparation can be prepared by producing the increased amount of laccase enzyme in the culture medium of the production host by means of gene technology or by optimising the culture conditions. The increased amount refers to an amount of laccase enzyme, which exceeds the
25 amount of laccase enzyme naturally produced by the natural host. By "spent culture medium" is here meant the culture medium of the host comprising the produced enzymes.

According to a preferred embodiment of the invention *Thielavia* and *Chaetomium* laccases can be produced in a filamentous fungus host, preferably in a *Trichoderma* host. The production is described in more detail in Example 4. The purification and characterization of recombinant laccases in terms of pH
30 optimum, thermal stability, and pI is described in Example 5. *Thielavia* laccase TaLcc2 had pH optimum on guaiacol at pH 5.5, TaLcc3 at pH 5.0 on guaiacol, TaLcc4 at pH 6.0 on DMP. CtLcc 1 had pH optimum on guaiacol at pH 5.0.

TaLcc2 enzyme functions at a broad pH range from pH 3 to 9, preferably at pH 4 to 8, most preferably at pH 4.5 to 6.5 determined on guaiacol. TaLcc3 enzyme functions at pH 3.5 to 7.5, preferably at pH 4 to 6.5 determined on guaiacol. TaLcc4 enzyme functions at pH 3.5 to 7.5, more preferably at pH 4 to 7, most preferably at pH 5 to 6.5 determined on DMP.

- 5 CtLcc1 enzyme functions at pH 3.5 to 8, preferably at pH 4 to 7, most preferably at pH 4.5 to 6 determined on guaiacol.

Of the mentioned pH ranges the first pH range means that 20 % or more of the maximal activity is on this region, the second pH range means that 40 % or more of the activity is on this region. The third region means that 80 % or more of the activity is on this region.

- 10 The specific activities were determined towards ABTS, DMP, syringaldazine and guaiacol as described in Example 6. The specific activity of TaLcc2 was highest on ABTS, 360 nkat/mg at pH 4.5, of TaLcc3 8.3 nkat/mg at pH 4.5, of TaLcc4 1000 nkat/mg at pH 4.5, respectively. The specific activity of CtLcc1 was 705 nkat/mg at pH 4.5.

- 15 Within the scope of the present invention are also laccase enzymes, which show at least 60 % identity to the amino acid sequence SEQ ID NO:41 (TaLcc2) and have a specific activity of at least 300, preferably at least 350 nkat /mg towards ABTS at pH 4.5, laccase enzymes, which show at least 58 % identity to the amino acid sequence SEQ ID NO:43 (TaLcc3) and have a specific activity of at least 7, preferably at least 8 nkat /mg towards ABTS at pH 4.5, laccase enzymes, which show at least 78 % identity to the amino acid sequence SEQ ID NO:45 (TaLcc4) and have a specific activity of at least 900, preferably at least 1000 nkat /mg towards ABTS at pH 4.5.

20 Within the scope of the present invention are also laccase enzymes, which show at least 74 % identity to the amino acid sequence SEQ ID NO:47 (CtLcc1) and has a specific activity of at least 600, preferably at least 700 nkat /mg towards ABTS at pH 4.5.

- 25 The production of laccase can also be improved by optimising the culture conditions and the culture medium of a wild or a recombinant strain. The carbon/nitrogen ratio can be optimised to be the best for the production of enzyme. The growing conditions, pH, temperature, mixing and air supply can be optimised to be the best possible for the enzyme production in question. In fermentation, inducers of laccase production, such as veratryl alcohol, xylydine, or lignin or other aromatic compounds can also be used. The way and the time of adding the inducers, as well as their concentration can be optimised.

The term "enzyme preparation" denotes here to any enzyme product, which contains at least one laccase enzyme. Thus, such an enzyme preparation may be a spent culture medium or filtrate containing one or more laccases or one or more laccases and other enzymes, an isolated laccase enzyme or a mixture of one or more laccase enzymes or a mixture of one or more laccase enzymes and one or more other enzymes.

5 In addition to the laccase activity, such a preparation may contain additives, such as mediators, 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. The enzyme preparation may be in the form of liquid, powder or granulate.

10 The enzyme preparation may comprise in addition to laccase, one or more other enzymes, which may be for example amylases, cellulases and/or peroxidases. Alternatively, before, during or after the laccase treatment of the present invention, another enzyme treatment may be carried out. The enzyme treatment may comprise, for example, one or more amylase treatments, one or more cellulase treatments and/or one or more peroxidase treatments. Which other enzymes are included to the enzyme preparation or are used in the enzyme treatment, depends on the application.

15 The enzyme preparation may comprise one or more laccase enzymes of the present invention or other laccase enzymes together with one or more laccase enzymes of the present invention. For example, laccase enzymes having different properties may be combined to make the enzyme preparation more useful for different conditions.

By "mediators" are here meant additives which are often needed for enhancing the effect of laccases.

20 Many of the prior art laccases do not function or do not function effectively in the absence of mediators. Also the laccases obtainable from *Thielavia* or *Chaetomium*, function more effectively in the presence of mediators. Suitable mediators include, for example methylsyringate, acetosyringon, ethylsyringate, butylsyringate and laurylsyringate, propionic acid-phenothiazine (PPT) 2,2'azinobis-3-ethylbenzthiazole-6-sulphonate (ABTS), 2,2,6,6-tetramethyl-1-piperidinyloxy (Tempo), 1-hydroxybenzotriazole (HBT),
25 violuric acid, N-hydroxy-acetanilide (NHA). The mediator may be used in the range 0.1 to 100 mg/ g or 0.1 to 100 mg/l, preferably 1 to 10 mg/g or 1 to 10 mg/l of the treated material depending on the application.

Denim bleaching

30 The enzymes of the present invention are in particular suitable for denim bleaching. By "increasing lightness" of denim is here meant a visible and measurable increase in the lightness in denim fabric. By "increasing lightness" of denim is meant in particular increasing lightness of denim on the face side of

denim. The increase can be measured for example by measuring the colour as reflectance values with a chromameter using L*a*b* color space coordinates as described in Examples 7 – 10.

5 “Bleached look” means the effects, which are obtained on denim fabric in the prior art by means of bleaching chemicals, e.g. sodium hypochlorite. So far the “chlorine bleaching” has been the most effective bleaching method for denim dyed with Indigo since almost all shades have been obtained with it. If a “white bleaching” effect has been desirable, the bleaching has been carried out 2 to 3 times one after the other in different treatment baths, or by using high concentrations of hypochlorite. Bleaching with glucose, sulphinic acid derivatives or laccases have been suggested for denim treatment to replace sodium hypochlorite.

10 To “increase the lightness” of denim fabric, according to the prior art, treatment with various bleaching chemicals or enzymes is carried out. Bleaching is often done after treatment with cellulases or pumice stones or both.

15 When using the laccases of the present invention, if more whitish effect is desired, higher dosages can be used or the enzyme treatment can be repeated or combined with other bleaching methods. The laccase treatment of the present invention can be combined also with any other bleaching treatment, with one or more chemical bleaching treatments or with one or more other enzyme treatments having capability of increasing lightness of denim.

The denim treatment according to the invention comprises generally the following steps:

- 20
- desized or optionally desized and cellulase treated denim is contacted in aqueous medium with an effective amount of laccase enzyme under suitable conditions for the function of the enzyme; and
 - one or more rinses with water are carried out.

25 The laccase treatment is preferably carried out on cellulase treated denim. Laccase treatment is followed by one or more rinses with hot or cold water optionally with detergents. Enzyme inactivation is usually not needed after laccase treatment since it does not reduce the strength of fabric, but if needed it is carried out by methods well known to a person skilled in the art. The treatment is typically carried out in an equipment normally used for wet processes in textile industry, such as industrial machines used for
30 washing, cellulase treatment, dyeing or finishing.

By “denim” is in connection of this invention meant denim fabric, usually denim jeans.

Performance of the laccase preparations of the present invention in denim bleaching was exemplified at different pH-values as described in the Example 7. Recombinant laccase preparations produced using *Trichoderma* as a host were tested for their ability to bleach denim and compared to a commercial laccase preparation DeniLite II Base from Novozymes.

Both CtLcc1 and TaLcc2 laccases were more efficient in decolorisation of indigo dye of denim compared to the prior art laccase at pH values 6 and 7 as can be seen in Table 18 and in Figure 8. The look of the denim fabric was in particular at pH 6 much lighter.

The ability of the laccases of the present invention to bleach denim at different temperatures was tested and compared to the prior art laccase as described in Example 8.

CtLcc1 and in particular TaLcc2 were more efficient in decolorization of denim (higher increase of lightness) compared to the prior art laccase at 40 to 50 °C. The two enzymes are thus very suitable for use in applications where low temperatures are preferred. However, CtLcc1 was more effective also at 60 °C and functions thus at broad temperature range.

According to a preferred embodiment of this invention denim treatment by the laccases of the present invention is carried out at the temperature of 30 to 80°C, preferably at the temperature of 40 to 70 °C, more preferably at the temperature of 40 to 60°C. The pH during the treatment may be in the range from pH 3 to 9, preferably from pH 4 to 8, most preferably from pH 5 to 7. The treatment may be carried out in 15 minutes to 2 hours, preferably in 30 minutes to 90 minutes, more preferably in 30 minutes to 60 minutes.

The dosage used in the treatment can be 2 to 500 nkat, more preferably 20 to 200, most preferably 20 to 100 nkat/ g fabric.

By the laccase enzyme of the present invention any kind of denim fabric can be treated. Advantageously the denim is Indigo dyed denim. By "Indigo dyed" is here meant that the denim to be treated is dyed with Indigo, with derivatives of Indigo or denim dyed with Indigo together with some other dye, for example indigo-dyed denim with sulphur bottom.

The denim fabric may be cellulase treated or stone washed, or both, or the denim fabric may be treated by laccase of the present invention already after desizing. Higher increasing of lightness of denim can be obtained when laccase treatment is carried out on cellulase treated fabric.

The "desizing" process is normally the first wet treatment of jeans and means the removal of starch or other sizing agents applied usually to the warp yarns to prevent damage during the weaving process. Alpha-amylases are used to remove starch-based size for improved and uniform wet processing. After desizing the jeans are normally rinsed with water.

- 5 The term "abraded" means here the appearance of denim fabric when it has been treated by cellulase enzymes or stone washed, or both. As a result of uneven dye removal there are contrasts between dyed areas and areas from which dye has been removed. Synonymous expressions are "stone washed look" or "worn look". The cellulase treatment may be done using neutral or acid cellulases or both. If a fabric is not cellulase treated or stone washed, the appearance of the fabric is said to be "dull", since the
10 fashionable contrasts would be missing.

Stain removal

The laccase enzymes of the present invention can be used also for stain removal under similar conditions as in denim bleaching.

- 15 According to a preferred embodiment of this invention denim treatment by the laccases of the present invention is carried out at the temperature of 30 to 80°C, preferably at the temperature of 40 to 70 °C, more preferably at the temperature of 40 to 60°C. The pH during the treatment may be in the range from pH 3 to 9, preferably from pH 4 to 8, most preferably from pH 5 to 7. The treatment may be carried out in 15 minutes to 2 hours, preferably in 30 minutes to 90 minutes, more preferably in 30 minutes to 60 minutes.

- 20 The dosage used in the treatment can be 0.2 to 2000 nkat/g of fabric, preferably 1 to 500, more preferably from 2 to 200 nkat/g of fabric.

- The laccases of the present invention and Denilite II Base laccase preparations were tested for their ability to remove stains as is described in Example 9. In the tests artificially soiled test cloths for grass soiling and for tea soiling were used with or without the mediator (methyl syringate). The dosages of the
25 enzymes were 20 and 200 nkat/ g of fabric and the test was run at 40, 50 or 60°C and pH 6 for 60 min.

As can be seen in Tables 21 and 22 and in Figures 10 to 13 CtLcc1 laccase was effective in removal of grass and tea soiling with mediator at 60°C and TaLcc2 laccase at 50°C. The effect was also seen at 40°C.

Decolorization of dyes

The laccase enzymes of the present invention can be used also in decolorization of dyes. Dye-house effluents, for example cannot be discharged to natural waters without degrading the dyes and/or decolorizing them. The decolorization can be carried out under similar conditions as used in denim bleaching. Suitable dosage of the enzyme and treatment time depends on the amount of the dye to be decolorized and the treatment conditions.

According to a preferred embodiment of this invention decolorization of dyes is carried out at the temperature of 30 to 80°C, preferably at the temperature of 40 to 70 °C, more preferably at the temperature of 40 to 60°C. The pH during the treatment may be in the range from pH 3 to 9, preferably from pH 4 to 8, most preferably from pH 5 to 7.

The enzyme dosages and treatment times can be tested and chosen to be most suitable for the application. As guidance can be used dosages of 0.2 to 2000 nkat/ l of the treatment solution. The treatment time is preferably 15 min to 24 hours, more preferably 30 min to 12 hours. If the treatment is carried out at lower temperature, for example 18 to 30 °C the treatment time may be longer.

As described in Example 10 the laccases of the present invention were tested for their ability to decolourize different dyes in the presence or absence of a mediator. CtLcc1 and TaLcc2 laccases were able to decolorize Indigocarmine and Remazol Brilliant Blue very effectively. Also Cibacron Brilliant Red 3B-P was partly decolorized.

Other applications

Since the laccases of the present invention have high oxidizing capacity of various substrates, they are well suited for many industrial applications. Such applications are for example the manufacture of fibre products and applications of forest industry, applications in cosmetic industry and in industry preparing personal care and other applications. In these applications, the temperature and pH are on the area where the laccases of the present invention function. The dosage and treatment time can be chosen depending on the application and material to be treated.

Mediators may be needed as additives to enhance the effect of the laccases of the present invention. In addition, it is essential that enough oxygen is brought to the reaction. If needed, oxygen can be added either by bringing air or oxygen or air enriched with oxygen to the reaction mixture.

The laccases of the present invention are suitable for use in textile industry, for treating man-made or natural fibers or their combinations. The enzyme is suitable for treating cellulosic fibers as well as proteinaceous fibers, such as wool or silk.

5 The laccases of the present invention are suitable for use in forest industry. Lignin-containing fibres can be brought into contact with the laccase. Due to the laccase treatment, the strength properties of the fibres improve, which can be utilised, for example, in the manufacture of fibre boards, in paper or cardboard products and composites, which are made of mechanically ground lignin-containing fibres. Wood fibers can be treated with laccases of the present invention also to functionize them or glue the fibers.

10 The laccases of the present invention are also well suited to depolymerization of various compounds. By using the laccases of the present invention lignin in kraft pulp can be depolymerised thereby producing a pulp with lower lignin content. Laccase can thus be used for bleaching of pulp to decrease the use of bleaching chemicals. As a result of the better bleachability of the pulp after laccase treatment, there is a reduction of the subsequent consumption of bleaching chemicals, which when chlorine containing chemicals are used, leads to a reduced formation of environmentally undesired organo-chlorine compounds.

15 The laccases of the present invention can be used also for polymering compounds, such as lignin, to produce high molecular weight compounds.

20 Because of the high oxidizing capacity of the enzyme it can be used for oxidizing of dyes or dye precursors or chromophoric compounds in cosmetic industry or in industry preparing products for personal care. The oxidation of the dyes leads to decolorization of the compounds. This effect can be used for example in hair dyeing or when whitening teeth. To carry out hair dyeing dye precursors or modifiers are usually needed.

25 The laccase according to the invention can also be used to improve the runnability of paper machines. The laccase can be used to improve the runnability of paper machines by polymerising compounds originating from lignin and extractives and by decreasing the detrimental growth of microbes in the paper machine.

30 Further possible applications where laccase enzymes of the present invention can be used are methods for improving doughs in baking applications, methods for clarifying beer and wine, use in improval of the production of fuel ethanol from renewable raw materials and use in various bioremediative processes as well as use in hard-surface cleaning or in detergent formulations.

In general, in the mentioned applications the treatment temperature is preferably 30 to 80 °C, more preferably 40 to 70 °C, although reactions can be carried out also at lower temperatures. The pH may be 3 to 9, preferably 4 to 7. The treatment time may be 15 min to 24 hours, preferably 30 min to 2 hours. The dosage may be 0.1 to 2000, preferably 1 to 1000, more preferably 2 to 200 nkat/ g or l of the material to be treated. A suitable amount of mediator may be added.

Compositions for the mentioned applications comprise the enzyme or enzyme preparation of the present invention in an effective amount and optionally additives suitable for the application in question. Compositions for textile industry may comprise for example a suitable amount of surface active agents, buffers, stabilizers and preservatives, compositions for forest industry may comprise for example a suitable amount of buffers, stabilizers and preservatives. In all compositions should be avoided substances harmful for environment and for human (or animal) use. In particular compositions for cosmetic industry and industry for personal care products should not contain harmful effects on skin or as ingested.

The present invention provides composition for the treatment of denim comprising a laccase enzyme or an enzyme preparation according to the invention. The present invention provides also a composition for removal of stain, a composition for bleaching of pulp, a composition for treating of fibre for textile industry, a composition for treating of fibre for forest industry, a composition for treating of wool, a composition for treating of hair, a composition for treating of dye house effluent, and a composition for decolorizing of dyes comprising a laccase enzyme or an enzyme preparation according to the invention.

The following examples are intended for illustration of the present invention and should not be interpreted as limiting the present invention in any way.

EXAMPLE 1. Production and purification of the *Thielavia arenaria* and *Chaetomium thermophilum* laccase

Production of the *Thielavia arenaria* and *Chaetomium thermophilum* laccase

Various strains from the culture collection of Roal Oy were screened for their ability to produce laccases with indicators Remazol Brilliant Blue R-478, tannic acid, and guaiacol as described in Kiiskinen et al. (2004). *Thielavia arenaria* ALKO4197 showed positive reactions on guaiacol and Remazol Brilliant Blue R-478, and *Chaetomium thermophilum* ALKO4265 showed strong positive reaction, when 5 mM ABTS solution in 25 mM succinate buffer (pH 4.5) or in 25 mM McIlvaine buffer (pH 6.0) was dropped onto fresh mycelium on agar plates.

Both fungi were maintained on PD agar (Difco) at +4°C. The inoculation and production medium contained: 25 g/l glucose (AnalaR), 27.5 g/l Bacto yeast extract (Difco), 0.5 mg/ml Indulin AT (Sigma), 0.04 l/l mineral solution (1.0 g/l $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (Riedel-de Haën), 1.0 g/l $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (Riedel-de Haën), 0.1 g/l $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ (Merck), 0.16 g/l $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (Merck), 1.0 g/l Na_2EDTA (Riedel-de Haën)). Glucose was sterilized separately and combined aseptically to the medium.

- The *Thielavia arenaria* ALKO4197 strain was cultivated in 50 or 200 ml volume on a rotary shaker (200 rpm) at temperature of 37°C. The medium was inoculated with 5 or 20 ml of well-grown mycelia.
- 10 The laccase activity was followed up to eight days and the highest laccase activity (about 20 nkat/ml) was reached after six days of cultivation (Figure 1A). Six parallel cultivations were made. Cells were removed from the fermentation broth by centrifugation (10 000 g for 10 min, at +4°C) and the culture filtrate was further purified.
- 15 The *C. thermophilum* ALKO4265 strain was cultivated in 50 or 200 ml volume on a rotary shaker (200 rpm) at temperature of 42°C. The medium was inoculated with 5 or 20 ml of well-grown mycelia. The laccase activity was followed up to four days and the highest laccase activity (about 170 nkat/ml) was reached after three days of cultivation (Figure 1B). Six parallel cultivations were made. Cells were removed from the fermentation broth by centrifugation (10 000 g for 10 min, at +4°C) and the culture
- 20 filtrate was further purified.

Purification of the *Thielavia* and *Chaetomium* laccases

- Concentrated culture filtrate of the crude *Thielavia laccase* was first loaded on Q Sepharose FF column (Pharmacia, V=26 ml), which was pre equilibrated with 10 mM Tris HCL, pH 8.5. Proteins were eluted with an increasing salt gradient (0-500 mM Na_2SO_4 in the equilibrating buffer, within 5 column volumes). Laccase active fractions eluted at 70-150 mM salt concentration and they were pooled and loaded on Sephacryl S100 gel filtration resin (Pharmacia, V=160 ml), which was equilibrated with 20 mM Tris-buffer, pH 7.0, containing 200 mM NaCl. Purification was followed by SDS-PAGE stained
- 25 with Coomassie brilliant Blue (Figure 2A). Laccase positive fractions were pooled and concentrated. Salts were removed and buffer changed to 20 mM Tris buffer, pH 7.0. In order to obtain high purity samples an additional Resource Q anion exchange step was included. The sample was loaded onto a Resource Q column (Pharmacia, V= 1ml), which was equilibrated with 10 mM Tris HCl pH 8.5. Proteins were eluted with a linear 1-300 mM Na_2SO_4 salt gradient within 12 column volumes.
- 30

The culture supernatant of *C. thermophilum* was concentrated and the buffer changed to the binding buffer by ultrafiltration (MWCO 10 000). Proteins were bound to DEAE Sepharose FF (Pharmacia, column volume 25 ml) at 20 mM Tris-buffer pH 8.0. Proteins were eluted with a sodium sulphate gradient (0-500 mM). The laccase positive fractions eluted at 150 -200 mM salt concentration, and they were pooled and further purified with hydrophobic interaction chromatography (Phenyl Sepharose FF, Pharmacia, column volume 22 ml). Proteins were bound at 500 mM sodium sulphate concentration, at 20 mM Tris buffer pH 7.0, and eluted with a decreasing salt gradient (500 - 0 mM). The laccase positive fractions eluted with 20 mM Tris buffer. Purity of the fractions was analyzed by SDS-PAGE and subsequent Coomassie staining (Figure 2B.).

10 Enzyme activity assay

The laccase activity from the culture supernatant was measured using ABTS as substrate. The activity assay was carried out in accordance with the method developed by Niku-Paavola *et al.* (1988). The sample was diluted with 0.025 M succinate buffer, pH 4.5. 0.350 ml of ABTS solution (11 g/l) was added to 1.15 ml of the dilution, and the reaction was followed for 2 minutes by the Perkin Elmer Lambda 20 spectrophotometer at a wavelength of 436 nm. The activity is expressed as nano katal.

Determination of protein contents

The protein contents were determined by the DC Protein Assay kit of Bio-Rad, based on a method developed by Lowry *et al.* (1951). The assays were carried out according to the supplier's instructions, and the intensity of the colour formed in the reaction was measured on a wavelength of 750 nm using the Perkin Elmer Lambda 20 spectrophotometer. A standard curve was defined using bovine serum albumin in concentrations of 0.25-1.25 g/l (BSA, Bio-Rad).

25 EXAMPLE 2. Characterization of the purified *C. thermophilum* laccase

Molecular weight and pI

Molecular weight of the *T. arenaria* and *C. thermophilum* laccases were determined on SDS-PAGE according to Laemmli (1970). The gels used in the SDS-PAGE analysis were ready-made 12% Tris HCl gels (BioRad). Protein bands were visualized by staining with Coomassie Brilliant Blue (R 350; Pharmacia) and compared with molecular weight markers (Prestained Protein Marker Broad Range #7708S; New England BioLabs, Beverly, Mass.). The molecular weight of the both laccases was approximately 80 kDa. The isoelectric point of the laccases was determined with isoelectric focusing

within the pH range of 3-9 (Pharmalyte IEF, Pharmacia) on a LKB 2117 Multiphor II Electrophoresis System (LKB Pharmacia, Bromma, Sweden) according to the manufacturer's instructions. Bands containing laccase activity were visualized by staining the gel with 2 mM ABTS in 25 mM succinate buffer (pH 4.5) and proteins by Coomassie Blue staining. The purified *Thielavia* laccase showed multiple
5 bands in isoelectric focusing at pIs 5.5, 5.9, 6.4, 6.8, and 6.9. The purified *C. thermophilum* laccase showed 3-4 bands in isoelectric focusing at pIs 4.1-4.3.

pH optimum

The pH-optimum of the *T. arenaria* and *C. thermophilum* laccases were determined in the universal
10 McIlvaine buffer within a pH range of 2.2-8.0 using guaiacol as substrate. The pH optima determined for the purified and crude *Thielavia* laccase are shown in Fig. 3A. As shown in Fig. 3A the pH optimum for *Thielavia* laccase is at 5.5, the enzyme shows substantially high activity still at pH 7, above which the activity starts to drop. The pH optimum of the purified and crude *C. thermophilum* laccase is at 5.0 (Fig. 3B).

15

Thermal stability

Thermal stability of the laccases were determined by incubating the enzyme solution (0.3 g l⁻¹) in 60 mM citrate buffer (pH 6). The residual enzyme activities were measured at on ABTS. As shown from the results the half lives of the *Thielavia* laccase was 26 and 5.5 hrs at 50, and 60 °C, respectively (Fig. 4A),
20 and for *C. thermophilum* 30 and 6 hrs 50, and 60 °C, respectively (Fig. 4B).

Specific activity

Specific activities of the purified *T. arenaria* and *C. thermophilum* laccases were determined towards different laccase substrates. The activities were determined towards ABTS (Niku-Paavola et al., 1988),
25 di-methoxy-phenol (DMP) (Schlosser et al., 1997), syringaldazine (Paszczyński et al., 1985), and guaiacol (Leonowicz & Grzywnowicz, 1981). For ABTS the activity measurements were carried out in 25 mM succinate buffer pH 4.5 at 25°C and for other substrates in 25 mM MES buffer, pH 5.5. Results are shown in Table 1.

Table 1. Specific activities of the purified wild type *Thielavia* (TaLcc) and *Chaetomium* (CtL) laccases.

Substrate	Spec.act TaLcc nkat/mg	Spec.act. CtL nkat/mg
ABTS	1020	705
DMP	260	290
syringaldazin	490	400
guaiacol	63	85

5 Inhibition of the laccase

The effect of various inhibitors on laccase activity was determined by measuring the oxygen consumption during the enzyme reaction with ABTS in sealed and fully filled Erlenmeyer flasks with an Orion Research 081010 oxygen electrode (Software: SensorLink™ PCM800; Orion, Espoo, Finland). The oxygen consumption rates were measured from solutions containing suitable amount of the laccase, 2 mM ABTS, and various inhibitors in different concentrations, in 50 mM citrate buffer (pH 5) in a 30 ml reaction volume

Table 2. Inhibition of the wild type *Thielavia* (TaLcc) and *Chaetomium* (CtL) laccases.

Compound	Concentration	Inhibition(%) TaLcc	Inhibition(%) CtL
EDTA	10 mM	0	0
NaN ₃	0.5 mM	99	100
KCN	0.1 mM	65	70
KCN	1 mM	ND	100
NaCl	0.1 mM	35	0
NaCl	1 mM	42	10
NaF	0.5 mM	ND	40
NaF	10 mM	ND	70

N-terminal and internal amino acid sequencing

The N-terminus of the protein as well as the internal peptides were sequenced according to Edman degradation chemistry (Edman and Begg, 1967) using PE Biosystems Procise Sequencer. For peptide preparation, the lyophilized protein was reduced with dithiotreitol, carboxymethylated with iodoacetamide and cleaved with sequencing grade trypsin (Promega) at enzyme/substrate mass ratios 1:100 for 12 hours at 37 C in 0.1 M ammoniumbicarbonate, pH 8.3 (Stone et al., 1988). Generated

peptides were separated by reversed-phase high performance liquid chromatography (RP-HPLC, Vydac C-18 column) with a linear acetonitrile gradient (0-60% acetonitrile in 0.1% trifluoroacetic acid). The internal peptide sequences for *Thielavia* laccase are shown in Table 3 (SEQ ID NO: 1 – 3). The N-terminus of the protein could not be obtained, because it was presumably blocked. Amino acid sequences obtained from the *Chaetomium*-laccase are shown in Table 4 (SEQ ID NO: 4 – 7). The sequences of the peptides 22.4 and 22.7 from *Chaetomium* were obtained after the corresponding laccase gene had been cloned.

Table 3. Internal peptide sequences determined from *Thielavia* -laccase (ALKO4197). The N-terminus of the protein was presumably blocked.

Peptide	Sequence	Comments
Peptide 1	YQGAPNTLPTNQGLPVPNH	An equal Ile signal can also be seen in the 12th cycle.
Peptide 2	ENWIGPDGVLK	
Peptide 3	(S) LFLAVGQR	(S), result unsure.

Table 4. N-terminal and internal peptide sequences of *C. thermophilum* laccase (ALKO4265) .

Peptide	Sequence	Comments
N-terminus	E (AD) GPGPCHTPANYACWAPGFD	In addition to Glu, equal Ala and Asp signals can be seen in the first cycle
Peptide 18.9	LTENDNWTGPDGVVK	
Peptide 22.4	DHNCLDLLDLVFPVPR	
Peptide 22.7	T (S) LGGTPT (L) FVXK	The amino acid in the first cycle can be Thr or Ser and on the seventh cycle Thr or Leu. X, result unsure.

EXAMPLE 3. Cloning of the *Thielavia arenaria* ALKO4197 and *Chaetomium thermophilum* ALKO4265 laccase genes

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) and Sambrook and Russell (2001).

The genomic libraries of *Thielavia arenaria* ALKO4197 and *Chaetomium thermophilum* ALKO4265 were made to Lambda DASH[®]II vector (Stratagene, USA) according to the instructions from the

supplier. The chromosomal DNAs, isolated by the method of Raeder and Broda (1985), were partially digested with *Sau3A*. The digested DNAs were size-fractionated and the fragments of the chosen size (9-23 kb) were dephosphorylated and ligated to the *Bam*HI digested lambda vector arms. The ligation mixtures were packaged using either Gigapack III XL (*Thielavia*) or Gigapack III Gold (*Chaetomium*) packaging extracts according to the manufacturer's instructions (Stratagene, USA). The titers of the *Thielavia* and *Chaetomium* genomic libraries were 1.2×10^6 and 3.6×10^6 pfu/ml and those of the amplified libraries were 1.1×10^{10} and 6.5×10^{10} pfu/ml, respectively.

The probes for screening the gene banks were amplified by PCR using the *Thielavia* ALKO4197 and *Chaetomium* ALKO4265 genomic DNAs as templates in the reactions. First, several primers (degenerate oligos) were planned and tested in PCR reactions (Table 5, SEQ ID NO: 8 – 31). The sequences of the homologous primers based on the amino acid sequences of the peptides from the purified TaLcc1 and CtLcc1 and the heterologous primers were planned according to the conserved laccase sequences (Fig. 5). The conserved sequences were identified by aligning the previously published amino acid sequences of *Neurospora*, *Podospora*, *Cryphonectria*, *Myceliophthora*, *Scytalidium* and *Coprinus* laccases (EMBL accession numbers P10574, P78722, Q03966, AAE68088, AAE68087, AAE63570, AAE63572, and AAE63571). In addition, a heterologous probe was amplified from the *N. crassa* laccase gene (genomic DNA from *N. crassa* strain ATCC9277 was used as a template), using primers POX12 and POX13 designed according to the published nucleotide sequence (Table 5). The combinations of the primers for the PCR reactions were selected according to the location of the peptide or the peptide homologue in the published laccase sequences. The PCR reaction mixtures contained 50 mM Tris-HCl, pH 9.0, 15 mM $(\text{NH}_4)_2\text{SO}_4$, 0.1% Triton X-100, 5 % DMSO, 1.5-3 mM MgCl_2 , 0.2 mM dNTPs, 5 μM each primer and 1-2 units of Dynazyme EXT DNA polymerase (Finnzymes, Finland) and 1-5 μg of the genomic DNA. The conditions for the PCR reactions were the following: 5 min initial denaturation at 95 °C, followed by 25-30 cycles of 1 min at 95 °C, 1 min annealing at 50 °C (*Thielavia* DNA as a template) or at 50 or 42°C (*Chaetomium* DNA as a template), 2 min extension at 72 °C and a final extension at 72 °C for 7-10 min.

Table 5. The oligonucleotides tested as PCR primers to amplify probes for screening of the laccase genes. Oligo, oligonucleotide; Oligo location, the amino acids of the peptide used in planning of the oligonucleotide sequence.

Oligo	Length (nts)	Degeneracy ^(a)	Sequence ^(b)	Peptide ^(c)	Oligo loc.
POX1	17	16	AAYTAYGCXTGYTGGGC (s)	Ct Lcc1 N-term	11-16
POX2	17	16	GCCCARCAXGCRTARTT (as)	Ct Lcc1 N-term	11-16
POX22	32	16	TGCCAYACSCCCGCGYAACTACGCTGCTGGGC (s) ^(e)	Ct Lcc1 N-term	6-16
POX3	17	16	GTCCARTTRTCRTTYTC (as)	Ct Lcc1 18.9	3-8
POX16	17	16	GARAAYGAYAAYTGGAC (s)	Ct Lcc1 18.9	3-8
POX23	32	8	GAGAACGAYAAGTGGACSGGCCCGAYGGCGT (s) ^(e)	Ct Lcc1 18.9	3-13
POX26	26	8	GAGAACTGGATCGGYCCCGAYGGYGT (s)	Ta Lcc1 2	1-9
POX27	17	48	GARAAYTGGATHGGXCC (s)	Ta Lcc1 2	1-6
POX28	20	16	CTCTTCCTCGCYGTSGGYCA (s)	Ta Lcc1 3	2-8
POX29	20	16	TGRCCSACRGCAGGAAGAG (as)	Ta Lcc1 3	2-8
POX30	20	8	TACCAAGGCGCYCCSAACAC (s)	Ta Lcc1 1	1-7
POX31	20	8	GTGTTSGGRGCRCCCTGGTA (as) ^(e)	Ta Lcc1 1	1-7
POX4	17	64	TGGTAYCAYWSXCAYTT (s)	Homol. I	1-6
POX5	17	64	AARTGXSWRTGRTACCA (as)	Homol. I	1-6
POX6	20	64	ATGCAYYTXCAYGGXCAYGA (s)	Homol. II	1-7
POX7	20	64	TCRTGXCCRTGXARRTGCAT (as)	Homol. II	1-7
POX8	17	64	CAYYTXCAYGGXCAYGA (s)	Homol. II	2-7
POX9	17	64	TCRTGXCCRTGXARRTG (as)	Homol. II	2-7
POX10	23	48	TGCCAXGCDATRTGRCARTGCAT (as)	Homol. III	1-8
POX11	20	48	TGCCAXGCDATRTGRCARTG (as)	Homol. III	2-8
POX12	17	Ncr codons	TGGTACCACTCGCATTT (s) ^(d)	Homol. I	1-6
POX13	17	Ncr codons	TCGTGGCCGTGCAGGTG (as) ^(d)	Homol. II	2-7
POX14	23	Ncr codons	TGCCAGGCAATGTGGCAGTGCAT (as) ^(d)	Homol. III	1-8
POX15	20	Ncr codons	TGCCAGGCAATGTGGCAGTG (as) ^(d)	Homol. III	2-8

^(a) To reduce degeneracy, some codons were chosen according to the fungal preference.

^(b) D = A or G or T, H = A or C or T, R = A or G, S = C or G, W = A or T, X = I (inositol) or C, Y = T or C; "s" in the parenthesis = sense strand, "as" in the parenthesis = antisense strand.

^(c) The peptide sequences are included in Fig. A.

^(d) *Neurospora crassa* codons were used (from sequence: EMBL M18334)

^(e) The codon usage chosen according to the xylanase genes *xyn11A*, *xyn11B* and *xyn11C* isolated from *C. thermophilum* ALKO4265 (EMBL AJ508931-508933).

DNA products having the expected sizes (calculated from the published fungal laccase sequences) were obtained from several reactions. In some of the PCR reactions, several bands were detected that had very similar sizes; e.g. three bands of about 0.2 kb were obtained with the primers POX8 and POX11 from the reactions with *Chaetomium* DNA. This suggested that several laccase genes can be found. The DNA fragments having the expected sizes were isolated from the most specific PCR reactions and they were cloned to pCR[®] Blunt-TOPO[®] vector (Invitrogen, USA). The inserts were characterized by sequencing and by performing Southern blot hybridizations to the genomic DNAs digested with several restriction enzymes.

The PCR products obtained from both the *Thielavia* and *Chaetomium* reactions were found to contain sequences from three different genes, according to the hybridization patterns and sequencing. Three

PCR fragments, each representing a different putative laccase gene (Table 6, SEQ ID NO: 32 – 37), were chosen from both *Thielavia* and *Chaetomium* reactions to be used as probes for screening the gene banks. The deduced amino acid sequences from all these probes had homology to several published laccase sequences (BLAST program, version 2.2.9 at NCBI, National Center for Biotechnology Information; Altschul et al., 1990). In addition to the homologous probes, the heterologous *N. crassa* laccase fragment was used for screening both the gene banks.

Table 6. The primers used in the PCR reactions and probes chosen for screening of the laccase genes. The genomic template DNA and the name of the plasmid containing the probe fragment are shown.

Gene	Forward primer	Reverse primer	Template DNA used in PCR reaction	Fragment obtained (kb)	Insert in plasmid
<i>Talcc1</i>	POX27	POX31	<i>T. arenaria</i> ALKO4197	1.0 kb	pALK1550
<i>Talcc2</i>	POX4	POX11	<i>T. arenaria</i> ALKO4197	1.3 kb	pALK1601
<i>Talcc3</i>	POX27	POX9	<i>T. arenaria</i> ALKO4197	1.3 kb	pALK1624
<i>Talcc4</i>	POX12	POX15	<i>N. crassa</i> ATCC9277	1.1 kb	–
<i>Ctlcc1</i>	POX8	POX11	<i>C. thermophilum</i> ALKO4265	0.2 kb	pALK1299
<i>Ctlcc2</i>	POX4	POX9	<i>C. thermophilum</i> ALKO4265	0.9 kb	pALK1295
<i>Ctlcc3</i>	POX8	POX11	<i>C. thermophilum</i> ALKO4265	0.25 kb	pALK1296

The *N. crassa* laccase fragment and the inserts from the plasmids listed in Table 6 were labeled by using digoxigenin according to the supplier's instructions (Roche, Germany). The amplified genomic libraries (8×10^4 – 1×10^6 plaques) were screened with the homologous probe fragments and with the *N. crassa* laccase fragment. The hybridization temperature for the filters was 68°C and the filters were washed 2x5 min at RT using 2xSSC–0.1% SDS followed by 2x15 min at 68°C using 0.1xSSC–0.1% SDS when the homologous probes were used. The filters probed with the *N. crassa* laccase fragment were washed 2x5 min at RT using 2xSSC–0.1% SDS followed by 2x15 min at 68°C using 2xSSC–0.1% SDS. Several positive plaques were obtained from each of the hybridizations. Some of the positive plaques were strongly hybridizing to the probe in question but, in addition, there was an amount of plaques hybridizing more weakly to the probes. This again suggested that there would be other laccase genes in the genomes, having cross-reaction to the probes used. From two to eight strongly hybridizing plaques were purified from each screening. The phage DNAs were isolated and characterized by Southern blot hybridizations. The chosen restriction fragments hybridizing to the probe were subcloned to pBluescript II KS+ or SK+ vectors and the relevant regions of the clones were sequenced.

A total of four laccase genes were cloned from *Thielavia arenaria* ALKO4197 and three from *Chaetomium thermophilum* ALKO4265. The Table 7 summarizes the information on the probes used for screening the genes, the phage clones from which the genes were isolated, the chosen restriction fragments containing the full-length genes with their promoter and terminator regions, the plasmid names, and the DSM deposit numbers for the *E. coli* strains carrying these plasmids.

Table 7. The probes used for cloning of laccase gene, the phage clone and the subclones chosen, the plasmid number and the number of the deposit of the corresponding *E. coli* strain.

Gene	Probe used in screening	Phage clone	The fragment subcloned to pBluescript II	Plasmid no	<i>E. coli</i> deposit no
<i>Talcc1</i>	pALK1550	F1	3.8 kb <i>SpeI</i>	pALK1342	DSM 15484
<i>Talcc2</i>	pALK1601	F9	4.2 kb <i>XbaI</i> - <i>SpeI</i>	pALK1347	DSM 15486
<i>Talcc3</i>	pALK1624	F1	4.3 kb <i>SmaI</i>	pALK1345	DSM 15485
<i>Talcc4</i>	<i>N. crassa</i> PCR probe	F14	5.0 kb <i>BglII</i>	pALK1664	DSM 15487
<i>Ctlcc1</i>	pALK1299	F6/4	3.7 kb <i>XhoI</i>	pALK1304	DSM 15075
<i>Ctlcc2</i>	pALK1295	F2/5	4.2 kb <i>XbaI</i>	pALK1305	DSM 15076
<i>Ctlcc3</i>	pALK1296	F3/7	3.5 kb <i>SacII</i> - <i>SalI</i>	pALK1685	DSM 16040

The sequences of the laccase genes are shown in Fig. 6. The relevant information on the genes and the deduced protein sequences are summarized in Table 8 and Table 9, respectively.

The peptide sequences of the purified TaLcc1 and CtLcc1 (Tables 3 and 4) were found from the deduced amino acid sequences of the clones containing the *Talcc1* and *Ctlcc1* genes (some inaccuracies were found from the peptide sequences after the deduced amino acid sequences were available). Thus, it could be concluded that the genes encoding the purified laccase proteins TaLcc1 and CtLcc1 were cloned. The synthesis of PCR fragments from the *Talcc1* gene was successful when the PCR primers were designed according to the TaLcc1 peptide sequences (POX28+POX31 and POX27+POX31). However, due to the inaccuracies in the peptide sequencing, the cloning of *Ctlcc1* succeeded only when the primers deriving from the homologous fungal laccase sequences were used. The *Thielavia* laccase gene *Talcc4* was obtained by using the *N. crassa* probe in the screening of the genomic library. No additional laccase genes were found from the plaques picked and purified from the *Chaetomium* library probed with the *N. crassa* laccase fragment.

Table 8. Summary on the laccase genes isolated from *Thielavia arenaria* ALKO4197 and *Chaetomium thermophilum* ALKO4265.

Laccase gene	Length with introns (bp) ^(a)	Coding region (bp) ^(b)	No of introns	Lengths of introns (bp)
<i>Talcc1</i>	2279	1851	6	51, 62, 91, 83, 79, 59
<i>Talcc2</i>	1957 ^(d)	1767	2	80, 107
<i>Talcc3</i>	2015	1833	3	65, 54, 60
<i>Talcc4</i>	1793	1719	1	71
<i>Ctlcc1</i>	2127	1821	5	50, 53, 50, 55, 95
<i>Ctlcc2</i>	1986	1797	3	49, 61, 79
<i>Ctlcc3</i>	2064 ^(c)	1869	3	58, 65, 69

^(a) The STOP codon is included.

^(b) The STOP codon is not included.

^(c) The other translation start site in *Ctlcc3*, deleting the first intron, would result in a gene length of 1958 bp and a coding region of 1821 bp (Fig. 6).

^(d) The other translation start site in *Talcc2* would result in a gene length of 1927 bp and a coding region of 1737 bp (Fig. 6).

Table 9. Summary of the deduced laccase sequences from *Thielavia arenaria* ALKO4197 and *Chaetomium thermophilum* ALKO4265. ss, signal sequence.

Laccase protein	No of aas	Length of ss NN/HMM ^(a)	C-term. tail ^(b)	Predicted MW (Da, ss not incl) ^(c)	Predicted pI (ss not incl)	Putative N-glycosylation sites ^(d)
<i>Talcc1</i>	617	21/21	DSGL + 13 aas	64 456	6.31	9
<i>Talcc2</i> ^(e)	589	29/24	DSGI	61 811/62 274	4.65/4.65	12
<i>Talcc3</i>	611	25/23	DSGL + 18 aas	62 703 /62 893	6.27/6.27	8
<i>Talcc4</i>	573	18/18	DSGV	61 072	4.31	9
<i>Ctlcc1</i>	607	20/20	DSGL + 13 aas	63 905	6.09	8
<i>Ctlcc2</i>	598	22/22	DSGL	64 162	6.15	9
<i>Ctlcc3</i> ^(f)	623	No ss found	DSGT	69 536	5.28	8

^(a) The prediction on the signal sequence was made using the program SignalP V2.0 (Nielsen et al., 1997; Nielsen and Krogh, 1998); the NN value was obtained using neural networks and HMM value using hidden Markov models.

^(b) The "consensus" amino acid sequence (DSGX) at the C-terminal end and the number of amino acids after the consensus sequence.

^(c) The predicted signal sequence and the C-terminal tail were not included. The prediction was made using the Compute pI/MW tool at ExPASy server (Gasteiger et al., 2003). The two values marked for *Talcc2* and *Talcc3* are calculated after deleting the two possible signal sequences.

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

^{1e} There are two possible translation start sites for the *Talcc2* gene. The predicted signal peptides and other values were obtained using the longer sequence. The predicted signal sequence would be 17 amino acids for the polypeptide encoded by the shorter gene (the deduced sequence 579 amino acids).

5 ^{1f} There are two possible translation start sites for the *Ctlcc3* gene. The deduced amino acid sequence for the shorter polypeptide is 607 amino acids, MW 62 029 Da and pI 4,65. No predicted signal sequence was detected from either of the deduced amino acid sequences.

10 The deduced amino acid sequences of *TaLcc1* and *CtLcc1* were found to be the most homologous to each other, as were also the *TaLcc3* and *CtLcc2* (also at the gene level, e.g. in the organization of introns of the respective genes). The identity value obtained for *TaLcc1* and *CtLcc1* using Needleman-Wunsch global alignment (EMBLOSUM62, Gap penalty 10.0, Extend penalty 0.5; European Molecular Biology Open Software Suite program package, version 2.9.0) was 69.5% and that for *TaLcc3* and *CtLcc2* was 15 67.3% (Table 10). The identity values of the other laccase proteins were lower, when aligned with each other and with *TaLcc1*, *CtLcc1*, *TaLcc3* and *CtLcc2* (Table 10).

20 Table 10. The identity values (%) obtained from alignment of the deduced amino acid sequences of the *Thielavia* ALKO4197 and *Chaetomium* ALKO4265 laccases (Needleman-Wunsch global alignment, EMBLOSUM62, Gap penalty 10.0, Extend penalty 0.5).

Laccase	Ta Lcc1	Ct Lcc1	Ta Lcc3	Ct Lcc2	Ta Lcc4	Ta Lcc2 ^{1a}	Ct Lcc3 ^{1a}
Ta Lcc1	100.0	69.5	47.8	47.1	34.7	34.4	28.8
Ct Lcc1		100.0	47.8	47.0	36.1	33.8	31.2
Ta Lcc3			100.0	67.3	35.6	37.5	28.4
Ct Lcc2				100.0	36.5	35.0	29.6
Ta Lcc4					100.0	42.4	31.2
Ta Lcc2						100.0	32.9
Ct Lcc3							100.0

^{1a} = The deduced *TaLcc2* and *CtLcc3* amino acid sequences starting from the first Met of the putative sequences (Fig. 6) were used in the alignments.

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The highest homologies of the deduced *TaLcc1* and *CtLcc1* sequences (BLAST program, version 2.2.9 at NCBI, National Center for Biotechnology Information; Altschul et al., 1990) were to the laccases from *Melanocarpus albomyces*, *Podospora anserina* and *Neurospora crassa* (EMBL accession numbers CAE00180, LAC2_PODAN, LAC1_NEUCR/XP_323881/KSNCLO). The highest identities of *TaLcc1* and *CtLcc1* to the laccases in the patent database were to laccases from *Myceliophthora thermophila* (EP 0765394 B1) and *Scytalidium thermophilum* (US 5,750,388). The other deduced laccase sequences did not have as high identities to the previously published sequences. The highest identities of *TaLcc3* and 30

CtLcc2 were to *Magnaporthe grisea* hypothetical protein (EAA57158.1) and to *Colletotrichum lagenarium* laccase (BAB32575). The highest homologies of the other laccases to the previously published sequences were as follows: TaLcc2 to *N. crassa* hypothetical protein (XP_330977), TaLcc4 to *Gibberella zeae* hypothetical protein (EAA68613), CtLcc3 to *N. crassa* and *Magnaporthe grisea* hypothetical proteins (XP_324706 and EAA47633). Thus, also other fungal species have similar sequences but these sequences have not yet been identified as laccases. The sequences found from the databases, having at least 50% identity to the deduced amino acid sequences of the laccases from *Thielavia* ALKO4197 and *Chaetomium* ALKO4265, are shown in Table 11.

Table 11. The sequences with at least 50% identity (%) to the deduced amino acid sequences of *Thielavia* ALKO4197 and *Chaetomium* ALKO4265 laccases. The alignment was made using Needleman-Wunsch global alignment (EMBLOSUM62, Gap penalty 10.0, Extend penalty 0.5).

The amino acid sequence	Identity (%)
TaLcc1	100.0
Melanocarpus albomyces CAE001810	73.1
Myceliophthora thermophila	68.3
Podospora anserina LAC2_PODAN	66.7
Scytalidium thermophilum	62.6
Neurospora crassa LAC1_NEUCR	60.7
Neurospora crassa XP_323881	60.7
Neurospora crassa KSNCL0	60.6
Neurospora crassa LAC2_NEUCR	60.4
Cryphonectria parasitica LAC1_CRYPA	57.5
Gaeumannomyces graminis var tritici Lac3 CAD10749	51.0
TaLcc2	100.0
Neurospora crassa XP_330977	59.3
Botryotinia fuckeliana laccase 2 AAK77953	56.4
Gaeumannomyces graminis var tritici Lac1 CAD10747	53.0
Gaeumannomyces graminis var graminis CAD24841	52.6
Botryotinia fuckeliana laccase 1 AAK77952	50.2
TaLcc3	100.0
Magnaporthe grisea EAA57158	57.4
Colletotrichum lagenarium BAB32575	53.4
TaLcc4	100.0
Gibberella zeae EAA68613	77.4
Gibberella zeae XP_390780	77.4
Magnaporthe grisea EAA52662	60.1

Gaeumannomyces graminis var tritici Lac2 CAD10748	54.8
Magnaporthe grisea EAA48009	53.6
Gibberella zeae XP_389822	50.2
CtLcc1	100.0
Melanocarpus albomyces CAE001810	73.2
Podospora anserina LAC2_PODAN	68.4
Myceliophthora thermophila	67.6
Scytalidium thermophilum	66.5
Neurospora crassa XP_323881	62.7
Neurospora crassa KSNCL0	62.6
Neurospora crassa LAC2_NEUCR	62.4
Neurospora crassa LAC1_NEUCR	62.1
Cryphonectria parasitica LAC1_CRYPA	58.1
CtLcc2	100.0
Magnaporthe grisea EAA57158	54.5
Colletotrichum lagenarium BAB32575	53.4
CtLcc3	100.0
Neurospora crassa XP_324706	52.7
Magnaporthe grisea EAA47633	52.3

EXAMPLE 4. Production of recombinant laccases in *Trichoderma reesei*

- 5 Expression plasmids were constructed for production of the recombinant TaLcc1, TaLcc2, TaLcc3, TaLcc4, CtLcc1 and CtLcc2 proteins. The expression cassette was not constructed for production of CtLcc3 due to lack of a predicted signal sequence in the deduced amino acid sequence. The expression plasmids constructed are listed in Table 12. The laccase genes, including their own signal sequences, were exactly fused to the *T. reesei cbh1* (*cel7A*) promoter by PCR. The *cbh1* promoter, *cbh1* terminator, *amdS* marker and the *cbh1* 3' flanking region included were as described in Paloheimo *et al.* (2003). The linear expression cassettes (Fig. 7), were isolated from the vector backbones and were transformed to *T. reesei* A47 protoplasts. The transformations were performed as in Penttilä *et al.* (1987) with the modifications described in Karhunen *et al.* (1993). The transformants were purified on selection plates through single conidia prior to sporulating them on PD.

Table 12. The expression cassettes constructed to produce *Chaetomium thermophilum* ALK04265 and *Thielavia arenaria* ALK04197 laccases in *Trichoderma reesei*. The overall structure of the expression cassettes was as described in Fig. 7. The laccase genes were exactly fused to the *cbh1* promoter except in pALK1326 and pALK1327 where the *Ct lcc1* gene is fused to a carrier polypeptide (Cel6A CBD A+B or A+B+B') and a synthetic Kex2 linker (including the amino acids RDKR). Analogous constructs to these two plasmids, pALK1285 and pALK1286, are described in Paloheimo et al. (2003).

Laccase gene	Expression plasmid	Size of the expr. cassette ^(a)	Laccase terminator ^(b)	Carrier
<i>Ct lcc1</i>	pALK1321	10.1 kb	205 bp (<i>EcoRV</i>)	No carrier
<i>Ct lcc1</i>	pALK1326	10.3 kb	205 bp (<i>EcoRV</i>)	Cel6A CBD (A+B)
<i>Ct lcc1</i>	pALK1327	10.4 kb	205 bp (<i>EcoRV</i>)	Cel6A CBD (A+B+B')
<i>Ct lcc2</i>	pALK1340	9.8 kb	92 bp (<i>BamHI</i>)	No carrier
<i>Ct lcc3</i>	Not done			
<i>Ta lcc1</i>	pALK1667	10.1 kb	80 bp (<i>NcoI</i>)	No carrier
<i>Ta lcc2</i>	pALK1655 ^(c)	9.9 kb	168 bp (<i>XhoI</i>)	No carrier
<i>Ta lcc2</i>	pALK1656 ^(d)	9.9 kb	168 bp (<i>XhoI</i>)	No carrier
<i>Ta lcc3</i>	pALK1671	10.0 kb	232 bp (<i>MscI</i>)	No carrier
<i>Ta lcc4</i>	pALK1684	10.0 kb	481 bp (<i>EcoRV</i>)	No carrier

^(a) The expression cassette for *T. reesei* transformation was isolated from the vector backbone by using *EcoRI* digestion, except in the case of pALK1671 where *NotI* was used.

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

^(c) The *Ta lcc2* gene from the first putative translation start site was used (the length of the gene 1957 bp, including the introns and the STOP codon; Fig. 6 and Table 8).

^(d) The *Ta lcc2* gene from the second putative translation start site was used (the length of the gene 1927 bp, including the introns and the STOP codon; Fig. 6 and Table 8).

The laccase 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) buffered with 5% KH_2PO_4 and supplemented with 0.1 mM CuSO_4 at pH 6.0. The laccase activity was assayed using ABTS as a substrate as described in Example 1. Laccase activity was obtained from all the constructs. The possible targeting of the expression cassette to the *cbh1* (*cel7A*) locus was screened as a CBHI-negative phenotype by dot blot (Minifold I-SRC 96 dot blotter, Schleicher & Schuell, Dassel, Germany) or by Western blot. The detection of the CBHI protein was performed using the monoclonal antibodies CI-258 or CI-261 (Aho et al., 1991) and the ProtoBlot Western blot AP system (Promega). 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.

The chosen CBHI-negative transformants were cultivated in fermentors to obtain material for purification of the recombinant proteins (Example 5) and for the application tests (Examples 7 - 10).

EXAMPLE 5. Purification of the recombinant *Thielavia* and *Chaetomium* laccases

The heterologously produced *Thielavia arenaria* and *Chaetomium thermophilum* laccases were purified from the culture filtrates with common chromatographic means. The buffer of the culture filtrate was changed to the appropriate equilibrating buffer prior to the chromatographic step with gel filtration using Sephadex G25 resin (Pharmacia). The purification procedures for each laccase are summarized in Table 13.

Table 13. Purification of the heterologously produced *Thielavia arenaria* and *Chaetomium thermophilum* laccases. HIC hydrophobic interaction chromatography, EB equilibrium buffer.

Laccase	Chromatographic method/ Resin	Equilibration buffer			Elution protocol
CtLcc1	Anionexchange / DEAE Sepharose FF	20 mM Tris	HCl,		with a linear gradient of 0-250 mM Na ₂ SO ₄ in EB
		pH 8.0			
	HIC/ Phenyl Sepharose FF	20 mM Tris containing Na ₂ SO ₄	HCl pH 7.0, 500 mM,		with a linear gradient of 200-0 mM Na ₂ SO ₄ in EB
	Anionexchange/ Resource Q	10 mM imidazole, pH 7.3			with a linear gradient of 0-150 mM Na ₂ SO ₄ in EB
TaLcc1	Anionexchange / DEAE Sepharose FF	5 mM Tris	HCl,		with a linear gradient of 0-350 mM Na ₂ SO ₄ in EB
		pH 8.5			
	Anionexchange/ Resource Q	5 mM Tris	HCl,		with a linear gradient of 0-200 mM Na ₂ SO ₄ in EB
	Gel Filtration/ Sephacryl S-100 HR	100 mM Tris 150 mM NaCl	HCl pH 7.3,		-
TaLcc2	Anionexchange / DEAE Sepharose FF	10 mM Tris	HCl,		with a linear gradient of 0-300 mM Na ₂ SO ₄ in EB
		pH 8.5			
	HIC/ Phenyl Sepharose FF	20 mM citrate, containing Na ₂ SO ₄	pH 7.0, 500 mM,		with a linear gradient of 500-0 mM Na ₂ SO ₄ in EB
	Anionexchange/ Resource Q	10 mM imidazole, pH 7.3			with a linear gradient of 0-150 mM Na ₂ SO ₄ in EB
	Gel Filtration/ Sephacryl S-100 HR	100 mM Tris 150 mM NaCl	HCl pH 7.0,		-
TaLcc3	Cationexcahnge/ CM Sepharose FF	20 mM acetate, pH 5.0			with a linear gradient of 0-100 mM Na ₂ SO ₄ in EB
	HIC/ Phenyl Sepharose FF	20 mM citrate, containing Na ₂ SO ₄	pH 6.0, 700 mM,		with a linear gradient of 700-0 mM Na ₂ SO ₄ in EB
	Cationexcahnge/ Resource S	10 mM acetate pH 5.0			with a linear gradient of 0-200 mM Na ₂ SO ₄ in EB
TaLcc4	Anionexchange / DEAE Sepharose FF	20 mM acetate pH 5.5			with a linear gradient of 120-400 mM Na ₂ SO ₄ in EB
	HIC/ Phenyl Sepharose FF	20 mM citrate, containing 1500	pH 6.0, mM,		with a linear gradient of 1500-900 mM

EXAMPLE 6. Characterization of the *Thielavia* and *Chaetomium* laccases

The purified recombinant *Thielavia* and *Chaetomium* laccases were characterized in terms of pH optimum, thermal stability, and pI as described in Example 2. The molecular weight was determined by MALDI-TOF mass spectrometry on a Ultraflex™ time-of-flight instrument (BrukerDaltonics, Germany) as previously described (Palonen *et al.*, 2003). The redox-potentials of the T1 coppers of for CtLcc, and TaLcc2 laccases were determined by photometric copper titration in 0.1 M KH₂PO₄ (pH 6.0) as described by Xu *et al.* (1996) using the redox titrant couple K₃Fe(CN)₆/K₄Fe(CN)₆. The redox potential of TaLcc1 was determined with a combined Pt-AgCl/KCl microelectrode at pH 5.0 according to Sigoillot *et al* (2004). The characterization results are collected to Table 14.

Table 14. Summary of the characteristics of the recombinant *Thielavia* and *Chaetomium* laccases. nd = not determined.

Laccase	pH optimum on guaiacol or DMP	T _{1/2} (60°C) (hrs)	PI	Number of pI isoforms	MW (MALDI-TOF)	E° mV
CtLcc1	5.0 guaiacol	7	4.0-4.3	3-4	71 670	480
TaLcc1	6.0 guaiacol	5	5.5-6.9	6-7	71 890	560
TaLcc2	5.5 guaiacol	0.5	3.5	1	75 618	450
TaLcc3	5.0 guaiacol	3.5	7.0-8.0	2	70 050	nd
TaLcc4	6.0 DMP	< 5min	3.0	1	nd	nd

The **inhibition effect** of different compounds on the activity of the laccases was determined as described in Example 2. except with TaLcc4, with which the inhibition was analyzed using spectroscopic activity assay. Instead of following oxygen consumption in the ABTS reaction, the enzyme activity was determined spectrophotometrically. Because the activity of TaLcc3 was very low with all tested substrates the inhibition experiments with this enzyme were not carried out. Results are shown in Table 15.

Table 15. Inhibition of the recombinant *Thielavia* and *Chaetomium* laccases by various compounds. Inhibition tested by spectrophotometric ABTS assay with TaLcc4, the inhibition of the other laccases determined by oxygen consumption measurements.

Compound	Concentr. (mM)	Inhibition (%)			
		CtLcc1	TaLcc1	TaLcc2	TaLcc4
EDTA	10	0	5	0	2
NaN3	0.5	100	95	95	95
KCN	0.1	70	60	30	44
	1	100	90	70	90
NaCl	0.1	0	0	20	5
	1	10	0	30	20

Specific activities of the purified *Thielavia* and *Chaetomium* laccases were determined towards ABTS, dimethoxy phenol (DMP), syringaldazine, and guaiacol as described in Example 2. The ABTS activity measurements were carried out in 25 mM succinate buffer pH 4.5 at 25°C, and the other activities in 25 mM MES buffer, pH 5.5. The results are shown in Table 16.

Table 16. Specific activities of the *Thielavia* and *Chaetomium* laccases compared to the specific activities of a well-known fungal laccase from *Melanocarpus albomyces*. MaL *Melanocarpus albomyces* laccase.

Substrate	MaL nkat/mg	CtLcc1 nkat/mg	TaLcc1 nkat/mg	TaLcc2 nkat/mg	TaLcc3 nkat/mg	TaLcc4 nkat/mg
ABTS	840	705	910	360	8.3	1000
DMP	290	290	285	75	2.1	110
Syringald	380	400	340	120	3.6	52
Guaiacol	90	85	61	40	0	5

Kinetic parameters of the *Thielavia* and *Chaetomium* laccases

The kinetic parameters, Michaelis-Menten constant K_m , turn-over number k_{cat} and the specificity constant (k_{cat}/K_m) were determined on ABTS and 2,6-dimethoxy phenol (DMP), and syringaldazin. The measurements on ABTS were done in 25 mM succinate buffer, pH 4.5. On syringaldazin and DMP 40 mM MES buffer, pH 6 was used.. All activity assays were carried out at 25°C. Kinetic parameters were estimated by a nonlinear regression curve fit. The results are shown in Table 4. The values were compared to those of *Melanocarpus albomyces* MaL, laccase.

Table 17. Kinetic parameters of the *Thielavia* and *Chaetomium* laccases determined on ABTS, syringaldazin, and DMP, and compared to the values of MaL.

	CtLcc1	TaLcc1	TaLcc2	TaLcc3	TaLcc4	MaL
ABTS						
K_m (μM)	330	75	30	1040	2470	270
k_{cat} (min^{-1})	4480	4130	640	37	8610	4690
k_{cat}/K_m ($\text{M}^{-1}\text{min}^{-1}$)	$1.36 \cdot 10^8$	$5.51 \cdot 10^7$	$3.52 \cdot 10^7$	$3.48 \cdot 10^4$	$3.48 \cdot 10^6$	$1.8 \cdot 10^7$
DMP						
K_m (μM)	4.6	17	30	14	1900	5
k_{cat} (min^{-1})	2500	4030	520	5	1590	4160
k_{cat}/K_m ($\text{M}^{-1}\text{min}^{-1}$)	$5.42 \cdot 10^8$	$2.37 \cdot 10^8$	$1.72 \cdot 10^7$	$3.57 \cdot 10^5$	$8.37 \cdot 10^6$	$8.1 \cdot 10^8$
Syringaldazin						
K_m (μM)	2.4	4.3	6.3	4.3	115	1.3
k_{cat} (min^{-1})	2490	1940	450	12	930	4710
k_{cat}/K_m ($\text{M}^{-1}\text{min}^{-1}$)	$1.04 \cdot 10^9$	$4.51 \cdot 10^8$	$7.12 \cdot 10^7$	$2.79 \cdot 10^6$	$7.96 \cdot 10^6$	$3.6 \cdot 10^9$

5

The biochemical data presented here clearly indicates that the recombinant CtLcc1 is the same protein as the wild type *Chaetomium* laccase purified from the culture supernatant and the recombinant TaLcc1 is the same protein as the wild type *Thielavia* laccase purified from the culture supernatant.

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EXAMPLE 7. Performance of laccase preparations in denim bleaching at different pH values

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The recombinant laccase preparations produced using *Trichoderma* as a host were used in all the application tests, in Examples 7 - 10. The recombinant laccases CtLcc1, TaLcc2 and TaLcc4, derived from strains RF5469, RF5573 and RF5687, respectively, were tested for their ability to bleach denim. The commercial laccase preparation DeniLite II Base from Novozymes was used as comparison.

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Lee Cooper jeans (MASI Company Oy, Finland) that were desized and treated with neutral ECOSTONE® cellulase were used as test material. Laccase treatments were performed in LP-2 Launder Ometer as follows. About 10 g of denim swatches (15 x 14 cm) were loaded into 1.2 liter containers containing 200 ml Mc Ilvaine's citrate phosphate buffer pH 5, 6 or 7 and the containers were tempered. Enzyme with or without the mediator (methyl syringate, DeniLite II Assist, Novozymes) was added as laccase activity units. Enzyme was dosed 200 nkat/g and the mediator 10 mg/g on the weight of fabric. Enzyme activity was measured with ABTS substrate (Example 1) but using citrate phosphate buffer in all examples 7 -10. The Launder Ometer was run at 50°C for 30 min and after that the temperature in Launder was raised to 80 °C for 10 min. The swatches were carefully rinsed with warm water, dried half-dry in a tumbler and after that air dried.

The bleaching effect was evaluated by measuring the colour as reflectance values with the Minolta Chromameter CM 1000 (Minolta Co.) using L*a*b* color space coordinates (illuminant D65). The colour from both sides of the swatches was measured before and after the laccase treatment. Each measurement was the average of several measurements.

5

Table 18 and Fig. 8 show that both CtLcc1 and TaLcc2 laccases were more efficient in decolorization of indigo dye of denim compared to DeniLite II Base at pH values 6 and 7. At pH 6 the look of the denim fabric was distinctly much lighter with these two laccases than with DeniLite also by visual evaluation. Without the mediator the laccases did not have notable effect on denim (Table 19).

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Table 18. Colour measurements of the face side of denim treated with laccase preparations and the mediator in Launder at pH 5 - 7. L* indicates lightness, -b* is the blue direction, +b* is the yellow direction.

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Prep.	Enzyme nkat/g	Mediator mg/g	Conditions	Before laccase treatment		After laccase treatment		Increase of L*
				L*	b*	L*	b*	
CtLcc1	200	10	30 min, 50°C, pH 5	29.16	-18.45	35.07	-18.38	5.91
TaLcc2	200	10	30 min, 50°C, pH 5	28.29	-18.47	33.68	-17.91	5.39
TaLcc4	200	10	30 min, 50°C, pH 5	28.16	-18.70	29.17	-18.47	1.01
DeniLite	200	10	30 min, 50°C, pH 5	28.41	-18.70	35.70	-17.59	7.29
CtLcc1	200	10	30 min, 50°C, pH 6	28.89	-18.66	40.08	-17.18	11.19
TaLcc2	200	10	30 min, 50°C, pH 6	28.44	-18.52	39.01	-17.46	10.57
TaLcc4	200	10	30 min, 50°C, pH 6	28.20	-18.47	29.55	-18.11	1.35
Denilite	200	10	30 min, 50°C, pH 6	26.98	-18.67	34.16	-17.82	7.18
CtLcc1	200	10	30 min, 50°C, pH 7	28.38	-18.94	36.69	-17.88	8.31
TaLcc2	200	10	30 min, 50°C, pH 7	28.59	-18.91	35.85	-18.27	7.26
TaLcc4	200	10	30 min, 50°C, pH 7	27.84	-18.63	28.31	-18.33	0.47
Denilite	200	10	30 min, 50°C, pH 7	28.67	-18.99	34.51	-17.75	5.84

Table 19. Colour measurements of the face side of denim treated with laccase preparations without the mediator or mediator alone in Launder at pH 5 - 7. L* indicates lightness, -b* is the blue direction, +b* is the yellow direction.

Prep.	Enzyme nkat/g	Mediator mg/g	Conditions	Before laccase treatment		After laccase treatment		Increase of L*
				L*	b*	L*	B*	
CtLcc1	200	0	30 min, 50°C, pH 5	28.42	-18.44	28.88	-18.65	0.46
TaLcc2	200	0	30 min, 50°C, pH 5	30.13	-18.62	30.11	-18.63	-0.02
TaLcc4	200	0	30 min, 50°C, pH 5	29.44	-18.69	29.40	-18.70	-0.04
DeniLite	200	0	30 min, 50°C, pH 5	29.18	-18.55	29.30	-18.34	0.12
Mediator	0	10	30 min, 50°C, pH 5	29.85	-18.58	29.90	-18.15	0.05
CtLcc1	200	0	30 min, 50°C, pH 6	28.96	-18.60	28.83	-18.53	-0.13
TaLcc2	200	0	30 min, 50°C, pH 6	28.87	-18.71	29.17	-18.51	0.30
TaLcc4	200	0	30 min, 50°C, pH 6	27.44	-18.55	27.68	-18.76	0.24
DeniLite	200	0	30 min, 50°C, pH 6	28.55	-18.48	28.77	-18.52	0.22
Mediator	0	10	30 min, 50°C, pH 6	28.68	-18.40	28.9	-18.37	0.22
CtLcc1	200	0	30 min, 50°C, pH 7	28.59	-18.89	29.32	-18.52	0.73
TaLcc2	200	0	30 min, 50°C, pH 7	27.47	-18.82	28.24	-18.30	0.77
TaLcc4	200	0	30 min, 50°C, pH 7	28.79	-18.71	29.29	-18.89	0.50
Denilite	200	0	30 min, 50°C, pH 7	27.82	-18.93	29.78	-18.31	1.96
Mediator	0	10	30 min, 50°C, pH 7	29.00	-18.94	30.06	-18.46	1.06

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EXAMPLE 8. Performance of laccase preparations in denim bleaching at different temperatures

- 10 Laccases CtLcc1, TaLcc1 (Example 7) and TaLcc2 (strain RF5573) were tested for their ability to bleach denim at different temperatures compared to commercial laccase preparation DeniLite II Base from Novozymes.

15 The test system and denim were as in Example 7, except that the conditions during the laccase and mediator treatment in Launder were 30 min, pH 6 and temperature 30 - 70°C (DeniLite II Base also at 80°C) and the enzyme was inactivated by alkaline treatment instead of raising the temperature in Launder as follows. After removing swatches from the containers they were soaked in warm water containing NaOH (pH 11.5) for 10 min and rinsed carefully with warm water. The swatches were dried half-dry in a tumbler and after that air dried. The bleaching effect was evaluated by measuring the colour
20 as reflectance values as in Example 7.

Table 20 and Fig. 9 show that CtLcc1 and especially TaLcc2 laccases were more efficient in decolorization of denim (highest increase of lightness) compared to the commercial laccase Denilite II Base at 40 – 50 °C and pH 6. TaLcc2 laccase is the most suitable enzyme for applications performed at low temperatures. CtLcc1 and TaLcc2 had also better bleaching effect at their optimal temperatures than DeniLite II base at its optimum

Table 20. Colour measurements of the face side of denim treated with laccase preparations and the mediator in Launder at different temperatures. L* indicates lightness, -b* is the blue direction, +b* is the yellow direction.

Prep.	Enzyme nkat/g	Mediator mg/g	Conditions	Before laccase Treatment		After laccase treatment		Increase of L*
				L*	b*	L*	b*	
CtLcc1	200	10	30 min, 30°C, pH 6	29.34	-18.99	31.78	-19.15	2.44
TaLcc2	200	10	30 min. 30°C, pH 6	29.54	-18.77	33.99	-19.33	4.45
Denilite	200	10	30 min, 30°C, pH 6	29.56	-18.60	32.73	-18.77	3.17
CtLcc1	200	10	30 min, 40°C, pH 6	28.71	-18.61	34.43	-18.77	5.72
TaLcc2	200	10	30 min, 40°C, pH 6	28.93	-18.48	37.08	-18.53	8.15
TaLcc4	200	10	30 min, 40°C, pH 6	29.11	-18.92	29.23	-18.44	0.12
DeniLite	200	10	30 min, 40°C, pH 6	28.87	-18.90	32.94	-19.14	4.07
CtLcc1	200	10	30 min, 50°C, pH 6	28.52	-18.97	36.78	-18.96	8.26
TaLcc2	200	10	30 min, 50°C, pH 6	28.47	-19.05	37.47	-18.48	9.00
Denilite	200	10	30 min, 50°C, pH 6	28.41	-19.10	34.67	-19.07	6.26
CtLcc1	200	10	30 min, 60°C, pH 6	28.88	-19.01	37.40	-18.29	8.52
TaLcc2	200	10	30 min, 60°C, pH 6	29.25	-18.76	36.07	-18.26	6.82
Denilite	200	10	30 min, 60°C, pH 6	29.06	-18.99	35.92	-18.33	6.86
CtLcc1	200	10	30 min, 70°C, pH 6	28.93	-18.95	34.16	-17.91	5.23
TaLcc2	200	10	30 min, 70°C, pH 6	28.3	-19.27	32.84	-17.94	4.54
Denilite	200	10	30 min, 70°C, pH 6	29.05	-19.15	36.72	-17.35	7.67
Denilite	200	10	30 min, 80°C, pH 6	29.28	-18.97	35.33	-17.02	6.05

Example 9. Stain removal with laccases

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Laccases CtLcc1, TaLcc2, TaLcc4 and Denilite II Base (Example 7) were tested for their ability to remove stains. The following artificially soiled test cloths were used: grass soiling (Art.164, EMPA Testmaterialen, Germany), tea soiling (Art. 167, EMPA Testmaterialen, Germany). The fabric was cut in 5.8 x 5.8 cm swatches. Laccase treatments were performed in LP-2 Launder Ometer as follows. About 5 g of soiled fabrics were loaded into 1.2 liter containers containing 150 ml Mc Ilvaine's citrate phosphate

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buffer pH 6 and the containers were tempered. Enzyme with or without the mediator (methyl syringate, DeniLite II Assist, Novozymes) was added as laccase activity units (Example 7). Enzyme was dosed 200 nkat/g and the mediator 10 mg/g on the weight of fabric, except at 40°C dosages of 20 nkat/g and 2 mg/g were also used. The Launder Ometer was run at 40, 50 or 60°C and pH 6 for 60 min. After that the swatches were carefully rinsed under running water and in shake flasks containing warm water and dried in the air.

The stain removal effect was evaluated by measuring the colour as reflectance values using L*a*b* color space coordinates (Example 7). The colour of the swatches was measured before and after the laccase treatment.

The results of stain removal tests are shown in Tables 21- 22 and Figures 10 - 13. CtLcc1 laccase was effective in removal of grass soiling with the mediator at 60°C and TaLcc2 laccase at 50°C , that can be seen in increased lightness and especially in reduced greenness values in Fig.10, and also clearly by visual estimation. Similar trend can be seen at 40°C (Fig. 12). CtLcc1 laccase had some effect without the mediator too, especially at 60°C. TaLcc4 laccase had a slight effect on grass (greenness reduced ca. 2 units) at 50°C. Without the mediator the efficiency in stain removal with laccases was low, especially at 40°C.

CtLcc1 laccase was effective in removal of tea soiling with the mediator at 60°C and TaLcc2 laccase at 50°C , that can be seen in reduced redness and especially in increased lightness values in fig.11, and also clearly by visual estimation. Same trend can be seen at 40°C (Fig. 13). Without the mediator the laccases did not have a notable effect on tea stain, especially at 40°C.

Table 21. Colour measurements of stain removal test with laccases at 50 and 60°C. L* indicates lightness, -b* is the blue direction, +b* is the yellow direction, +a* is the red direction, -a* is the green direction). Untreated artificially soiled test cloth and mediator and buffer controls were used for comparison.

Sample	Enz. dosage nkat/g	Mediator mg/g	Conditions	Grass			Tea		
				L*	a*	b*	L*	a*	b*
Artificially soiled cloth (untreated)	-	-	-	78.32	-	10.18	69.27	8.56	25.80
CtLcc1, RF5469	200	10	60 min, 60°C, pH 6	79.92	-1.15	18.55	79.76	3.52	22.16
CtLcc1, RF5469	200	0	60 min, 60°C, pH 6	79.04	-3.69	18.12	77.10	4.84	21.10
Mediator only	0	10	60 min, 60°C, pH 6	77.98	-6.80	19.26	75.54	5.00	20.5
Buffer only	0	0	60 min, 60°C, pH 6	77.93	-6.70	19.31	75.55	4.94	20.58
TaLcc2, RF5573	200	10	60 min, 50°C, pH 6	79.50	-1.15	17.94	77.73	3.99	22.89
TaLcc4, RF5687	200	10	60 min, 50°C, pH 6	78.62	-4.00	17.16	75.13	5.19	22.44
Mediator only	0	10	60 min, 50°C, pH 6	77.95	-6.45	18.56	75.97	4.84	20.47

Table 22. Colour measurements of stain removal test with laccases at 40°C. L* indicates lightness, -b* is the blue direction, +b* is the yellow direction, +a* is the red direction, -a* is the green direction). Untreated artificially soiled test cloth and mediator and buffer controls were used for comparison.

Sample	Enz.dosage nkat/g	Mediator mg/g	Conditions	Grass			Tea		
				L*	a*	b*	L*	a*	b*
Artificially soiled cloth (untreated)	-	-	-						
CtLcc1, RF5469	200	10	60 min, 40°C, pH 6	78.18	-8.88	25.29	69.36	8.65	25.8
TaLcc2, RF5573	200	10	60 min, 40°C, pH 6	79.74	0.17	16.48	77.70	4.26	24.50
TaLcc2, RF5571	200	10	60 min, 40°C, pH 6	79.19	-0.26	16.99	77.04	4.41	24.36
TaLcc4, RF5687	200	10	60 min, 40°C, pH 6	79.24	-0.31	16.72	77.28	4.36	24.32
Mediator only	0	10	60 min, 40°C, pH 6	78.94	-3.77	16.77	74.56	5.58	23.55
CtLcc1, RF5469	200	0	60 min, 40°C, pH 6	78.88	-6.19	18.28	74.72	5.63	22.16
TaLcc2, RF5573	200	0	60 min, 40°C, pH 6	80.17	-4.14	17.45	76.22	5.37	22.68
TaLcc4, RF5687	200	0	60 min, 40°C, pH 6	80.01	-4.90	17.63	76.09	5.37	22.59
Mediator only	0	10	60 min, 40°C, pH 6	79.84	-4.98	17.62	76.74	5.05	21.67
Buffer only	0	0	60 min, 40°C, pH 6	80.1	-5.67	17.68	76.25	5.11	22.15
CtLcc1, RF5469	20	2	60 min, 40°C, pH 6	79.66	-5.79	18.45	76.00	5.22	22.74
TaLcc1, RF5598	20	2	60 min, 40°C, pH 6	79.94	-1.20	16.17	76.34	5.10	23.89
TaLcc2, RF5573	20	2	60 min, 40°C, pH 6	80.16	-0.73	16.20	77.17	4.65	24.40
TaLcc4, RF5687	20	2	60 min, 40°C, pH 6	79.25	-2.14	16.41	75.61	5.24	23.66
Mediator only	0	2	60 min, 40°C, pH 6	78.53	-6.07	19.33	75.09	5.73	22.08
			60 min, 40°C, pH 6	79.32	-6.43	18.64	74.71	5.94	22.99

EXAMPLE 10. Decolorization of dyes using laccase preparations

The recombinant laccases CtLcc1, TaLcc2 and TaLcc4, derived from *Trichoderma* strains (Example 7) were tested for their ability to decolorize different dyes in the presence of the methyl syringate mediator (Example 7) or without it. The experiments were carried out in 100 ml shake flasks containing 50 ml of dye dissolved in citrate phosphate buffer pH 6. Dye concentration 5 mg/50 ml was used. Enzyme was dosed 100 nkat per 50 ml and the mediator 5 mg per 50 ml. Control samples contained only dye solution. The shake flasks were incubated at 50°C for 30, 60 and 120 minutes. Samples of 3.5 ml were taken in test tubes for visual evaluation.

The results are shown in Table 23 and 24. CtLcc1 and TaLcc2 laccases were able to decolourize Indigocarmine and Remazol Brilliant Blue (Reactive Blue 19) to great extend or completely and Cibacron Brilliant Red 3B-P partly in the presence of the mediator. Degradation of Indigocarmine was fast, and the blue colour had turned to light yellow in already 30 min or earlier. The reaction seemed to be completed after 60 min with all dyes, since no visually detectable changes in the colours of the samples were observed any more.

Table 23. Decolorization of dyes with CtLcc1 laccase. Treatment time 30 and 60 min. - no visually detectable change, + visually detectable fading of the colour, ++ considerable fading of the colour, +++ complete/almost complete decolorization.

Dye 5 mg/50 ml	Enz. dosage nkat/50 ml	Mediator mg/50 ml	Time 30 min	Time 60 min
Cibacron Brilliant Red 3B-P (Ciba-Geigy)	100	0	-	-
Cibacron Brilliant Red 3B-P (Ciba-Geigy)	100	5	+	+
Remazol Brilliant Blue (Sigma)	100	0	-	-
Remazol Brilliant Blue (Sigma)	100	5	++	+++
Indigocarmine (Merck)	100	0	-	-
Indigocarmine (Merck)	100	5	+++	+++

Table 24. Decolorization of dyes with TaLcc2 laccase. Treatment time 30 and 60 min. - no visually detectable change, + visually detectable fading of the colour, ++ considerable fading of the colour, +++ complete/almost complete decolorization.

Dye 5 mg/50 ml	Enz. dosage nkat/50 ml	Mediator mg/50 ml	Time 30 min	Time 60 min
Cibacron Brilliant Red 3B-P (Ciba-Geigy)	100	0	-	--
Cibacron Brilliant Red 3B-P (Ciba-Geigy)	100	5	+	+
Remazol Brilliant blue (Sigma)	100	0	-	-
Remazol Brilliant blue (Sigma)	100	5	++	+++
Indigocarmine (Merck)	100	0	-	--
Indigocarmine (Merck)	100	5	+++	+++

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WHAT IS CLAIMED IS:

1. A laccase enzyme, characterized in that it comprises an amino acid sequence selected from the group comprising SEQ ID 41 (TaLcc2) or a sequence showing at least 60 % identity to the sequence SEQ ID NO: 41, SEQ ID 43 (TaLcc3) or a sequence showing at least 58 % identity to the sequence SEQ ID NO: 43, SEQ ID 45 (TaLcc4) or a sequence showing at least 78 % identity to the sequence SEQ ID NO: 45, SEQ ID 47 (CtLcc1) or a sequence showing at least 74 % identity to the sequence SEQ ID NO: 47, SEQ ID 49 (CtLcc2) or a sequence showing at least 55 % identity to the sequence SEQ ID NO: 49 and SEQ ID 51 (CtLcc3) or a sequence showing at least 53 % identity to the sequence SEQ ID NO: 51.
2. The laccase enzyme according to claim 1, wherein the enzyme is obtainable from a microbial source.
3. The laccase enzyme according to claim 1 or 2, wherein the enzyme is obtainable from a filamentous fungus.
4. The laccase enzyme according to claim 1, wherein the enzyme comprises the amino acid sequence SEQ ID 41 (TaLcc2) or a sequence showing at least 60 % identity to the sequence SEQ ID NO: 41.
5. The laccase enzyme according to claim 1 to 4, wherein the enzyme is obtainable from the genus *Thielavia*.
6. The laccase enzyme according to any one of claims 1 to 5, wherein the enzyme is obtainable from the deposited strain CBS 116071.
7. The laccase enzyme according to claim 5 or 6, wherein the enzyme is encoded by pALK1347 deposited in *E. coli* under the number DSM 15486.
8. The enzyme according to any one claims 4 to 6, wherein the enzyme functions at pH 3 to 9, preferably at pH 4 to 8, most preferably at pH 4.5 to 6.5 under suitable conditions.
9. The enzyme according to any one of claims 4 to 8, wherein the pH optimum of the enzyme is about pH 5.5 under suitable conditions.
10. The enzyme according to any one of claims 4 to 9, wherein the enzyme is effective at temperatures 30 to 80 °C, preferably at temperatures 40 to 60°C, more preferably at temperatures 40 to 50°C.
11. The laccase enzyme according any one of claims 4 to 10, wherein the enzyme is capable of bleaching denim.

12. The laccase enzyme according any one of claims 4 to 11, wherein the enzyme is most effective in bleaching denim at the temperature of about 40 to 50°C.
13. The laccase enzyme according any one of claims 4 to 12, wherein the enzyme is most effective in bleaching denim at about pH 6.
- 5 14. The laccase enzyme according any one of claims 4 to 13, wherein the enzyme is effective in stain removal.
15. The laccase enzyme according any one of claims 4 to 14, wherein the enzyme is capable of decolorizing dyes.
- 10 16. The laccase enzyme according to claim 4 to 15, wherein the enzyme is most effective in the presence of one or more mediators.
17. The laccase enzyme according to claim 1, wherein the enzyme comprises the amino acid sequence SEQ ID 43 (TaLcc3) or a sequence showing at least 58 % identity to the sequence SEQ ID NO: 43.
- 15 18. The laccase enzyme according to any one of claims 1 to 3 or 17, wherein the enzyme is obtainable from the strains of genus *Thielavia*.
19. The laccase enzyme according to claim 17 or 18, wherein the enzyme is obtainable from the deposited strain CBS 116071.
20. The laccase enzyme according to any one of claims 17 or 19, wherein the enzyme is encoded by pALK1345 deposited in *E. coli* under the number DSM 15485.
- 20 21. The enzyme according to any one claims 17 to 20, wherein the enzyme functions at pH 3.5 to 7.5, preferably at pH 4 to 6.5 under suitable conditions.
22. The enzyme according to any one of claims 17 to 21, wherein the pH optimum of the enzyme is about pH 5.0 under suitable conditions.
- 25 23. The laccase enzyme according to claim 1, wherein the enzyme comprises the amino acid sequence SEQ ID 45 (TaLcc4) or a sequence showing at least 78 % identity to the sequence SEQ ID NO: 45
24. The laccase enzyme according to any one of claims 1 to 3 or 23, wherein the enzyme is obtainable from the strains of genus *Thielavia*.
- 30 25. The laccase enzyme according to claim 23 or 24, wherein the enzyme is obtainable from the deposited strain CBS 116071.

26. The laccase enzyme according to any one of claims 23 to 25, wherein the enzyme is encoded by pALK1664 deposited in *E. coli* under the number DSM 15487.
27. The enzyme according to any one of claims 23 to 26, wherein the enzyme functions at pH 3.5 to 7.5, preferably at pH 4 to 7, most preferably at pH 5 to 6.5 under suitable conditions.
- 5 28. The enzyme according to any one of claims 23 to 27, wherein the pH optimum of the enzyme is about pH 6 under suitable conditions.
29. The laccase enzyme according to claim 1, wherein the enzyme comprises the amino acid sequence SEQ ID 47 (CtLcc1) or a sequence showing at least 74 % identity to the sequence SEQ ID NO: 47.
- 10 30. The laccase enzyme according to any one of claims 1 to 3 or 29, wherein the enzyme is obtainable from the strains of genus *Chaetomium*.
31. The laccase enzyme according to claim 29 or 30, wherein the enzyme is obtainable from the deposited strain CBS 730.95.
- 15 32. The laccase enzyme according to any one of claims 29 to 31, wherein the enzyme is encoded by pALK1304 deposited in *E. coli* under the number DSM 15075.
33. The enzyme according to any one claims 29 to 32, wherein the enzyme functions at pH 3.5 to 8, preferably at pH 4 to 7, most preferably at pH 4.5 to 6 under suitable conditions.
34. The enzyme according to any one of claims 29 to 33, wherein the pH optimum of the enzyme is about pH 5.0 under suitable conditions.
- 20 35. The enzyme according to any one of claims 29 to 34, wherein the enzyme is effective at temperatures 30 to 80 °C, preferably at temperatures 40 to 70°C, more preferably at temperature 50 to 60°C.
36. The laccase enzyme according any one of claims 29 to 35, wherein the enzyme is capable of bleaching denim.
- 25 37. The laccase enzyme according any one of claims 29 to 36, wherein the enzyme is most effective in bleaching denim at the temperature of about 50 to 60°C.
38. The laccase enzyme according any one of claims 29 to 37, wherein the enzyme is most effective in bleaching denim at about pH 6.
- 30 39. The laccase enzyme according any one of claims 29 to 38, wherein the enzyme is effective in stain removal.

40. The laccase enzyme according any one of claims 29 to 39, wherein the enzyme is capable of decolorizing dyes.
41. The laccase enzyme according to any one of claims 29 to 39, wherein the enzyme is most effective in the presence of one or more mediators.
- 5 42. The laccase enzyme according to claim 1, wherein the enzyme comprises the amino acid sequence SEQ ID 49 (CtLcc2) or a sequence showing at least 55 % identity to the sequence SEQ ID NO: 49.
43. The laccase enzyme according to any one of claims 1 to 3 or 42, wherein the enzyme is obtainable from the strains of genus *Chaetomium*.
- 10 44. The laccase enzyme according to claim 42 or 43, wherein the enzyme is obtainable from the deposited strain CBS 730.95.
45. The laccase enzyme according to any one of claims 42 to 44, wherein the enzyme is encoded by pALK1305 deposited in *E. coli* under the number DSM 15076.
- 15 46. The laccase enzyme according to claim 1, wherein the enzyme comprises the amino acid sequence SEQ ID 51 (CtLcc3) or a sequence showing at least 53 % identity to the sequence SEQ ID NO: 59.
47. The laccase enzyme according to any one of claims 1 to 3 or 46, wherein the enzyme is obtainable from the strains of genus *Chaetomium*.
- 20 48. The laccase enzyme according to claim 46 or 47, wherein the enzyme is obtainable from the deposited strain CBS 730.95.
49. The laccase enzyme according to any one of claims 46 to 48, wherein the enzyme is encoded by pALK1685 deposited in *E. coli* under the number DSM 16040.
50. The laccase enzyme according to any one of the preceding claims, wherein the enzyme lacks the signal sequence.
- 25 51. The laccase enzyme according to any one of the preceding claims, wherein the enzyme lacks the tail.
52. The laccase enzyme according to any one of the preceding claims, wherein the enzyme is produced in a heterologous production host.
- 30 53. The laccase enzyme according to any one of the preceding claims, wherein the laccase is produced in a microbial host.

54. The laccase enzyme according to any one of the preceding claims, wherein the laccase is produced in a filamentous fungus host.
55. The laccase enzyme according to any one of the preceding claims, wherein the enzyme is produced in a host of the genus *Trichoderma* or *Aspergillus*.
- 5 56. A nucleic acid sequence, c h a r a c t e r i z e d in that it encodes the enzyme as defined in any one of claims 1 to 55.
57. A vector, c h a r a c t e r i z e d in that it comprises the nucleic acid sequence according to claim 56.
- 10 58. The vector according to claim 57, wherein the nucleic acid sequence has been operably linked to expression control sequences allowing expression in prokaryotic or eukaryotic host cells.
59. A host cell, c h a r a c t e r i z e d in that the nucleic acid sequence according to claim 56 or the vector according to claim 57 or 58 has been introduced into it.
60. The host cell according to claim 59, wherein the host cell is a microbial host cell.
- 15 61. The host cell according to claim 59 or 60, wherein the host cell belongs to the genus *Trichoderma* or *Aspergillus*.
62. A process for the production of a polypeptide having laccase activity, comprising the steps of culturing the host cell according to any one of claims 59 to 61, and recovering the polypeptide.
- 20 63. A polypeptide having laccase activity encoded by the nucleic acid sequence according to claim 56, or by the vector according to claim 57 or 58 and which is obtainable by the process according to claim 62.
64. A process for obtaining an enzyme preparation comprising a polypeptide according to claim 63 comprising the steps of culturing a host cell according to any one of claims 59 to 61 and
25 either recovering the polypeptide from the cells or separating the cells from the culture medium of the host and obtaining the supernatant.
65. An enzyme preparation obtainable by the process according to claim 64.
66. An enzyme preparation, which comprises the laccase enzyme according to any one of claims 1 to 55.

67. The enzyme preparation according to claim 65 or 66, wherein the enzyme preparation further comprises a suitable additive(s) selected from the group comprising stabilizers, buffers, mediators and preservatives.
- 5 68. The enzyme preparation according to any one of claims 65 to 67, wherein the enzyme preparation is the spent culture medium of the production host.
69. The enzyme preparation according to any one of claims 65 to 68, wherein the enzyme preparation is in the form of liquid, powder or granulate.
- 10 70. A method for treating denim, which comprises contacting denim in an aqueous medium with a laccase enzyme according to any one of claims 1 to 55 or with an enzyme preparation according to any one of claims 65 to 69 under suitable conditions for the function of the enzyme.
71. The method according claim 70, wherein the treatment is carried out in the presence of a suitable mediator.
- 15 72. The method according claim 70 or 71 wherein the treatment is carried out at the temperature of 30 to 80°C, preferably at the temperature 40 to 70 °C, more preferably at the temperature of 40 to 60°C.
73. The method according to any one of claims 70 to 72, wherein the treatment is carried out at pH range from pH 3 to 8, preferably at pH 4 to 7, most preferably at pH 4.5 to 6.5.
- 20 74. The method according any one of claims 70 to 73, wherein the treatment is carried out in 15 minutes to 2 hours, preferably in 30 minutes to 90 minutes, most preferably in 30 minutes to 60 minutes.
75. The method according any one of claims 70 to 74, wherein the dosage of enzyme used in the treatment is 2 to 500 nkat, preferably 20 to 200 nkat, more preferably 20 to 100 nkat/ g fabric.
- 25 76. The method according any one of claims 70 to 75, wherein the treatment is repeated one or more times.
77. A method for stain removal, which comprises contacting material to be treated with a laccase enzyme according to any one of claims 1 to 55 or with an enzyme preparation according to any one of claims claim 65 to 69 under suitable conditions for the function of the enzyme.

78. A method of bleaching pulp, which comprises the step of contacting said pulp with a laccase enzyme according to any one of claims 1 to 55 or with an enzyme preparation according to any one of claims 65 to 69 under suitable conditions for the function of the enzyme.
- 5 79. A method for treating natural or man-made fibre, which comprises contacting fibre with a laccase enzyme according to any one of claims 1 to 55 or with an enzyme preparation according to any one of claims 65 to 69 under suitable conditions for the function of the enzyme.
- 10 80. A method for treating lignocellulosic fibre, which comprises contacting fibre with a laccase enzyme according to any one of claims 1 to 55 or with an enzyme preparation according to any one of claims 65 to 69 under suitable conditions for the function of the enzyme.
81. A method for treating wool, which comprises contacting wool with a laccase enzyme according to any one of claims 1 to 55 or with an enzyme preparation according to any one of claims 65 to 69 under suitable conditions for the function of the enzyme.
- 15 82. A method for treating hair, which comprises contacting hair with a laccase enzyme according to any one of claims 1 to 55 or with an enzyme preparation according to any one of claims 65 to 69 under suitable conditions for the function of the enzyme.
- 20 83. A method for treating dye house effluents, which comprises contacting dye house effluents with a laccase enzyme according to any one of claims 1 to 55 or with an enzyme preparation according to any one of claims 65 to 69 under suitable conditions for the function of the enzyme.
84. A method for decolorizing of dyes, which comprises contacting dyes or dye containing material with a laccase enzyme according to any one of claims 1 to 55 or with an enzyme preparation according to any one of claims 65 to 69 under suitable conditions for the function of the enzyme.
- 25 85. Use of a laccase enzyme according to any one of claims 1 to 55 or an enzyme preparation according to any one of claims claim 65 to 69 for denim treatment, for removing stain, for bleaching of pulp, for treating of fibers, for treating of wool, for treating of hair, for treating of dye house effluents or for decolorization of dyes or dye containing material.

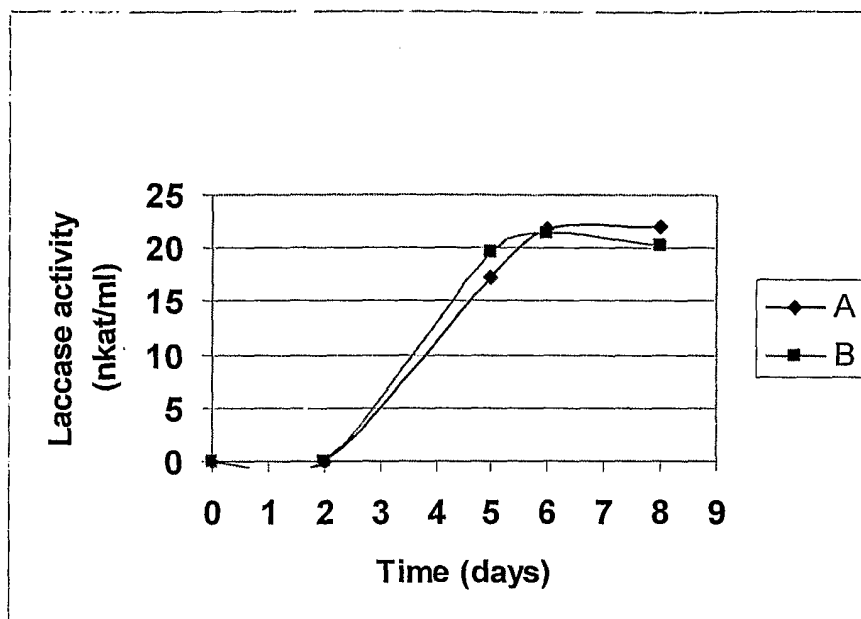


Fig. 1A

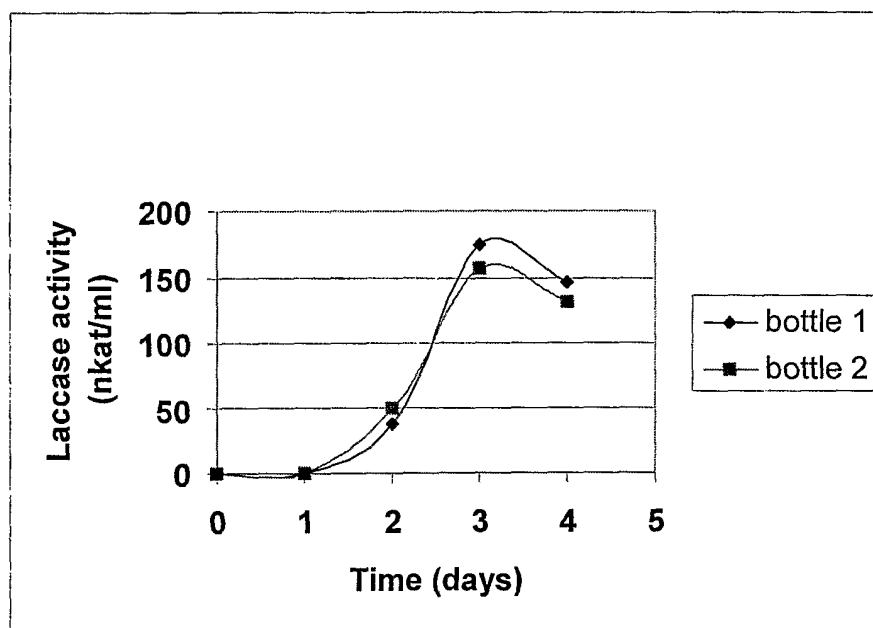


Fig. 1B

1. 2. 3. 4. 5. 6. 7.

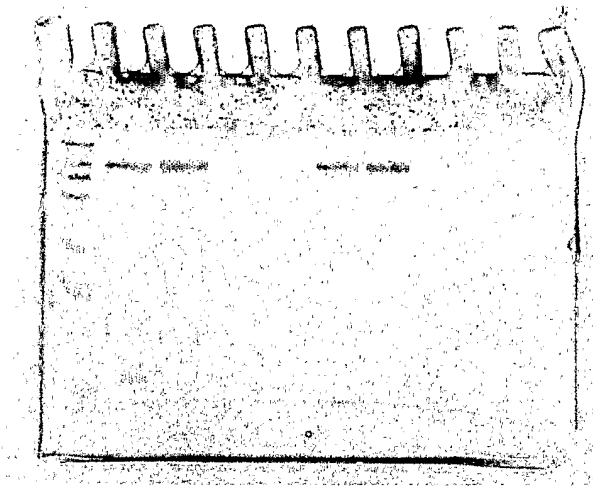


Fig. 2A

1 2 3 4 5 6 7 8 9 10

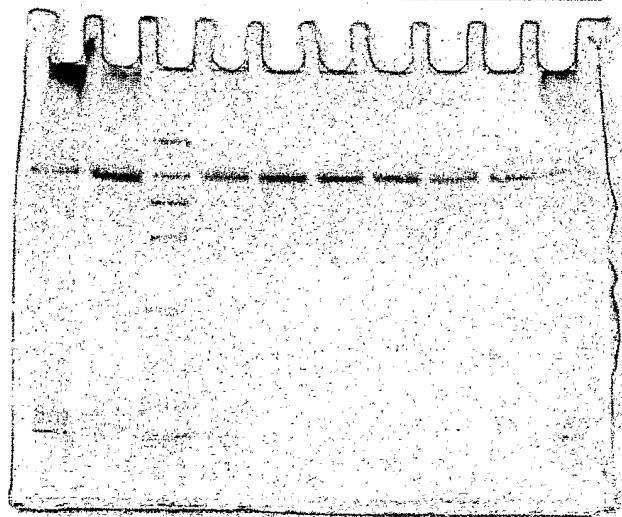
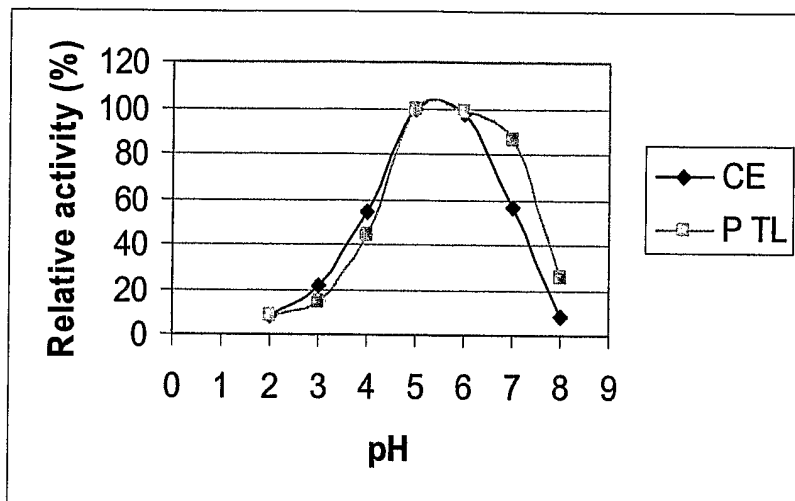
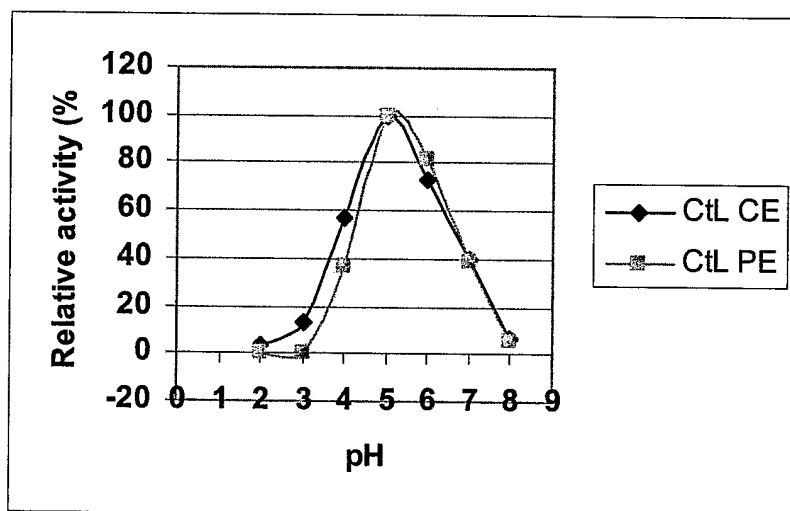
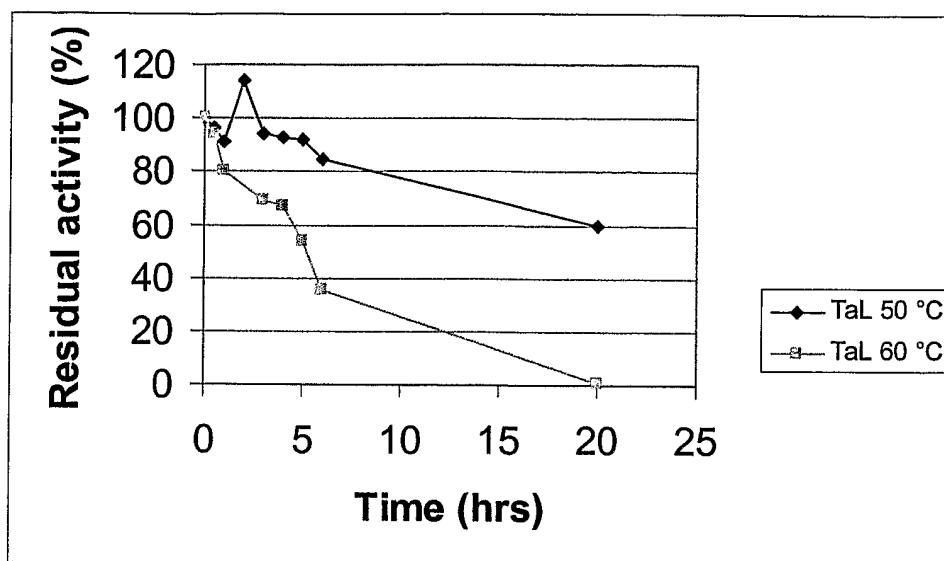
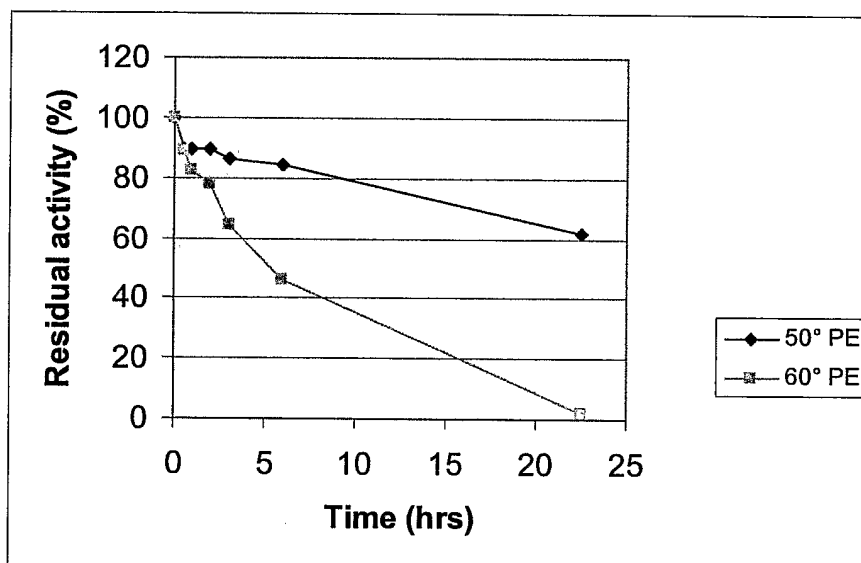


Fig. 2B

**Fig. 3A****Fig. 3B**

**Fig. 4A****Fig. 4B**

Peptide I

1	2	3	4	5	6
W	Y	H	S	H	F
TGG	TAC	CAC	AGA	CAC	TTC
	T	T	TCC	T	T
			T		
			G		

Peptide II

1	2	3	4	5	6	7
(M)	H	L	H	G	H	D
ATG	CAC	CTA	CAC	GGA	CAC	GAC
	T	T	C	T	C	T
			T		T	
			G		G	

Peptide III

1	2	3	4	5	6	7	8
(M)	H	C	H	I	A	W	H
ATG	CAC	TGC	CAC	ATA	GCA	TGG	CAC
	T	T	T	C	C		T
				T	T		
					G		

Fig. 5A

Peptide 1

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
Y	Q	G	A	P	N	T	L	P	T	N	Q/I	G	L	P	V	P	N	H
TAC	CAA	GGA	GCA	CCA	AAC	ACA	CTA	CCA	ACA	AAC	CAA/ATA	GGA	CTA	CCA	GTA	CCA	AAC	CAC
T	G	C	C	C	T	C	T	C	C	C	T	G	C	C	T	C	C	T
		G	G	G		G	G	G	G			T	G	G	G	G	G	
		T	T	T		T	T	T	T				T	T	T	T	T	

Peptide 2

1	2	3	4	5	6	7	8	9	10	11
E	N	W	I	G	P	D	G	V	L	K
GAA	AAC	TGG	ATA	GGA	CCA	GAC	GGA	GTA	CTA	AAA
G	T		C	C	C	T	C	C	T	C
			T	G	G		G	G	G	
				T	T		T	T	T	

Peptide 3

1	2	3	4	5	6	7	8	9
(S)	L	F	L	A	V	G	Q	R
AGA	CTA	TTC	CTA	GCA	GTA	GGA	CAA	AGA
TCC	T	C	T	T	C	C	C	G
G	G		G	G	G	G		G
T	T		T	T	T	T		T

Fig. 5B

N-terminal peptide

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
E	G	P	G	P	C	H	T	P	A	N	Y	A	C	W	A	P	G	F	D
GAA	GGT	CCT	GGT	CCT	TGT	CAT	ACT	CCT	GCT	AAT	TAT	GCT	TGT	TGG	GCT	CCT	GGT	TTT	GAT
G	C	C	C	C	C	C	C	C	C	C	C	C	C		C	C	C	C	C
	A	A	A	A			A	A	A			A			A	A	A		
	G	G	G	G			G	G	G			G			G	G	G		

Peptide 18.9

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
L	T	E	N	D	N	W	T	G	P	D	G	V	V	K
TTT	ACT	GAA	AAT	GAT	AAT	TGG	ACT	GGT	CCT	GAT	GGT	GTT	GTT	AAA
C C	C	G	C	C	C			C	C	C	C	C	C	C
A	A						A	A	A		A	A	A	
G	G						G	G	G		G	G	G	

Fig. 5C

Ta lcc1 and the deduced amino acid sequence

1	ggatccccgggtcagtcctatataaggggctgagtggtccagctcttccatgcctttgattc	
61	tcttgaatcaccaggacactcgggcggttcagtccttgcataaactcgggtcttcccttcc	
121	tctcactgcttttcttcgctcagatatatttcaggcgacctcaaacagctcgccatcATG	
1		<u>M</u>
181	AAGTCTTGGGCCCGCCGCTGGCGCTCATGGTGGGCATTCTCAGCCCTCATGCTGCCGCC	
2	K S W A A A V A L M V G I L S P H A A A	
241	GCACCTCCTGCAAACCCGGTCCAGAGAGACATGCTCCAGGTCCTTGAGGCGAGACAGTCT	
22	A P P A N P V Q R D M L Q V L E A R Q S	
301	GGCCCGACTTGCAACACCCCGTCCAATCGTGGTGGTGGACCAATGGTTTCGACATCAAC	
42	G P T C N T P S N R A C W T N G F D I N	
361	ACCGACTATGAAGTCAGCACTCCTAATACCGGACGTAAGTGTGGCCgtaagcttccctcc	
62	T D Y E V S T P N T G R T V A intron 1 (51 bp)	
421	ctttaaggaggcagagctaggactaacaagcaccagTACCAACTTACCCTCACTGAGAAA	
77		Y Q L T L T E K
481	GAGAACTGGATCGGTCCCGATGGCGTTCTCAAGAATGTGGTGATGTTGGTCAATGgtacg	
85	E...N...W...I...G...P...D...G...V...L...K...N V V M L V N D	Peptide 2
541	ttgatgtccaattctgtataaagagaagaaacgtgctgatacgctcccttcgtctagACA	
104	intron 2 (62 bp)	K
601	AGATTATAGgtatgttgtcaaaccgctgtaaccccaaccgccaagacctggaggctcct	
105	I I G intron 3 (91 bp)	
661	cgcttgacgtgtgtgtacaatatgctgacctcgccgccagGGCCAACCATCCGCGCGAAC	
108		P T I R A N
721	TGGGGTGACAAATATCGAAGTCACTGTGCATCAACAATCTCAAACCAATGGgtacgaccac	
114	W G D N I E V T V I N N L K T N G	
781	ttgaatcatcccgggcctacccttaacacaaaatctcaacgtgcatccgatctgacgtat	
	intron 4 (83 bp)	
841	tatatccatctagTACCTCGATGCACTGGCATGGCCTTCGTCAGCTGGGTAACGTTTCA	
131	T S M H W H G L R Q L G N V F N	Cu-binding
901	ACGACGGTGCCAACGGCGTGACTGAGTGCCAATCCCGCCCAAGGAGGGCGCAAGACGT	
147	D G A N G V T E C P I P P K G G R K T Y	
961	ACAAGTTCCGTGCGACACAGTATGGCACCAGCTGGTATCACTCCCACTTCTCGGCCAGT	
167	K F R A T Q Y G T S W Y H S H F S A Q Y	Cu-binding
1021	ACGGCAACGGCGTGGTGGCACCATCCAGATCGACGGCCCTGCCTCTCTGCCATATGACA	
187	G N G V V G T I Q I D G P A S L P Y D I	
1081	TTGATCTGGGCGTGTTCCTCTCATGGACTACTACTACAGGTGGGCCGATGAGCTGGTGC	
207	D L G V F P L M D Y Y Y R S A D E L V H	
1141	ACTTCACCCAGAGCAACGGCGCCCCGCCAAGCGACAACGTCTCTTCAATGGCACCGCC	
227	F T Q S N G A P P S D N V L F N G T A R	
1201	GTCACCCTGAGACGGGGGCGAGCCAGTGGTACAACGTACGCTGACTCCAGGCAAGCGAC	
247	H P E T G A G Q W Y N V T L T P G K R H	
1261	ACCGCCTGCGCATCATCAACACGTGACCGACAACCACTTTTCAAGGTGTCGCTTGTGGCC	
267	R L R I I N T S T D N H F Q V S L V G H	

Fig. 6A I

1321	ACAACATGACCGTCATTGCCACCGACATGGTCCCCGTCAACGCCTTTACTGTCAGCAGCC	
287	N M T V I A T D M V P V N A F T V S S.....L	
1381	TATTCCTCGCCGTAGGCCAGCGATACGATGTCACCATCGACGCCAATAGCCCGGTGGGCA	Peptide 3
307	F.....A.V.G.Q.R Y D V T I D A N S P V G N	
1441	ACTACTGGTTCAACGTGACTTTTCGGCGATGGGTTGTGCGGCTCCAGTAACAACAAATTCC	
327	Y W F N V T F G D G L C G S S N N K F P	
1501	CAGCCGCCATCTTCCGCTACCAGGGCGCCCCGCTACGCTCCCGACGGATCAGGGTCTAC	Peptide 1
347	A A I F R Y.Q.G.A.P.A.T.L.P.T.D.Q.G.L.P	
1561	CCGTGCCCAATCACATGTGTTTGGACAACCTGAACCTAACTCCTGTGGTGACACGGAGCG	
367	V.....P.N...H M C L D N L N L T P V V T R S A	
1621	CGCCCGTCAACAACCTTGTCAAGCGTCCGTCCAACACGCTGGGCGTCACTCTCGATATCG	
387	P V N N F V K R P S N T L G V T L D I G	
1681	GCGGCACGCCGCTCTTGTGTGGAAGGTCAACGGCAGCGCCATCAACGTCGACTGGGGCA	
407	G T P L F V W K V N G S A I N V D W G K	
1741	AGCCGATCCTTGACTATGTCATGAGCGGCAACACGAGCTACCCGGTCAGCGATAACATTG	
427	P I L D Y V M S G N T S Y P V S D N I V	
1801	TGCAGGTGGACGCTGTTGACCAGgtacgccccctcttgaagccccctagcagttcacgctag	
447	Q V D A V D Q intron 5 (79 bp)	
1861	tatacaatacaagtacatgtctaacacttccctccctattcagTGGACTTACTGGCTGATC	
454	W T Y W L I	
1921	GAGAACGACCCGACCAATCCCATTGTGTCAGCTTGCCGCACCCGATGCATCTGCACgtacgt	Cu-binding
460	E N D P T N P I V S L P H P M H L H	
1981	tcaaacctccccccacccccacttcatacaaaatatactgacaaatcgacagGGCCACG	
478	intron 6 (59 bp) G H D	
2041	ACTTCCTCGTCTTGGGCCGATCACCCGACGAGCTCCCCAGCGGGGGTCCGTACATCT	
481	F L V L G R S P D E L P S A G V R H I F	
2101	TTGACCCGGCCAAGGACCTGCCCGGCTTAAGGGCAACAACCCCGTGCGGCGGGACGTGA	
501	D P A K D L P R L K G N N P V R R D V T	
2161	CGATGCTTCCGGCGGGGCGGCTGGCTGCTGCTGGCGTTCAAGACGGACAACCCGGGCGCAT	
521	M L P A G G W L L L A F K T D N P G A W	
2221	GGCTGTTCCACTGCCACATTGCGTGGCAGCTGTGCGGCGGCCTGTTCGGTCGACTTCCTCG	Cu-binding
541	L F H C H I A W H V S G G L S V D F L E	
2281	AGCGGCCCAACGACCTTCGCACGCAGCTCAACAGCAACGCCAAGCGCGCCGACCGCGACG	
561	R P N D L R T Q L N S N A K R A D R D D	
2341	ACTTCAACCGCTCTGCCGCGAGTGAACGCCTACTGGCCTACCAACCCGTTCCCCAAGA	
581	F N R V C R E W N A Y W P T N P F P K I	
2401	TCGACTCGGGCTTGAGGCACCGGTTTGTGAGGAGAGCGAGTGGATGGTTTCGCTAAactg	
601	D S G L R H R F V E E S E W M V R *	
2461	cctggctgtgccaattgatttgatgggtacatgtacctgttggtgttactgttgacgagg	
2521	ctgtgtaagtaccatggcaaggggtgttttcaggggtgctctggggtaattggcacagt	
2581	acatggaggggtctgggggtgggtatatacaaggcttgctgctccgtttttatcttttggt	
2641	tgattaagactttcttctgtctgatgtacgagtcaggccgcc	

Fig. 6A II

Ta lcc2 and the deduced amino acid sequence

1 ggatcccgtagtcacaagacccagcaccagggttaaccatcctatacggggcaagatct
 61 accacttgacgcatatattgaagtccaatacatcgtcactgtagtagtcatgggtgtct
 121 gtaagttccggcagggactgtagtactcgacgggttcctctccttccagttagccctcgt
 181 agagcatgttgacgttacgataggtttccccccccccatttgagctgtgatatactttc
 241 aagttgttgaaagccttttagctgagcataacgatcgtggctgacgaaagcctccgtgcgg
 301 cacacctaataattacgattccgaatgctcctccaaggagttgtcatatgatactattgat
 361 gtgagcttcaggggagcaggcccattacggaaggtagaccagggacaactccttctcggc
 421 ttaaccgggcccggatcgtggaatgcctcgccaaagactcattgcgaggcgacatggagcc
 481 aagcccaccttctcttgaggctctgatattgatgctgtacgagtataaagtcgatgatct
 541 cctgcaatgtcccagagcttatccaagcagctcttggaccaaggaagccatcggctactc

 601 aacaggcgcgccacattcgttcaactttgaattggtagcaccATGTTAAACCGAGACACT
 1 M L T E T L
 661 CAAGTCAAGAAACATGTTGCAATCAATCCTTGCTCTTCTTGCCACCGCCCTGGGCTCATC
 7 K S R N M L Q S I L A L L A T A L G S S
 721 TGCCTTCGCCATCCCTGAACTCCCAGAGGTCTCGCTTCAGCCCCGGCAAGCTTGCGAGAA
 27 A F A I P E L P E V S L Q P R Q A C E N
 781 CACTGCAACGTCTCGCCAATGCTGGGGTGACCTTTCAATCGACACCAACTACTATGAAGT
 47 T A T S R Q C W G D L S I D T N Y Y E V
 841 TCTTCCCAGCACCGGGCGCCCGCAGGAGTATTGGCTGAGTGTCTGGAAGGCCCTTG
 67 L P S T G R P P Q E Y W L S V V E G P C
 901 TGCCCCAGACGGATATAACAGGACCTGTATGACCTTTAACGGCACCGTGCCGGGGCCAAAC
 87 A P D G Y N R T C M T F N G T V P G P T
 961 CCTCTTCGCCGATTGGGGCGACGAGCTCGTTATCCATGTACAAACAACATGGTCAACAA
 107 L F A D W G D E L V I H V T N N M V N N
 1021 TGGCACCGGATCCACTGGCACGGGATTGCGATGCTGAATAACACGCTCAACGACGGCGT
 127 G T A I H W H G I R M L N N T L N D G V Cu-binding
 1081 TCCCGGAGTGACGCAGTGCGCCATCGCCCCGGGCGAGTCCATGACATACCGGTTCAACGT
 147 P G V T Q C A I A P G E S M T Y R F N V
 1141 CACCCAGTACGGCTCGACTTGGTACCACAGCCACTTCAGCCTCCAGTACGCCGAGGGTCT
 167 T Q Y G S T W Y H S H F S L Q Y A E G L Cu-binding
 1201 CTTCGGGGGCATGATTCTCCGGGGGCCAGCACGGCCAACTGGGACGAGGACCTCGGAGT
 187 F G G M I L R G P S T A N W D E D L G V
 1261 CTTGTTTCTGCAGGACTGGTCACACGTGCAAGCCTTCACTAGGTGGCATGAAGCGAAAGC
 207 L F L Q D W S H V E A F T R W H E A K A
 1321 CGGGTTTCTCCATCACTCGATGGCGGCCTGATCAACGGCACAAACACATTGACTGCTC
 227 G F P P S L D G G L I N G T N T F D C S
 1381 CACACTGTGCGCGACGGACCCCAAGTGCACCGGCAATGGCAAGAAGTTCGAGACCGTCTT
 247 T L S P T D P K C T G N G K K F E T V F
 1441 CGAACCCGGCAAGAAGTATCTTATCCGCCTCATCAACGTGGCAATCGACGGAGTGTTCCTCA
 267 E P G K K Y L I R L I N V A I D G V F Q
 1501 GTTCAGCATCGACGGACACAGCCTCACCGTCATAGCGACGGATTTGGTGCCGATTGTCCC
 287 F S I D G H S L T V I A T D L V P I V P

Fig. 6B I

1561 CTACACGACCGACAGCGTCCAGATCACCATCGGCCAGCGTTATGATATCATTGTGCGAGGC
 307 Y T T D S V Q I T I G Q R Y D I I V E A
 1621 AAACGCAACGCCGGGAACTACTGGATGCGGGCCGACTGGGTACAGCCTGCGTGACCAA
 327 N A T P G N Y W M R A D W V T A C V T N
 1681 CGATCACCCCGAGCACATGACGGGCATCGTTCGCTATGACGCGAGCAGCATCGACCCCC
 347 D H P E H M T G I V R Y D A S S I D P P
 1741 GACGTCCGAAAGCAACGTACAAAAACCAGCAGCTGCCTCGGCGAGCCGAATGAGAAAAAC
 367 T S E S N V T K T S S C L G E P N E K T
 1801 GATCCCGCATTGTGCTCGATGTCACCAACATTGGCGGCACCAACGTCGAGGAACTGTC
 387 I P H L S L D V T N I G G T N V E E L S
 1861 CTTTGACACCACCTCTGGCGACTACTTCCAGTGGACTCTCAATACGAGCAGCCTGGTGTCT
 407 F D T T S G D Y F Q W T L N T S S L V L
 1921 CGATTGGGGGAACCCGACGATGGCCCGAATCTTCAACGGGGATGCCATCTTCCCCACCGA
 427 D W G N P T M A R I F N G D A I F P T E
 1981 GTACAACGTCTGTGCCGTCAAC**gtgagt**ctccccatgtttgcagtaacccccgaacccta
 447 Y N V V A V N *intron 1 (80 bp)*
 2041 gagagtgcggcgattt**cgcta**atcatggttccttatccg**cag**AAAACCGGCACTGGACCG
 454 K T G T G P
 2101 GAATGGACAGTCCTAGTGATTCAAGATCAAAGCAATTTGCC**gtaagc**ctcccaaattccc
 460 E W T V L V I Q D Q S N L P
 2161 aatccacttgtccctgccccttgcagtcaggtgcgctgcgtgggaatgggatccc**agctg**
intron 2 (107 bp)
 2221 **acgt**caaggcttttttcattt**gttgcag**CATTGCGCATCCGATCCACCTGCACGGCCACG
 474 I A **H** P I **H L H G H** D Cu-binding
 2281 ACTTTTGGGTGTGGCGGCCGAGGAGGGCGTCTTCAATGGCAACATTAGCAGCTTCAACA
 485 F W V L A A E E G V F N G N I S S F N T
 2341 CGAGGAACCCGGCGCGCGCGACGTCGCCACGCTGCCGGGCAGAGGCTACCTGGCCATCG
 505 R N P A R R D V A T L P G R G Y L A I A
 2401 CCTTCCAGATCGACAACCCGGGCACCTGGCTGACCCACTGCCACATTGCCTGGCACGCCA
 525 F Q I D N P G T W L T **H C H I A W H** A S Cu-binding
 2461 GCCAGGGCCTTTGCTCGAGTTTGTCGAGAGCCAGTCCGAGATTGTGACCGACGAGGTCA
 545 Q G L S L E F V E S Q S E I V T D E V S
 2521 GCCGCGGCGTGTTTAACGACGTCTGCGCTTCTGGCGGGCACATGACCCCTTTGGGAGC
 565 R G V F N D V C A S W R A H D P L W E Q
 2581 AGGAGGACTCGGGAATTTGAatctgagggcgctcgtttctttccttccggtgtttgagagt
 585 E D S G I *
 2641 aagatatgtatcatgaaatttctttattctttatctttttgttctttctttggtccgtgtt
 2701 gtctgtctctgcgggttagaatacacgaggaaacccaactgggtgttgagtggaactcttcg
 2761 ggctcgagcccaacgctcattgtttccctagaagttgtctttgtttctggttcaccactc
 2821 caccatttgtacgggtagttgacggattgttcggctcactcaaccggctgtaagtgttg
 2881 gagatgtttcaactctcaatctcggaggttgggctgcggctcctgcatgaggggatggt
 2941 cgcagtatgtgtgttagtacagaaaaacacagcataaagcaatacgaactgtgtgcccg
 3001 tccgtacctaaggaacaaaccggtcaacaagacaccagtgaaaggttatcagtcgaagc
 3061 cactcgggttgaaatttctcagctc

Fig. 6B II

Ta lcc3 and the deduced amino acid sequence

1 acggctgcggttatcggagcacgttgcatggcaatgtccatactcttactgagaaaagag
 61 ttcgggtcaaggacggtgctggggaatagatgcccgctctaggaattccggtagtggcggt
 121 ctttcggaaatataaccgagcaggacgttcagcccatcgctccctgtgtatataaagcatg
 181 aagttattctatgtagaatcctagactatcttagaagcgaaccttcgtcgcagatgctt

 241 cgtgttttccatcattacttcccgtagtactagtATGGTCTGCCTTCAGTATCAGCTCAC
 1 M V C L Q Y Q L T
 301 AGCAGCCTGCTTTGCTGTGTAGCGGTTCGTACCCACCCAGCTTCCTGCTCTCCTGTCCA
 10 A A C F A V L A V V T T P A S C S P V Q
 361 GCCGAGCCCCGTTACGATCTCTTACCAAGACAAACAATAATCCCCGGCGGTAAGCCATG
 30 P S P V H D L L P R Q T I I P G G K P C
 421 TGGTCAAAATAATGCCACGAATAGGGGGTGTGGAAAAACAACCTGGAACATCACCACCGA
 50 G Q N N A T N R G C W K N N W N I T T D
 481 CTATGAAGTCGACACGCCCCCTGCGTTCAACACCAGAGTGgtatgtccttgctcataata
 70 Y E V D T P P A F N T R V
 541 agtggctagcctcgaagatcaggccaaagactgataggagctcagTATGACCTTCACATC
 1 intron 1 (65 bp) Y D L H I
 601 ACCAATGTACCAACTGGCTCGGCCCTGATGGGGTTCGGAAGCCTGCCATGCTTATAAAC
 6 T N V T N W L G P D G V R K P A M L I N
 661 GgtgtgttgccagagcccacaagatcctccaaggtcacttctgacaacaaggcagGCTCA
 26 G intron 2 (54 bp) S
 721 TTCCCTGGTCCCACAATCTCAGCCGACTGGGGCGACTACATTATTGTCAATGTCCACAAT
 2 F P G P T I S A D W G D Y I I V N V H N
 781 GACATGCAAGATAACGGgtaagaaatgcagcacggtacccccggcatcctcaaacctgct
 22 D M Q D N G intron 3 (60 bp)
 841 tgctaacacataggttagAACGTCAATCCATTGGCATGGAATCCGTGAGCTTGGCGAGAGC
 1 T S I H W H G I R Q L G E S Cu-binding
 901 AACCAGGACGGCGCAAACGGCGTAACGGAATGCCCCATTCTCCCGGATCCAGCAAAACC
 15 N Q D G A N G V T E C P I P P G S S K T
 961 TACGACTTCCATGTGACGCAGTACGGCACCTCGTGGTACCACAGCCACTACTCCAACCAG
 35 Y D F H V T Q Y G T S W Y H S H Y S N Q Cu-binding
 1021 TACGGCAATGGGGTTCGTGGGCGCCCTGATCGTGC GCGGCCCGGCATCAGCCAACACGAC
 55 Y G N G V V G A L I V R G P A S A N Y D
 1081 ATCGACCTCGGTCCCTACCTGATCAGTACTACTACTACGAAACCGCAGACCGCCTCCAT
 75 I D L G P Y L I S D Y Y Y E T A D R L H
 1141 CTCCGAGCCGAGCTGGTCAGCAACGGCCACCGCCAGACAGCGACAACATCCTGTTCCGC
 95 L R A E L V S N G P P P D S D N I L F R
 1201 GGCAAAAACATCAACCCCAAGCGTGCAGGCAGCGGCTCCTACGACCGCCTCGTCTAACC
 115 G K N I N P K R A G S G S Y D R L V L T
 1261 CCCGGCAAGAAGCATCTAATCCGGCTCATCAACGCCAGCGTGGACAACCTCCTTCGTCATC
 135 P G K K H L I R L I N A S V D N S F V I
 1321 TCCCTCGTCGGCCACAACCTTACCCTCATCTCCACCGACATGGTCCCCATACCCCTGTG
 155 S L V G H N F T V I S T D M V P I T P V

Fig. 6C I

1381 GTGCGCAGCTCCTTATTCATGGGCGTCGCCAGCGGTACGACGTCATTGTCTGAAGCCAAC
 175 V R S S L F M G V G Q R Y D V I V E A N
 1441 CAGCCCGTGGGCAACTACTGGCTCAACGCGACGCTCGAGGCCAGAACAACTGCGGGCAT
 195 Q P V G N Y W L N A T L E A Q N N C G H
 1501 TCGGTCAACCCCTTCCCCGCGGCTATTGTCCAGTACGAGGGCGCCAGCTCCACCGCCCTG
 215 S V N P F P A A I V Q Y E G A S S T A L
 1561 CCGACCAACCGCGGGACGCCCTGACGGCGACATGCAATGGCGAGAAAGGGTTTTTCGCCC
 235 P T N R G T P L T A T C N G E K G F S P
 1621 ATTGTTAAGCGGACAGTTTCAAGTTCCCTGTTCCAGCCGTCTACCCTGCCAGTCAGCCTT
 255 I V K R T V S S S L F Q P S T L P V S L
 1681 GAATTCCCCACTACAGACCGGGCAGGTGTTTGTAGTGGCGGGTTAAGAACACGCCGATA
 275 E F P T T D R G Q V F E W R V K N T P I
 1741 AGCGTCTGAATGGGAGCATCCCGTGCTGGAGTACATTTTGCAGGGGAACACCTCCTTCCCG
 295 S V E W E H P V L E Y I L Q G N T S F P
 1801 GCAAAGGCCAATCTCATTGAAGTCCCGCAGGCAAATGTCTGGACGTTCTGGGTGATTGAG
 315 A K A N L I E V P Q A N V W T F W V I Q
 1861 AACGGGTTTGGGCTGCCGACCCGATTACCTGCATGGGCATGATTTCTGGTGCTTGGG
 335 N G F G L P **H** P I **H L H G H** D F L V L G Cu-binding
 1921 GTCGGCAACGGGACTTTTGTATGCTGCGTCTATGAGGGGGCTGTTGAATTTTAATAATCCT
 355 V G N G T F D A A S M R G L L N F N N P
 1981 GTTCGTAGGGATGTCTGAGCAGATGCCTGGCAATGGGTGGCTCGTGATTGCGTTCAAGACG
 375 V R R D V E Q M P G N G W L V I A F K T
 2041 GATAATCCGGGGTGTCTGGTTGATGCATTGCCATATTGGCTGGCATGTGGCTATGGGGCTG
 395 D N P G C W L M **H C H I G W H** V A M G L Cu-binding
 2101 GGGATACAGTTTTTGGAAACGGAGGAGTGATATCCTAGCGCTCATGAAGCTGGATCAGATG
 415 G I Q F L E R R S D I L A L M K L D Q M
 2161 GTGCCCAATTGTGAGGCTTGGAGGGCCTATGCAAGGACGAGTCCATATCTGCCAAAGCTT
 435 V P N C E A W R A Y A R T S P Y L P K L
 2221 GATTCTGGGGCTGAAAAGGGGGTGGAGATGAGGGAGGGGATGGAGCCGGCTGTGAGGCGC
 455 D S G L K R G V E M R E G M E P A V R R
 2281 ATTGGTTAGattgaggtatcgctactcaacttgcttccaagctggctatggctatgagtg
 475 I G *
 2341 ttcaggaagaagaggggaatctcactattcttgcagcatgcccactaagataaaaaggaa
 2401 ctgaaaaagaactcaccattgtgcgggttgaacgacatgggaccgcctgaccgctgaag
 2461 tgacatcattactatcttcaccacgctgagcattcgaattcccagacgcccttggtagt
 2521 gccatttggatggcttaccgtgactctggacgaaagaggtgcgcaagactccgcatggct
 2581 aggggtcatatcgccaatggcgctcatcccgccagggattaaggcagctcataaaccctac
 2641 tttggtctcttgcttacaacgccttctgacattaggacatggaatagacatcattact
 2701 attgaagatgtttggacacatgcaatgcgtactttgagttgctatcaaaaagcatgaaac
 2761 agcgatgtaggtgaggtactcaatagatatggcggttaagcagaaataccaatcatttca
 2821 ggagataataaccgtcaactcacgcagggcttcaacgttgagtggtgacgacaggcccc
 2881 tgacgtcttggttgctggagggatcgctcaggggttaaaaataagggttaggggttcaagtc
 2941 ttccgtcaactccctaaggaggcaaggatcc

Fig. 6C II

Ta lcc4 and the deduced amino acid sequence

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1  ggatccgggaaggtgactgagaagatgtccacgctatggcctcgccgtgtctctcaaagaga
61  tggcggttctctggctcgtgatgttcggatttgacgacggcggtgattggcaacgactttg
121  cgggacgttggatttggacactttgggggtttgacaatggcgagagcgagacaagtaggt
181  actaaccgctttcctccaggatttctgtgctgggaggcgccaggaggcgggctgacgat
241  ggacttgggttacaagggagtgaagatgtggcttacagggacgtacatctactatataagc
301  tgaacactgtggcacttctctgagaaaagcaatgggcagggtatctcagtagcatgttcagg
361  accccttaagttagaccatttcaacttacatgcttcttccaactcgtttctccctcgtcgg

421  cccttggaagtttagaccgggctcttacatgacaATGATTTCGCGTCTTCTTTTCACAAG
1      M I S R L L F T S

481  CAGTTTCACTGCTACACTGGCTTCTGCTCTGCCAGGTCTATTATACGCCCTCATTCAAG
10     S F T A T L A S A L P G L L Y A P H S S

541  CGCACTGCTGGCTCGTTCTCTCTGTAGCGGCAATACGGCGTCAACCCGGTCCCAGTGGTG
30     A L L A R S S C S G N T A S T R S Q W C

601  CGACTACTCCATCGACACCGACTATACGACAGAGCCCGTTGACACCGGGGTACTCGAGA
50     D Y S I D T D Y T T E P V D T G V T R E

661  GTACTGGCTTGAGTTAACGGACGTAACCGTGTGCGCAGACGGGGTGTGCGGGTCAGCGAT
70     Y W L E L T D V T V S P D G V S R S A M

721  GGCTGTCAATGGCTCCATCCCTGGTCCAACCATCTTCGCTGACTGGGGTGATACCGTCGT
90     A V N G S I P G P T I F A D W G D T V V

781  CGTTCACGTCACCAACTCGTTATCCACTTCTCTGAACGGAACAAGCATCCACTGGCATGG
110    V H V T N S L S T S L N G T S I H W H G   Cu-binding

841  CATCCGCCAAACTACACCAACCAGAACGACGGTGTGCTCCATTACGCAGTGTCTCTCT
130    I R Q N Y T N Q N D G V A S I T Q C P L

901  GGCGGTTGGCGAATCAACCACCTACACCTGGAAGCAACACAATATGGCTCATCTTGGTA
150    A V G E S T T Y T W K A T Q Y G S S W Y   Cu-binding

961  CCAATCTCACTTCAGCCTTCAGGCCTGGGAAGGCGTCTTTGGTGGGATCATCATCAATGG
170    H S H F S L Q A W E G V F G G I I I N G

1021 TCCTTCTACCGCCAACTATGACGAGGATCTTGGCATGCTTTTCTCAATGATTGGGATCA
190    P S T A N Y D E D L G M L F L N D W D H

1081 CCAAACTGTGCGATGAGCTCTATTCCAGTGTCTGAAACCTCTGGGCCACCCACCTTGGCCAA
210    Q T V D E L Y S S A E T S G P P T L A N

1141 CGGTCTCATCAACGGTACGAACGTCTACGGGAAGATGGTGATTTCATCCCAGACCGGAAC
230    G L I N G T N V Y G E D G D S S Q T G T

1201 AAGATTGGCTGTCTCATTACGTCTGGCACCTCATACCGCATGCGGCTGGTGAATGCCGC
250    R L A V S F T S G T S Y R M R L V N A A

1261 CGTCGACACGCATTGGAAATTCTCCATCGGCAACCACACAATGACTGTGATGGCCGCTGA
270    V D T H W K F S I G N H T M T V M A A D

1321 TCTCGTTCCGATTGAGCCATATGAAACGACGGTCTTAACAATGGGATGGgtaagtacct
290    L V P I E P Y E T T V L T I G M G

1381 acatcatcagaccacctacttattcggtttcttaggacacccgagactgagttccacata
      intron 1 (71 bp)

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Fig. 6D I

1441 gGACAAAGATACGACATAGTCGTACGGCAGATCAAGCTGATGTCGCGGATAACTTCTGG
 307 Q R Y D I V V T A D Q A D V A D N F W
 1501 ATGAGGGCTATCCCACAATCAGCATGCTCTGATAACGACAGCGCCGACAATATCAGAGGG
 326 M R A I P Q S A C S D N D S A D N I R G
 1561 ATCGTCTACTATGGTGATAGTCCCGGCACCCCTTCAACAACCTGGCTACGACTTCGAGGAC
 346 I V Y Y G D S P G T P S T T G Y D F E D
 1621 GCCTGCGATGATGAGACGGCCAACATCACCCCTTACATCTCCAAGACGGTTCTTCAGCA
 366 A C D D E T A N I T P Y I S K T V S S A
 1681 GAATGGAATGACTTGGAAACTGCCTCAGTCTCTAGGAACCTCGGCCGGCCTTTTCAAATGG
 386 E W N D L E T A S V S R N S A G L F K W
 1741 TATCTCAACAGCACGACCATGCTAGTTGACTGGGCAAAACCCACTCTCGAGATGGTGACG
 406 Y L N S T T M L V D W A K P T L E M V T
 1801 GACAACGTGACCGAATACGATTCCGGATGACGCCATCATTGAGCTGAATGAAGCCAACCAG
 426 D N V T E Y D S D D A I I E L N E A N Q
 1861 TGGGTGTACATGGTGGTTCAAACAACGCTCCAGGTTCCACACCCAATCCATCTGCACGGT
 446 W V Y M V V Q T T L Q V P **H** P I **H L H G** Cu-binding
 1921 CACGATTTCTTCATTCTCGCCCAAGGTTCCAGGGACATACGATTTCGTCGACAGTAACATTG
 466 **H** D F F I L A Q G S G T Y D S S T V T L
 1981 AAGACGGATAACCCACCCAGACGCGACACGGCCATGCTGCCATCGCAGGGATACTTGGTC
 486 K T D N P P R R D T A M L P S Q G Y L V
 2041 ATGGCCTGGGAGACGGACAACCCTGGCGTTTGGCTGATGCACTGTCACATCGGTTGGCAT
 506 M A W E T D N P G V W L M **H C H I G W H** Cu-binding
 2101 ACCTCAGAGGGCTTCGCCCTCCAGTTCATTGAGCGGAAGAGCGAGATCGCGTCGATCGTC
 526 T S E G F A L Q F I E R K S E I A S I V
 2161 AGTACGTCGACTATGAAAGATATTTGCACCAAGTGGGAGGAGTTTCAGGAGGAATATTCA
 546 S T S T M K D I C T K W E E F Q E E Y S
 2221 ATTGAACAAGAAGACTCTGGTGTATGAgatggatgtgctgtgaccattttgcgtcaaaga
 566 I E Q E D S G V *
 2281 catcctgcctgtcggtaactctgatttgccgtgtgaacttgggtttactaggtatattacag
 2341 atacctacatcattggacatgtacataaggtacttaagtatcccccgatgcatctgtca
 2401 tctgtcaagtcacctcaaccgttctagcccatcatgcacgtatgtacatgaatgtaagt
 2461 atggaacctagcacattagcaacagtttgcaaacgagatcggtcatggtcctaagagtca
 2521 tgattagggggcagcgcaggttcgacttggtgtttatacagttaatcgtgcgcccccggt
 2581 tacatattatgtaccctgcttacacatccatatataactaaggtgcttgaggtcggtgtac
 2641 cgctgttgagctcctagcattcattccagggagcgtggcaatgactggtatgtctgtggg
 2701 cgcgggcgtagatggtgggggtgggagatatc

Fig. 6D II

Ct lcc1 and the deduced amino acid sequence

1 ttcccgaaggcttcaactcacactcggctcctcactccaaccaagcctgtcgtatacacga
 61 actcgtcccgcgtctaccgggaggttgctgcacctgccttgacgacattccgaatctg
 121 ggaactaagacggcatcacctcgatatattggataggatccgagttttcgcgatctttg
 181 ttgccttactgaggagaaggtgcactctgggtgttgagtctgtcgtctatataacgacc
 241 agaccttgcgacagtgagttgactccagttcttcatcgtcaccatcagcatcagacgctt
 301 cagtgtcttgagcctcgttcttttaacaaatcgtcactcaatcatagttggcttggtgc

 361 catcagcatcATGAAGTTCCTAACCTATGCCACCGCCCTCCTGGGTGCCCTTACAGCCGT
 1 M K F L T Y A T A L L G A L T A V

 421 CGTAGGGGCAATCCCAACTCGTTCTGGCACCAAGAAAAAGTACATTAGAGATGGCCCTGG
 18 V G A I P T R S G T K K K Y I R D G P G N-term. peptide

 481 ACCCTGCCATACCCCAAGTAATCGGGCATGCTGGGCCCTGGGTTCGACATCAATACTGA
 38 P C H T P S N R A C W A P G F D I N T D

 541 CTACGAAGTCAACACACCCAACACGGGTGTTACCCGCAATgtaagtcttcatcgttacta
 58 Y E V N T P N T G V T R N intron 1 (50 bp)

 601 gtgcagtgaatttcgtgctgacagaggttagTACACTCTCACTCTGACGGAGGAAGACAAC
 71 Y T L T L T E E D N Peptide 18.9

 661 TGGACCGGCCCGGATGGGGTGGTCAAGGAAAAGATTATGCTGGTTAATGgtatgtcagcc
 81 W T G P D G V V K E K I M L V N G

 721 cctgagctaggtatgatgatgactaaccatataatgtgcagGCAAACTTTAGgtatgt
 98 intron 2 (53 bp) K T L G

 781 ctctcctggtgaagttaagctagatgatgtactaaccatagcagGCCCCGACCATTGAGGC
 102 intron 3 (50 bp) P T I E A

 841 CAACTGGGGTGACTGGATCGAAGTCAAGGTCATCAATAACCTCTTGACTAATGGgtatgt
 107 N W G D W I E V K V I N N L L T N G

 901 cttgttcttcccccttttagtggagtgcccaagctgactgactcccatagAACTTCCATCC
 125 intron 4 (55 bp) T S I H Cu-binding

 961 ACTGGCACGGCATTCACCAGAAGGGCAGTAACCTTCACGACGGTGCCAACGGCGTTACTG
 129 W H G I H Q K G S N L H D G A N G V T E

 1021 AATGCCCATCCCTCCCAACGGTGGCCAGCGCACCTATCGCTTCGGTGCCCGAGCAGTATG
 149 C P I P P N G G Q R T Y R F R A Q Q Y G

 1081 GCACCAGCTGGTACCACTCTCACTTCTCTGCTCAGTATGGCAATGGCATCGTCGGTCCCA
 169 T S W Y H S H F S A Q Y G N G I V G P I Cu-binding

 1141 TCGTTATCCACGGTCCAGCTTCTCTGCCCTATGACATCGACCTTGGCCCCCTTCCCTCTTA
 189 V I H G P A S L P Y D I D L G P F P L T

 1201 CGGACTACTACTACAAGAGCGCGGATGAGCTGGTGCGCCACACTCAGAACAACGGCCAC
 209 D Y Y Y K S A D E L V R H T Q N N G P P

 1261 CGTTCAGCGACAACGTCCTCTTCAACGGCACCGCGTTTCATCCCCAGACTGGCCATGGTC
 229 F S D N V L F N G T G V H P Q T G H G Q

 1321 AGTATGCTAAGGTGACCCTGACCCCGGGCAAGCGTCACCGCCTGCGCATCATCAACATGT
 249 Y A K V T L T P G K R H R L R I I N M S

 1381 CGACGGAGAACCCTTCCAGGTCTCTCTCGTCGGCCACCAGTTCACCGTCATTGCCGCTG
 269 T E N H F Q V S L V G H Q F T V I A A D

Fig. 6E I

1441 ACATGGTCCCCGTCCACTCCTACACTACTGACAGCCTGTTTCTCGCTGTCGGGCAGCGTT
 289 M V P V H S Y T T D S L F L A V G Q R Y

1501 ATGATGTCACCATCGACGCTTCGCAGACCCCCGGTAACTACTGGTTCAACGTCACCTTTG
 309 D V T I D A S Q T P G N Y W F N V T F G

1561 GCGGCGGTTTCGCTTGCGGTGGTTTCCTTCAACCCTAACCCAGCTGCCATCTTCCACTATG
 329 G G F A C G G S F N P N P A A I F H Y E

1621 AGGGTGCCCCGATGCTCTGCCGACTGACCCTGGTGTTCCTCCGCGGACCACAACCTGCT
 349 G A P D A L P T D P G V P P R D H N C L Peptide 22.4

1681 TGGACACTCTGGATCTCGTCCCTGTCGTACCTCGTAATGTTCAAGTCAACAGTTCGTCA
 369 D T L D L V P V V P R N V Q V N Q F V K

1741 AGAAGCCTGAGAACACCCTGCCTGTTGAGCTGTCCCTTGGTGGTACCCCGCTCTTCGTTT
 389 K P E N T L P V E L S L G G T P L F V W Peptide 22.7

1801 GGAAGGTCAACGGCAGCGCCATTGATGTTGACTGGGGCAACCCCGTCTTCAGTATGTGA
 409 K V N G S A I D V D W G N P V L Q Y V M

1861 TGGACCGCAACACCAGCTACCGCCAAGCCGACAACATCGTCGAGGTTAATGGTGTCAACC
 429 D R N T S Y R Q A D N I V E V N G V N Q

1921 AGTGGACTTACTGGCTGATTGAGAACGACCCCAATGGTGCCTTCAGCCTGCCCCACCCCA
 449 W T Y W L I E N D P N G A F S L P H P M Cu-binding

1981 TGCATCTTCACgtaagttatattccctctttctcttttacctacccccctttattgcagat
 469 H L H intron 5 (95 bp)

2041 tagtcccttcttttagcccatatctcagataaactaacctccttccagGGCCACGACTTCCT
 472 G H D F L

2101 CATTGTGCGCCGCTCCCCGACGTCCCGCCGGGCTCGAACCAGCGCTACAACCTTCGACCC
 477 I V G R S P D V P P G S N Q R Y N F D P

2161 CGCCACCGACATCTACCGCCTGCGCGGTGAGAACCCGACCCGTCGTGACGTGCCATGCT
 497 A T D I Y R L R G Q N P T R R D V A M L

2221 TCCTGCCGGCGGCTGGCTGCTGCTCGCCTTCCTCACCGACAACCCCGGTGCCTGGCTCTT
 517 P A G G W L L L A F L T D N P G A W L F

2281 CCACTGCCACATTGCTTGGCACGTGTGCGGCGGTCTGTCTGTGACTTCCTCGAGCGGCC
 537 H C H I A W H V S G G L S V D F L E R P Cu-binding

2341 GAACGACCTGCGCAACAGCATCCTCCAGCATGATAAGGACGAGTTCAACCGCGTGTGCAA
 557 N D L R N S I L Q H D K D E F N R V C N

2401 TGAGTGGCGCAGCTACTGGCCTAATAGTCCCATCCTAAGATTGACTCTGGTTTGAAGCA
 577 E W R T Y W P N S P H P K I D S G L K H

2461 CCGCTGGGTTGAGGAGAGTGAGTGGCTGGTTCGATAGgggggtgtgtgcagagttgggg
 597 R W V E E S E W L V R *

2521 gtgatttgggcatgggttttgagcgttgggatatgggcttgataatcattaatgtgtt
 2581 ttggttgggcatatttcggagttttggctatcggttatcgtagtggtcaatagtagtcaa
 2641 cccttctctatcgtaatagtaatcaacccttctctacacaatttgatatacgtagcgttg
 2701 atgacttgggactgaaggccaaacttaagttgggccatttttccggctcttttccctcatg
 2761 tgataggtaaatgagcctagtttgactatgtgtgctgttcagcactcactgcactatgt
 2821 acagccaagatgtccccaagtgttcgatatgtcaagttccagcattgacgatcagctctc
 2881 tatgttatcactttgcactcgccttttttatgctaagtgcctctaccactgaaaatccct
 2941 agccctgccaccgg

Fig. 6E II

Ct lcc2 and the deduced amino acid sequence

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1  tctagaagatatcggcagtcgcggtagcttgaatcatgagcggggatatacgagagaggg
61  agcagccccctcggttaacatgcggatgagaacatggcagcatcatcgattcctacgtcta
121 tatcttggagacgtataagaggaagtggatgacgataatcgtaggagatctactgaccg
181 ctctctcacggcatattatgaattcgtgactttatccctctctgacgtgggtttctgacaa

241 ctgccATGCCAGACGTAGTCTCATACTCCTTCTCCTCGCCTTCCTTGGGCTCCTACACT
1  M P R R S L I L L L L A F L G L L H L

301 TAACCCCTAGCAAACCCCATCAAGGCAAACCCAAACCCCTCCTCCTAGCCAGACAAACCA
20 T L A N P I K A N P N P F L L A R Q T I

361 TCACCCCTGGAGGCCAGCCCTGTGGCCAACATGGCCCAGAGAACAGACTCTGTTGGCGAA
40 T P G G Q P C G Q H G P E N R L C W R N

421 ACCTTTGGAATATCAGTACTGATCCGGACGTCAGCATCCCCCTGCCTACAACAATCGAT
60 L W N I S T D P D V S I P P A Y N N R Y

481 ATgtaagtctcctcaagcctctgaataactagtcgctcactgacatcatccagTATGATTTG
80 intron 1 (49 bp) Y D L

541 CACATCACCAATGAGACAAACTGGCTAGGGCCAGACGGCGTGCGGAAGCACGCTATGCTC
83 H I T N E T N W L G P D G V R K H A M L

601 ATCAACAgtcagtcctcccttgccgctgacccctcccttatcttctgtcagaggggctaac
103 I N N intron 2 (61 bp)

661 aacccctagATCAATTTCCCGGCCCTACCATTGAAGCAGAATGGGGTGACTATATCGTAGT
106 Q F P G P T I E A E W G D Y I V V

721 GAATGTCTACAATGACCTGGAGGACAACGGGtaagcctctcttccctcatttgggtactc
123 N V Y N D L E D N G intron 3 (79 bp)

781 gcctaacaccgtcaacaacttaccctcactaccgctgactccatccagGACATCCATCC
133 T S I H Cu-binding

841 ATTGGCATGGCATCCGCCAATTCGGCGAAAGCAACCAAGATGGCACCAATGGCGTCACCG
137 W H G I R Q F G E S N Q D G T N G V T E

901 AGTGCCCCATTCCACCGGGGCACATGAAAACATACAGTTTCCACGTCACCTCAGTACGGAA
157 C P I P P G H M K T Y S F H V T Q Y G T

961 CGTCTGGTACCACAGCCACTTCTCCAACCAGTACGGCAACGGCGTGCTCGGCGCACTGG
177 S W Y H S H F S N Q Y G N G V L G A L V Cu-binding

1021 TGGTCAAAGGCCCGGCTTCCGCCAACTACGACATCGACCTCGGCCCGTACATCATTAGCG
197 V K G P A S A N Y D I D L G P Y I I S D

1081 ACTACTACCACGAGACGGCCGACAGATTACATCTCCAGGCAGAGCTACTCCGCAACGGGC
217 Y Y H E T A D R L H L Q A E L L R N G P

1141 CCCCTCCAGACAGCGACAACATCCTCTTCAGGGGCAAGAACATCAACCCTGACGGCTCGG
237 P P D S D N I L F R G K N I N P D G S G

1201 GCCGCGGCTCTTATGACCGATTAACCTTCATCCCAGGCAAGAAACACCTGCTGCGTCTGA
257 R G S Y D R L T L I P G K K H L L R L I

1261 TCAACGCAAGCGTGGATAACTCCTTCACGGTCTCCCTCGTGGGACACAACCTTCACGGTCA
277 N A S V D N S F T V S L V G H N F T V I

```

Fig. 6F I

1321 TTGCGACCGATATGGTCCCGGTCCAGCCAACCGTCCGCGAGAGCCTGTTTCATGGCCGTCG
297 A T D M V P V Q P T V R R S L F M A V G

1381 GCCAGCGTTATGATGTTATTGTTACCGCCGACCAGCCCGTAGATAATTATTGGCTGAACG
317 Q R Y D V I V T A D Q P V D N Y W L N V

1441 TCACCCTGGAAGCAAACAACAACTGCGGCCGCTCTCGCAACCCCTACCCAGCAGCCATCA
337 T L E A N N N C G R S R N P Y P A A I I

1501 TCCACTACGAGGGAGCCAGCCCAACCGCCCTCCCCACCAACCGTGGCAGCCCCCTCGTAG
357 H Y E G A S P T A L P T N R G T P L V A

1561 CAACCTGCACCGGCGAGACAGGCTTCACCCCGTCGTGCCGCGGAATATCCCCCCTCAACT
377 T C T G E T G F T P V V P R N I P P N F

1621 TCTTCCGCCCTTCCGACATCGCCTCCAATACCTGCCAATCGGCCTCAACATTGTCAACC
397 F R P S D I A S N T L P I G L N I V N H

1681 ACACCACTAAAGGCCAAATCTTCTCCTGGCATGTGAAGAACACGCCTATTTCCGTGGAAT
417 T T K G Q I F S W H V K N T P I S V E W

1741 GGGGGCATCCAGTATTAGAGTACATCCTCGAAGGGAATTACTCCTTTCCCGCAGCGGTTA
437 G H P V L E Y I L E G N Y S F P A A V N

1801 ATCTGATTCAACTCAACCAAAAAGACACCTGGACACTCTTTTTGGTGCATAGTTCGTTAT
457 L I Q L N Q K D T W T L F L V H S S L S

1861 CATTACCGCACCCAATCCACCTCCACGGACACGACTTCCTCGTCCTGGGCCTGGGCAGCG
477 L P H P I H L H G H D F L V L G L G S G Cu-binding

1921 GCACCTTTGATCCTCAGACACATCTCCCTTGCTAAACTACTCAAACCCCGTCCGCGCAG
497 T F D P Q T H L P L L N Y S N P V R R D

1981 ACGTTGAACAGCTCCCCGGCTTGGGGTGGGCGCCATCGCGTTCAAGACGGATAACCCGG
517 V E Q L P G L G W A A I A F K T D N P G

2041 GCGTGTGGCTCATGCACTGCCATATTGGGTGGCATGTGGCGATGGGGCTGGGGGTGCAGT
537 V W L M H C H I G W H V A M G L G V Q F Cu-binding

2101 TTTTGGAGAGAGCGAGCGAGATGAGGGTGCTGATGAAGCTGGATCAGGTGGTGCCGAATT
557 L E R A S E M R V L M K L D Q V V P N C

2161 GTAACGCGTGGAGGGAGTACGAACGGGTGGGGAATTGGTTGCCTAGGGGGGATACGGATT
577 N A W R E Y E R V G N W L P R G D T D S

2221 CGGGGTTGTGAggggggtgaagagtgaggatgggacggttagacgtctggttatcagcc
597 G L *

2281 tggggatcgcatcatggccggtagatcctttcatcttggatccgcttatgttgtcatttg
2341 actgggatgctgcgtagaggctgagctctgttaacgccagtggtgtaaaactcagcag
2401 agtgccattttgaggcattgtgagatgttcggaccactatcacacaccttaacattcgat
2461 agccttacgttaaggtgcttgatcatggcctgaggatgtcgtcaccattgttccca

Fig. 6F II

Ct lcc3 and the deduced amino acid sequence

1 ccgcggctgatactaccagaaaacgccccagttacacatatcacgcgggaatctctgt
 61 acagggtgtctcaactgtgtcggttcggtccgctcctgggaacgacattagcaccacctctttg
 121 agccgtcataatatctttctttctttctttcccccgtgctacgcaatccagacaaccctg
 181 gcaaagattgcctattgagagctgggaaactgccaagatgggcaacgctgaccggcgatca
 241 tctgtcgagacagcattaacgctcaacagccaccggcaaccatccctagacactgccaac
 301 ttgctgcggtgtgccttgacactacatgtggcggtgacatggatacgatgttttcggatatac
 361 cagaaagcttcatccgcgataggtctgtctatctctctctttctgccccttctctgtctt
 421 ttccaggacccccctaaaatttcatatgctctcacatcggggcccagcatatctattccatg
 481 tctcccggttccatggtcttcatccccgtcacagattctgttcccttctgttattgcttc
 541 ttactggcagagtgtgttggttgggtgaaagaccacctgctgccccttctctagccatc

 601 **ATG**GGGGTCATTTTGGAGGATTTAAACAACATTGCGTCCGTGGTAGAGCAGGCTCTCAGT
 1 M G V I L E D L N N I A S V V E Q A L S

 661 ACCGTGGTAGAGAAGGTCACCTCCGCCCTATCACAGCTGGACACTA**ATG**Ggtacgtcagt
 21 T V V E K V T S A L S Q L D T N G

 721 gacaggtccactccccttgaatgtaccgaccctaactcagctatgtagATACTCGATCTG
 38 intron 1 (58 bp) Y S I W

 781 GGGAACTTTGCTTGCTCCTCATTGGCTCCCTTTTGGACAGATAACCCGCTGCCTGATGG
 42 G T L L A S S L A P F L T D N P L P D G

 841 ATATCCATGGGGCAACTTGACGGACTACGGCAACAACCCGTATCGTGAATGTCCACATAC
 62 Y P W G N L T D Y G N N P Y R E C P H T

 901 CGGCATTACTAGATCCTATCACTTCACCATCAGTCGGGGAGTCATTGCTCCAGATGGCTA
 82 G I T R S Y H F T I S R G V I A P D G Y

 961 CGAACGCGAGGTGCTCCTTGTGAACGGGGCCTTCCCGGGACCACTTATTGAAGCAAATTG
 102 E R E V L L V N G A F P G P L I E A N W

 1021 GGGAGACACCATCATTGTGAAAGTTTTCAACAATATATCAAACCCCGAAGAGGGGCACCTC
 122 G D T I I V K V F N N I S N P E E G T S

 1081 CATACTGGCACGGCTTCCTCCAGCACCACACTCCCTGGGAAGATGGCACTCCTGGCAT
 142 I **H W H** G F L Q H H T P W E D G T P G I Cu-binding

 1141 CACCCAATGTCCCATCCCTCGGGAAAAGCTTACACCTACAAGTTCAACGCCAGTCTGTA
 162 T Q C P I P S G K A Y T Y K F N A S L Y

 1201 CGGTACAACCTGGTATCATGCCCATTAATCAAGCCCAATATGCTGGTGGCATTGTTGGGCC
 182 G T T **W Y H A H** Y S A Q Y A G G I V G P Cu-binding

 1261 TATTGTTATCCATGGACCAACCAAGAAGGCTATGATATTGATGTCGGTCTGTCTATGCT
 202 I V I H G P T K E G Y D I D V G P V M L

 1321 GGGT**Gta**agctctatccgaccgggagatgactggcaactagatgtcacgaagactcata
 222 G D intron 2 (65 bp)

 1381 acttccacagATTGGTATCACCAAGAATACTACAATATCGTCAAGACAATGCTGAGCCCC
 224 W Y H Q E Y Y N I V K T M L S P

 1441 AGCGAAAGTCTCTCCGGGTTTATTCCGACAACAACCTGATCAACGGCAAGATGGACTTC
 240 S E S P L R V Y S D N N L I N G K M D F

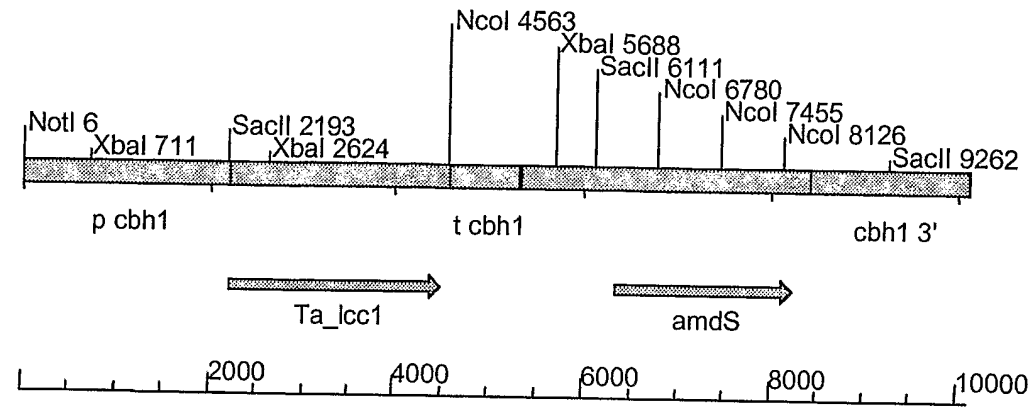
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 260 N C S T V S E D D P H R C T P N A G I S

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 280 K F R F Q A G Q V H R L R L I N L G G D

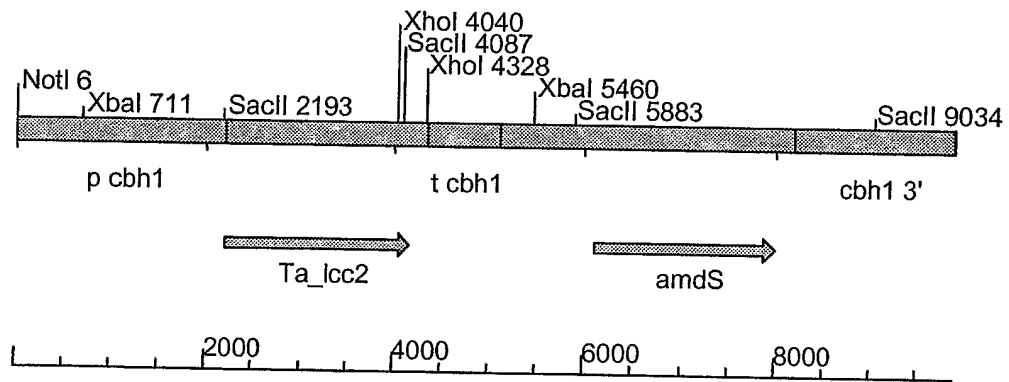
Fig. 6G I

1621 GGTATCCAGCGCTTCTCCATAGATGAGCACGTCCTAACCGTCATCGCCGAGGACTTTGTG
 300 G I Q R F S I D E H V L T V I A E D F V
 1681 CCGGTCAAGCCGTACAACACGACAGTGGTAGTGCTGGGCGTCGGCCAGCGCGCTGATGTT
 320 P V K P Y N T T V V V L G V G Q R A D V
 1741 TTGGTGAAGTCCCAATGCGGGAGGGCCCAAGTCTACGTTCTGGATGCGCTCCAGCCTCACG
 340 L V T A N A G G P K S T F W M R S S L T
 1801 ACATGCTCGCCGCGCCAGGCAGCCGAACGCTGTGGCTGTTGTGTGTATGATGAGGCGGAC
 360 T C S P A R Q P N A V A V V L Y D E A D
 1861 GAGAATGCGGTGCCAATAGCAAGCCGTGGGAGATACCGAATCCTGATGTGTGTGCTAAT
 380 E N A V P N S K P W E I P N P D V C A N
 1921 CTGCCGCTGGAGATCACCGAGCCACTGTACCCAATTCCGCTGCCTGAGCCGACCTTTACA
 400 L P L E I T E P L Y P I P L P E P T F T
 1981 GAGAGAATGGAGATCGAGATCTTCAAAAATGAGTCCAAGATTGGCTGTGGAAGTTCAAT
 420 E R M E I E I F K N E S K I W L W K F N
 2041 GATATATCAATGCGGACGCATTACAACAAGCCGGTGCTGCTGCTCGCCAACCAAGGAGAA
 440 D I S M R T H Y N K P V L L L A N Q G E
 2101 TATGACTACCCCGAAGAATGGAACGTCGTGAACACTATCAAAACGAGTCCGTCCGAAT
 460 Y D Y P E E W N V V N Y Y Q N E S V R I
 2161 GTTGTGAAAAACAACCTCTCCTACCCGtaggtataacctcagaggaatcttctgctcttgt
 480 V V K N N S P T P *intron 3 (69 bp)*
 2221 tgtccgcataccatgactaacccttgactcaacagCCACCCGATGCATCTCCACGGCCAC
 489 H P M H L H G H Cu-binding
 2281 AAC'TTTTACATCCTCCACGAGGGCCCCGGCGACTGGGATGGCACCATGGTCCGGCCAAGC
 497 N F Y I L H E G P G D W D G T M V R P S
 2341 AACCCCCACAGACGGGACGTCTACCTGGTACGCGGGTTTGGTCATCTTGTCTGCAATTCT
 517 N P H R R D V Y L V R G F G H L V L Q F
 2401 GATGGTGAACCTGGTACGTGTGCCGTAGTTCTCAGCCAGACTCCGTGTGCGCTTGGAGGA
 537 D G E P G T C A V V L S Q T P C A L G G
 2461 AGGCAATCTAACAACATAATAGGAGTCTGGGCTTCCACTGCCATATTGCATGGCACGCA
 557 R Q S N N I I G V W A F H C H I A W H A Cu-binding
 2521 TCGGGTGGTTTTCTAGCGACGCTTATTGTCCAGCCGGATACGGTTGAGAAATTCAACGTT
 577 S G G F L A T L I V Q P D T V E K F N V
 2581 CCGGAGGATGTGTGGAATAACTGCAACGCGTGGGATCACTACACAAAGCATAACGTTGTT
 597 P E D V W N N C N A W D H Y T K H N V V
 2641 GAGCAGATTGACAGTGGTACATAAAttttaggttcgtttcgttggccgagtgcggcagct
 617 E Q I D S G T *
 2701 gataggacacgggtttaaattccccgtcgaataatgtcgatgtagtttttgcataatatac
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 2821 gtgcacagccaaattgcacgaaaatgtgcttattgagcgaccgtgggcattatatcatc
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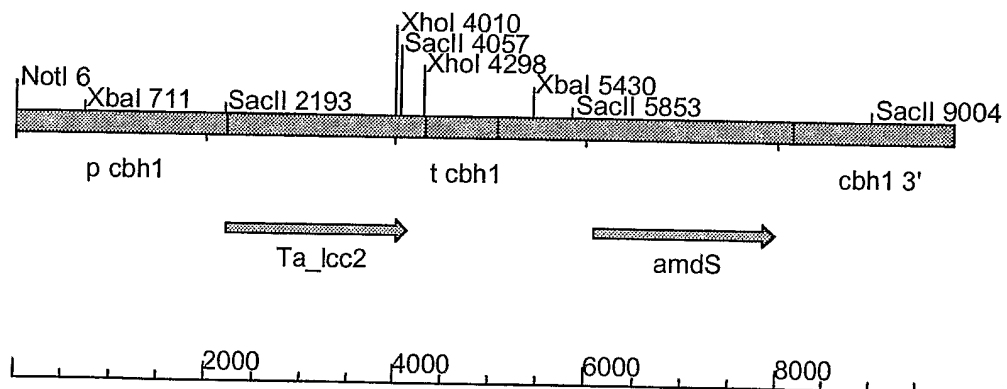
Fig. 6G II



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pALK1655 expression cassette (9.9 kb)



pALK1656 expression cassette (9.9 kb)

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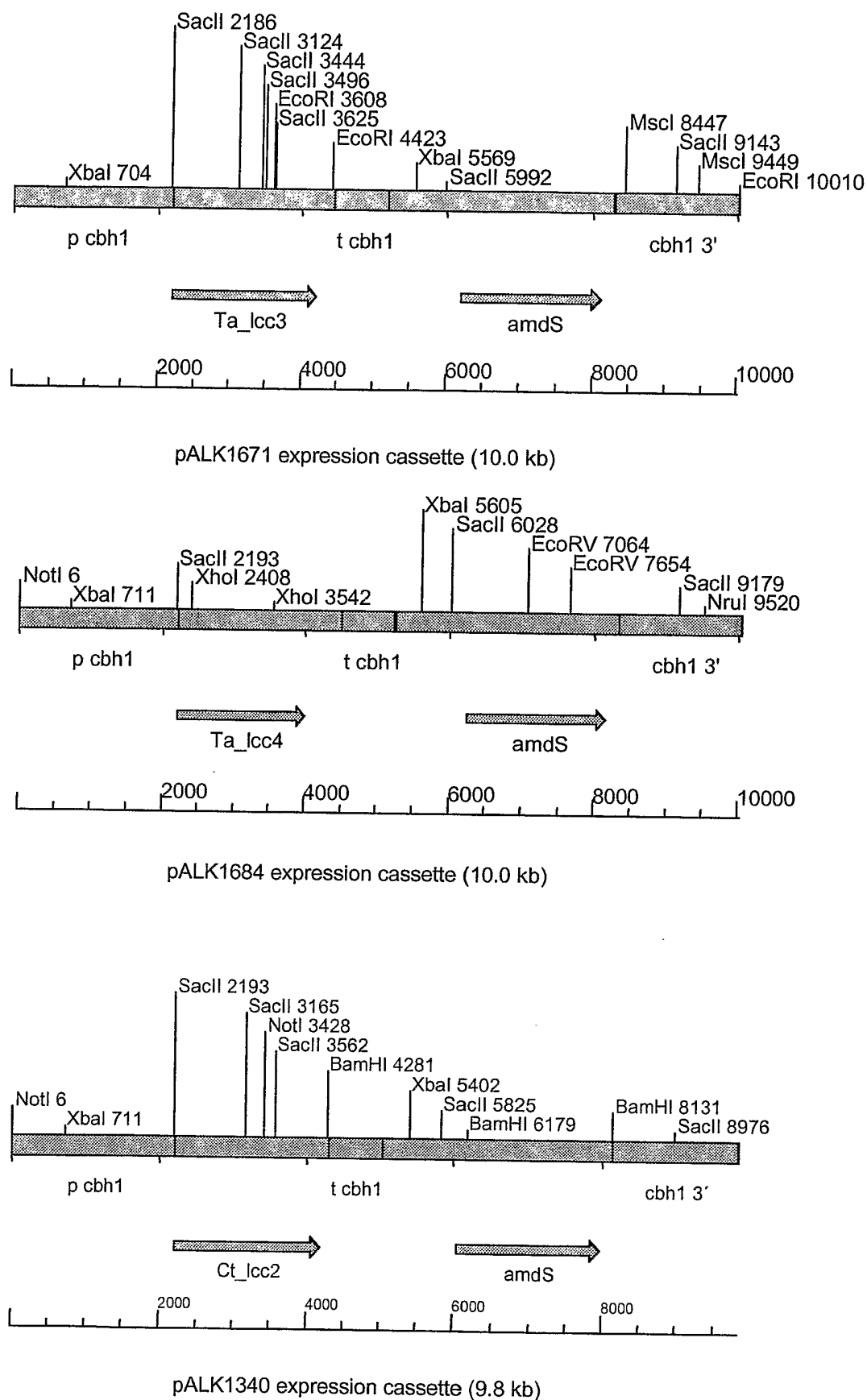


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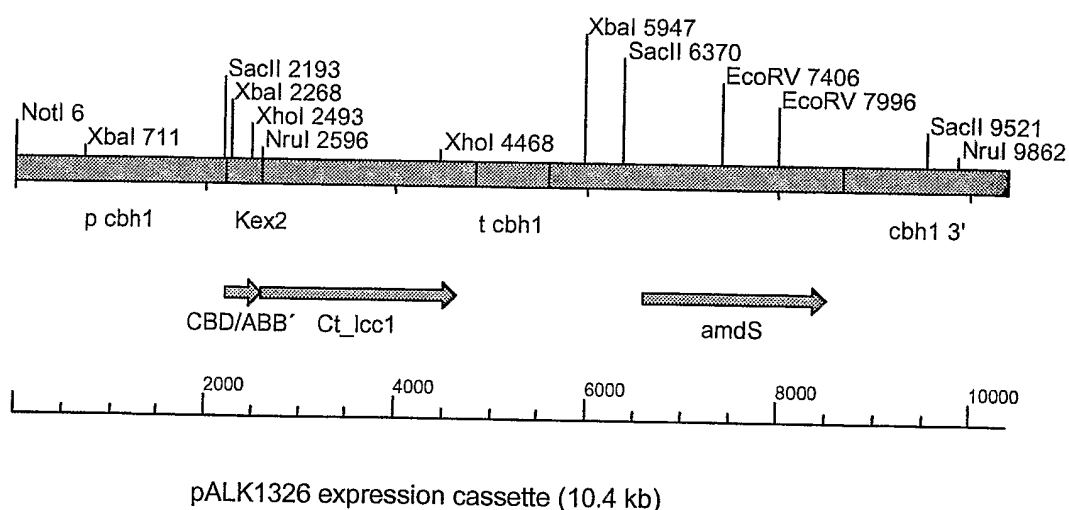
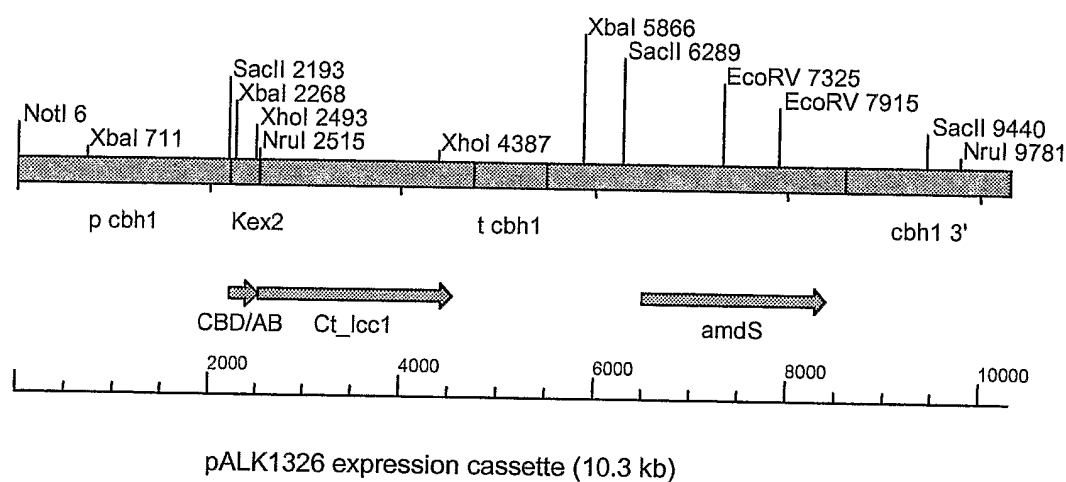
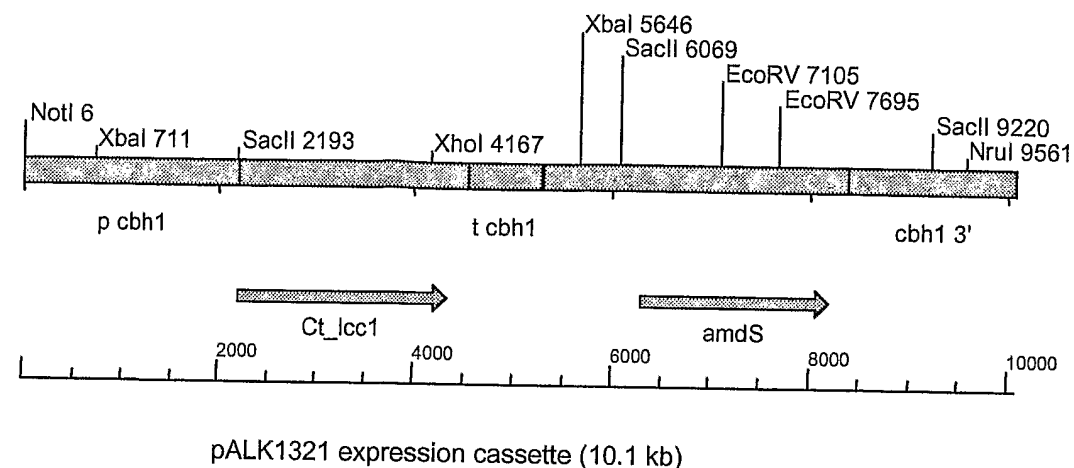
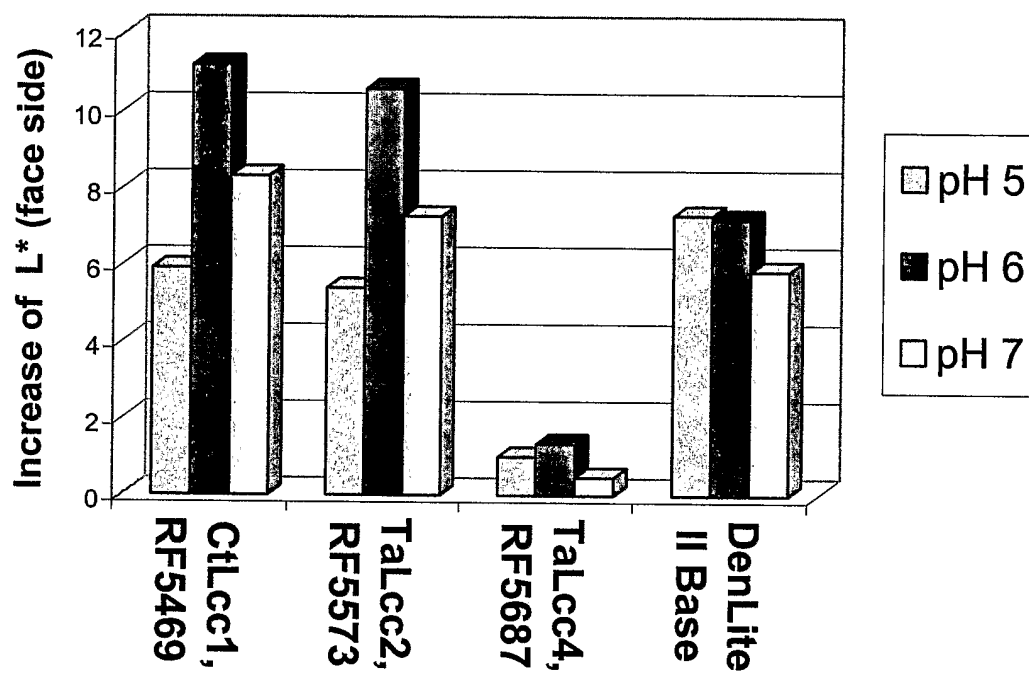
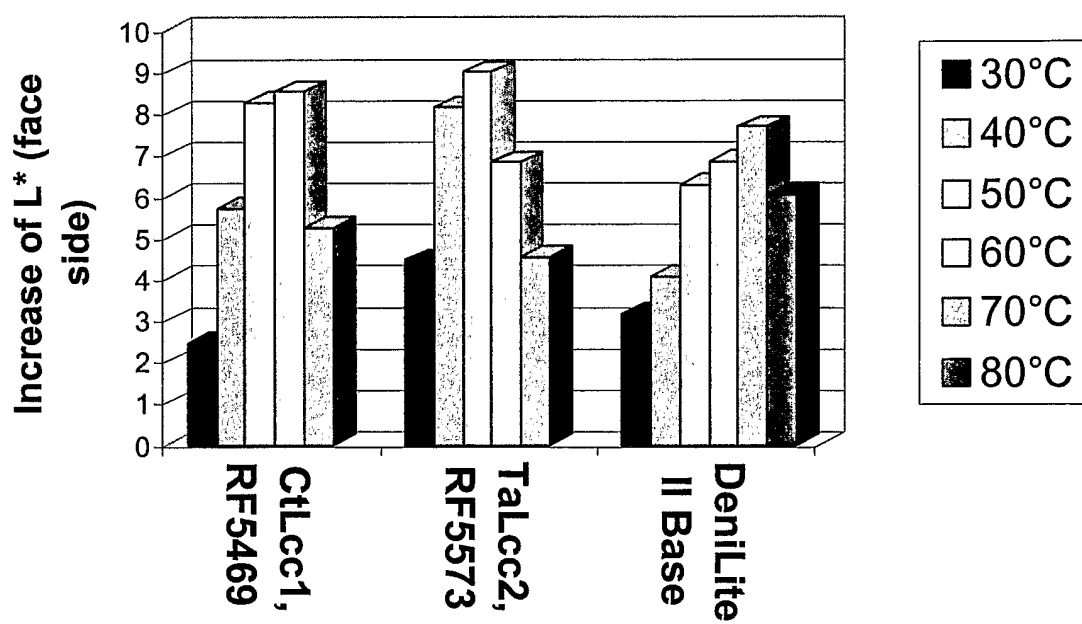
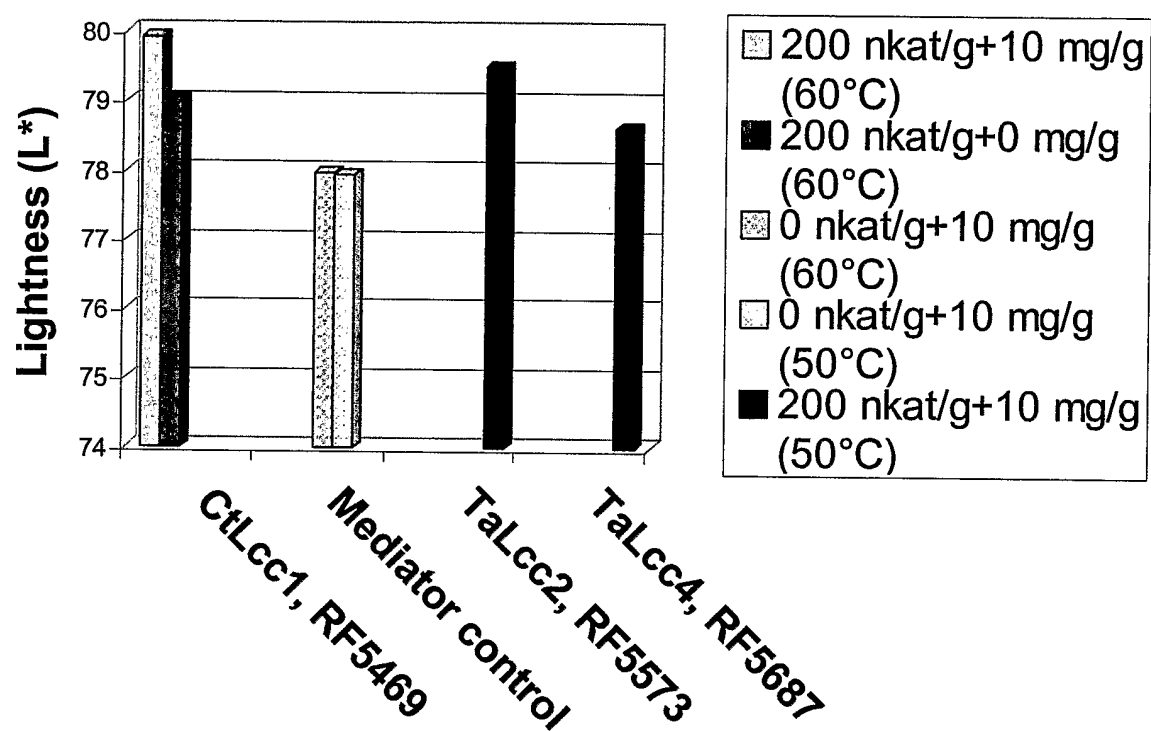
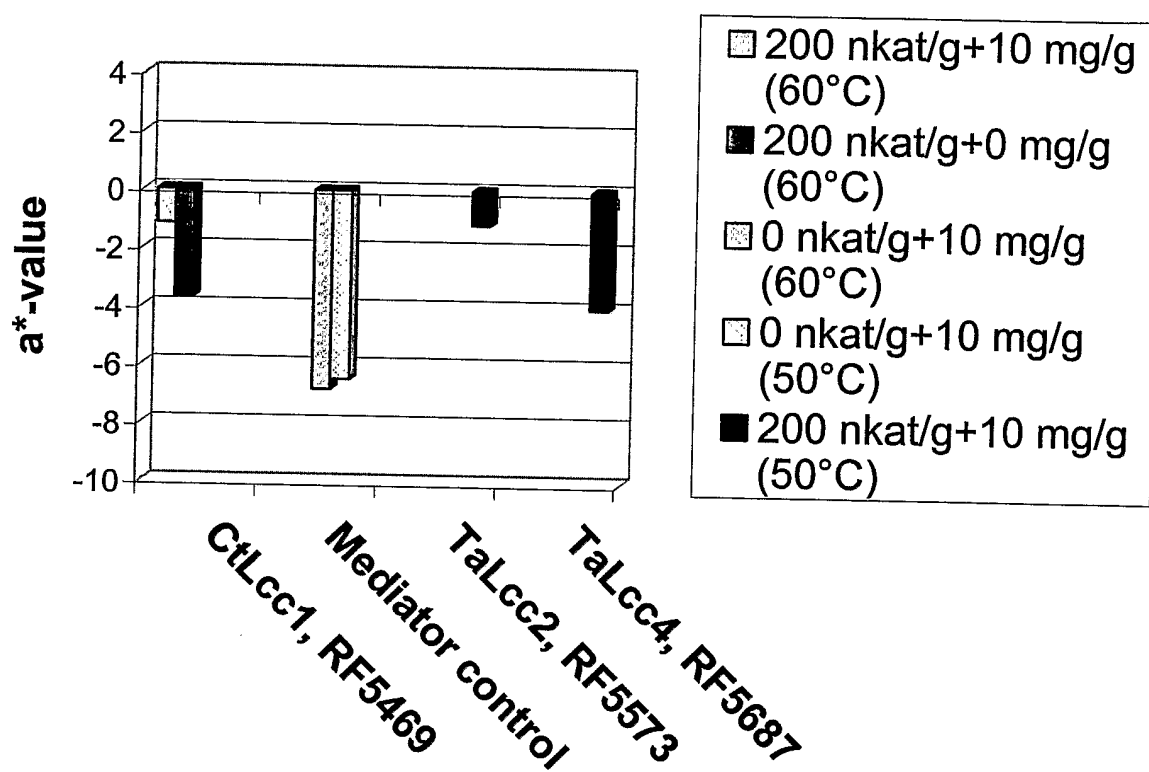


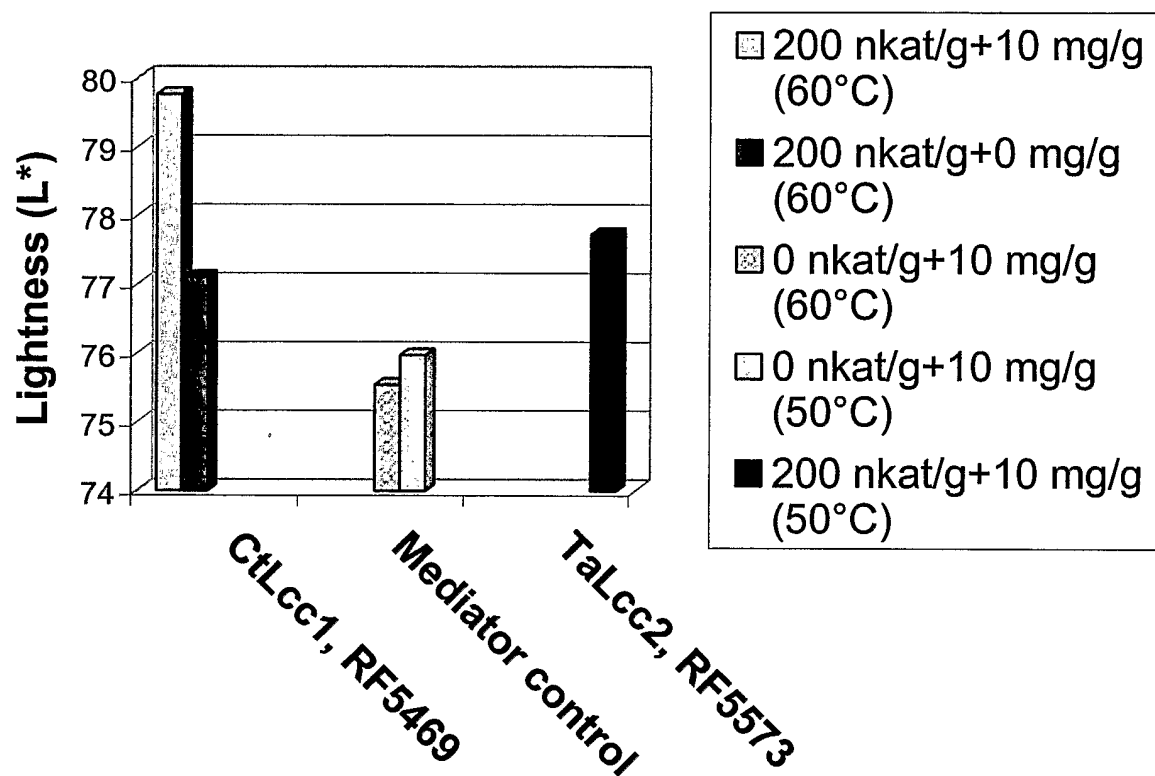
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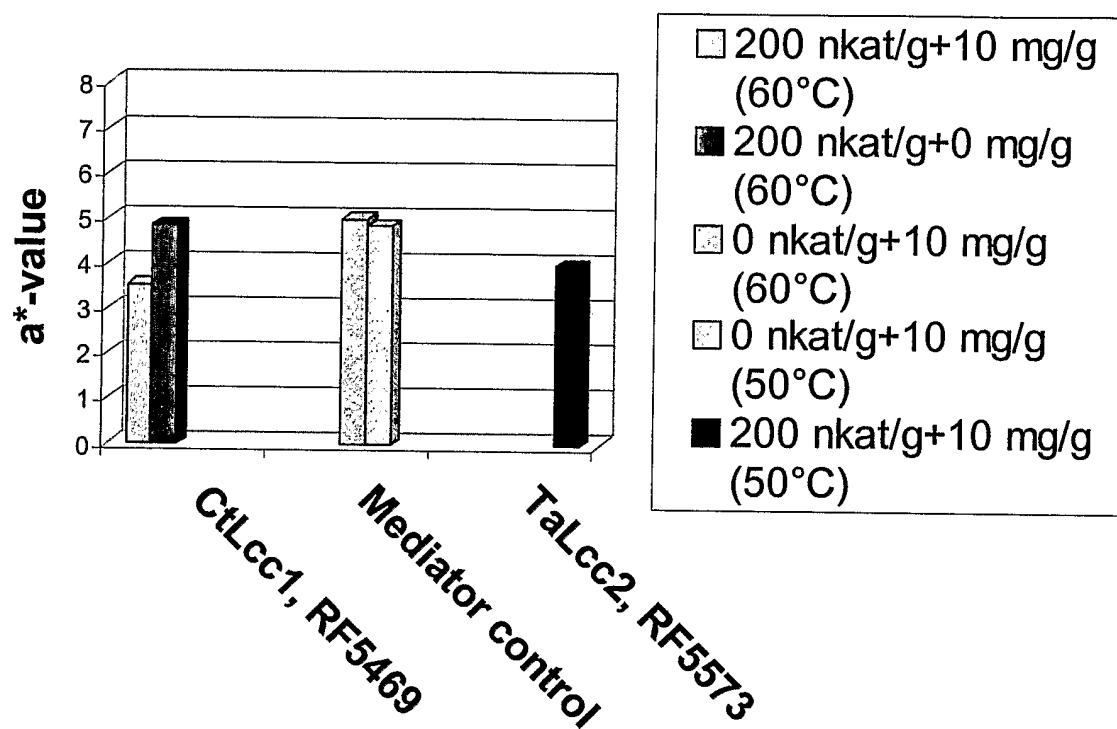
**Fig. 8**

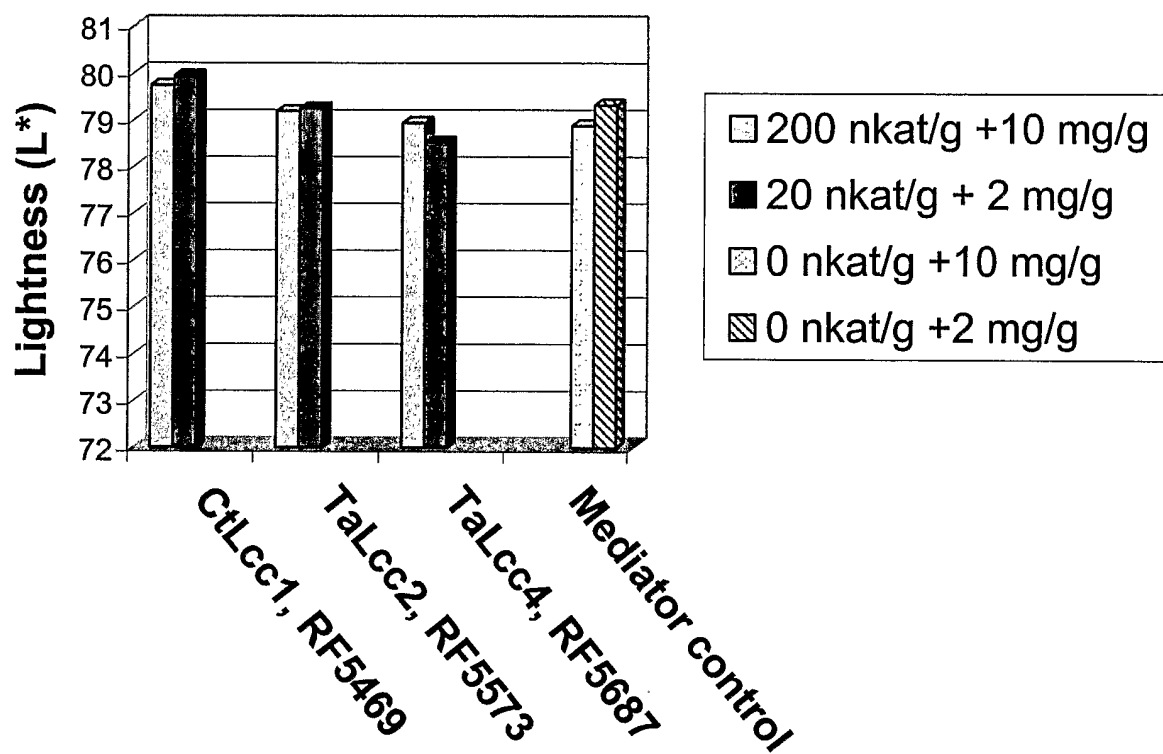
**Fig. 9**

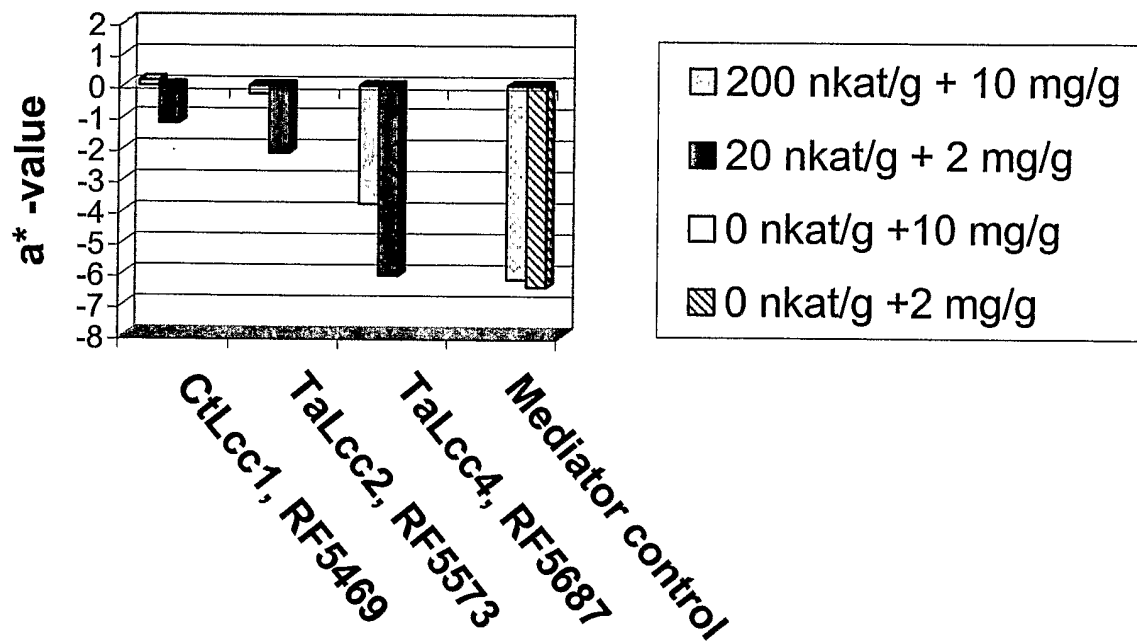
**Fig. 10A**

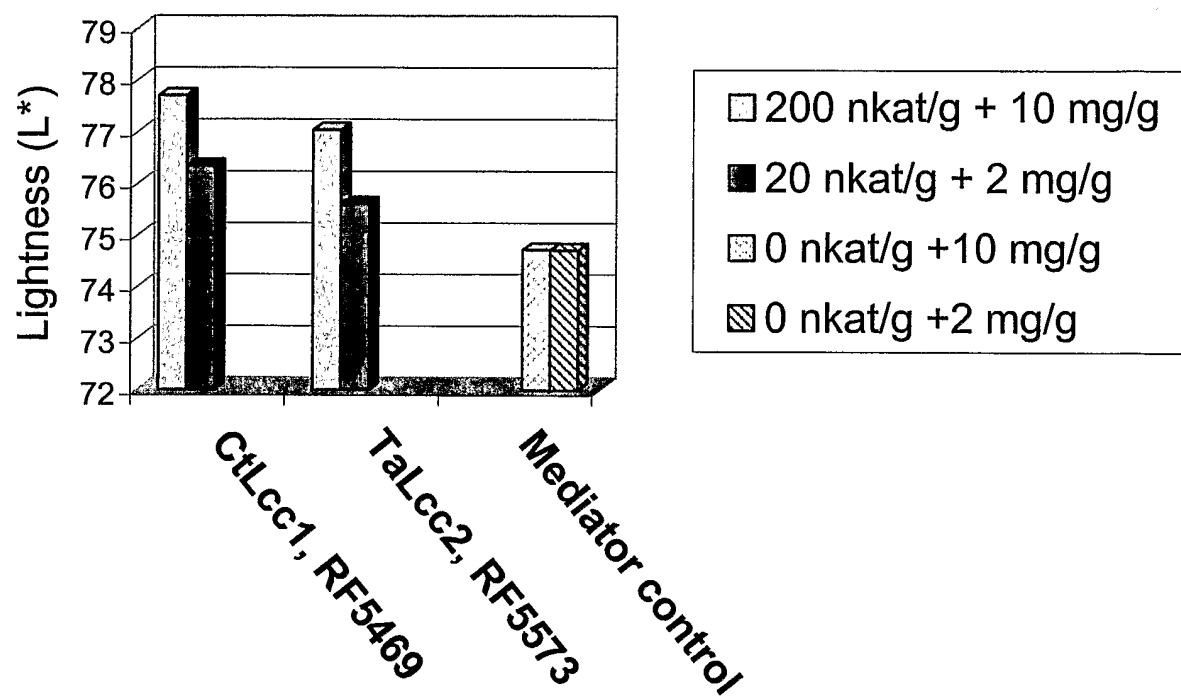
**Fig. 10B**

**Fig. 11A**

**Fig. 11B**

**Fig. 12A**

**Fig. 12B**

**Fig. 13A**

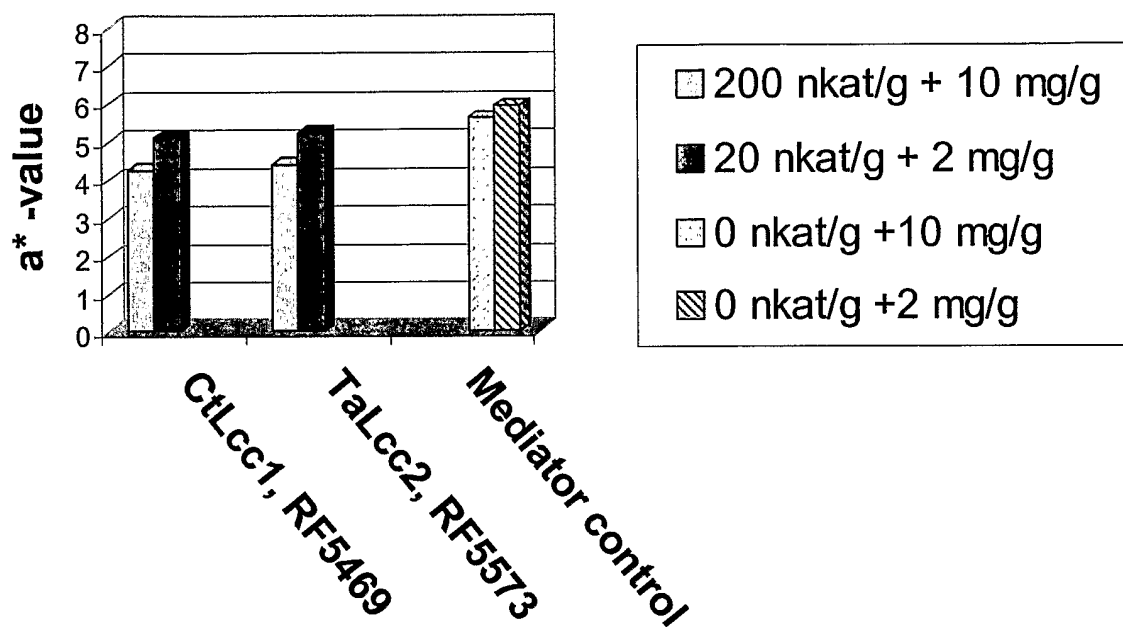


Fig. 13B

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 <222> (6)..(6)
 <223> n can be c or i

<400> 21
 aartgnsrwt grtacca

17

<210> 22
 <211> 20
 <212> DNA
 <213> artificial

<220>
 <223> Sequence of the oligonucleotide primer POX6

<220>
 <221> misc_feature
 <222> (9)..(9)
 <223> n can be c or i

<220>
 <221> misc_feature
 <222> (15)..(15)
 <223> n can be c or i

<400> 22
 atgcaytnc aygncayga

20

<210> 23
 <211> 20
 <212> DNA
 <213> artificial

<220>
 <223> Sequence of the oligonucleotide primer POX7

<220>
 <221> misc_feature
 <222> (6)..(6)
 <223> n can be c or i

<220>
 <221> misc_feature
 <222> (12)..(12)
 <223> n can be c or i

<400> 23
 tcrtgncrt gnarrtgc

20

<210> 24
 <211> 17
 <212> DNA
 <213> artificial

roa12sequence listing.txt

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<220>
<223> sequence of the oligonucleotide primer POX8

<220>
<221> misc_feature
<222> (6)..(6)
<223> n can be c or i

<220>
<221> misc_feature
<222> (12)..(12)
<223> n can be c or i

<400> 24
cayytncayg gncayga 17

<210> 25
<211> 17
<212> DNA
<213> artificial

<220>
<223> sequence of the oligonucleotide primer POX9

<220>
<221> misc_feature
<222> (6)..(6)
<223> n can be c or i

<220>
<221> misc_feature
<222> (12)..(12)
<223> n can be c or i

<400> 25
tcrtgncrcrt gnarrtg 17

<210> 26
<211> 23
<212> DNA
<213> artificial

<220>
<223> sequence of the oligonucleotide primer POX10

<220>
<221> misc_feature
<222> (6)..(6)
<223> n can be c or i

<400> 26
tgccangcda trtgrcartg cat 23

<210> 27
<211> 20
<212> DNA
<213> artificial

<220>
<223> sequence of the oligonucleotide primer POX11

<220>
<221> misc_feature
<222> (6)..(6)
<223> n can be i or c

<400> 27
tgccangcda trtgrcartg 20

<210> 28
<211> 17
<212> DNA
<213> artificial

<220>
<223> Sequene of the oligonucleotide primer POX12

<400> 28

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roal2sequence listing.txt

tggtaccact cgcattt 17

<210> 29
<211> 17
<212> DNA
<213> artificial

<220>
<223> Sequence of the oligonucleotide primer POX13

<400> 29
tcgtggccgt gcaggtg 17

<210> 30
<211> 23
<212> DNA
<213> artificial

<220>
<223> Sequence of the oligonucleotide primer POX14

<400> 30
tgccaggcaa tgtggcagt cat 23

<210> 31
<211> 20
<212> DNA
<213> artificial

<220>
<223> Sequence of the oligonucleotide primer POX15

<400> 31
tgccaggcaa tgtggcagt 20

<210> 32
<211> 1056
<212> DNA
<213> Thielavia arenaria ALK04197

<220>
<221> misc_feature
<222> (1)..(1056)
<223> Sequence of the PCR fragment obtained from Thielavia arenaria
ALK04197 using the primers POX27 and POX31

<400> 32
gagaactgga tcgggcccga tggcgttctc aagaatgtgg tgatgttggt caatggtacg 60
ttgatgtcca attctgtata aagagaagaa acgtgctgat acgctccctt cgtctagaca 120
agattatagg tatgttgta aacccgctgt aacccaacc gccaaacct ggaggctcct 180
cgcctggacg tgtgtgataa tatgctgacc tcgccgccag ggccaacct ccgcgcgaac 240
tggggtgaca atatcgaagt cactgtcatc aacaatctca aaaccaatgg gtacgaccac 300
ttgaatcatc ccgggcctac ccctaacaca aaatctcaac gtgcatccga tctgacgtat 360
tatatccatc tagtacctcg atgcactggc atggccttcg tcagctgggt aacgttttca 420
acgacggtgc caacggcgtg actgagtgc caatcccgcc caaaggaggg cgcaagacgt 480
acaagttccg tgcgacacag tatggcacca gctggatatca ctcccacttc tcggcccagt 540
acggcaacgg cgtggtcggc accatccaga tcgacggccc tgcctctctg ccatatgaca 600
ttgatctggg cgtgttccct ctcattggact actactacag gtcggccgat gagctggtgc 660
acttcaccca gagcaacggc gccccgcaa gcgacaacgt cctcttcaat ggcaccgccc 720
gtcaccctga gacgggggca ggccagtggg acaacgtcac gctgactcca ggcaagcgac 780
accgcctgcg catcatcaac acgtcgaccg acaaccactt tcagggtgtcg cttgtcggcc 840
acaacatgac cgtcattgcc accgacatgg tccccgtcaa cgcctttact gtcagcagcc 900
tattcctcgc cgtaggccag cgatacgtg tcaccatcga cgccaatagc ccggtgggca 960
actactggtt caacgtgact ttcggcgatg ggttggtgcg ctccagtaac aacaaattcc 1020

roal2sequence listing.txt 1056
cagccgccat cttccgctac cagggcgccc cgaaca

<210> 33
<211> 1296
<212> DNA
<213> Thielavia arenaria ALK04197

<220>
<221> misc_feature
<222> (1)..(1296)
<223> Sequence of the PCR fragment obtained from Thielavia arenaria
ALK04197 using the primers POX4 and POX11

<400> 33
tgggtaccaca gccacttcag cctccagtac gccgaggggtc tcttcggggg catgattctc 60
cggggggccca gcacggccaa ctgggacgag gacctcggag tcttgtttct gcaggactgg 120
tcacacgtcg aagccttcac taggtggcat gaagcgaaag ccgggtttcc tccatcactc 180
gatggcgggc tgatcaacgg cacaacaca ttgactgct ccacactgtc gccgacggac 240
cccaagtgca ccggcaatgg caagaagttc gagaccgtct tcgaaccgg caagaagtat 300
cttatccgcc tcatcaacgt ggcaatcgac ggagtgttcc agttcagcat cgacggacac 360
agcctcaccg tcatagcgac ggatttgggt ccgattgtcc cctacacgac cgacagcgct 420
cagatcacca tcggccagcg ttatgatata attgtcgagg caaacgcaac gccgggaaac 480
tactggatgc gggccgactg ggtcacagcc tgcgtgacca acgatcaccg cgagcacatg 540
acgggcatcg ttgcgtatga cgcgagcagc atcgaccccc cgacgtccga aagcaacgtc 600
acaaaaacca gcagctgcct cggcgagcgg aatgagaaaa cgatcccgca ttgtcgctc 660
gatgtcacca acattggcgg caccaacgtc gaggaactgt cctttgacac cacctctggc 720
gactacttcc agtggactct caatacgagc agcctgggtc tcgattgggg gaacccgacg 780
atggcccgaa tcttcaacgg ggtgcccac ttccccaccg agtacaacgt cgttgccgtc 840
aacgtgagtc tccccatgtt tgcagtaacc ccgaaccctt agagagtgcg gcgatttcgc 900
taatcatggt tccctatccg cagaaaaccg gcaactggacc ggaatggaca gtcctagtga 960
ttcaagatca aagcaatttg ccgtaagcct cccaaattcc caatccactt gtcctgccc 1020
ttgcaagtca ggtgcgctgc gtgggaatgg gatcccagct gacgtcaagg ctttttcatt 1080
tgtttgacg atttgcgcatc cgatccacct gcacggccac gacttttggg tgctggcggc 1140
cgaggagggc gtcttcaatg gcaacattag cagcttcaac acgaggaacc cggcgcggcg 1200
cgacgtcgcc acgtgcccgg gcagaggcta cctggccatc gccttccaga tcgacaaccc 1260
gggcaccttg ctgaccact gccacattgc ctggca 1296

<210> 34
<211> 1295
<212> DNA
<213> Thielavia arenaria ALK04197

<220>
<221> misc_feature
<222> (1)..(1295)
<223> Sequence of the PCR fragment obtained from Thielavia arenaria
ALK04197 using the primers POX27 and POX9

<400> 34
gagaactgga ttggccctga tggggttcgg aagcctgcc tgcattataaa cgggtgtgtg 60
ccagagccca caagatcctc caaggtcact tctgacaaca aggcaggctc attccctggg 120
cccacaatct cagccgactg gggcgactac attattgtca atgtccacaa tgacatgcaa 180
gataacgggt aagaatgca gcacgggtacc cccggcatcc tcaaacctgc ttgctaacac 240
ataggtagaa cgtcaatcca ttggcatgga atccgtcagc ttggcgagag caaccaggac 300
ggcgcaaacg gcgtaacgga atgccccatt cctccggat ccagcaaac ctacgacttc 360

roal2sequence listing.txt

```

catgtgacgc agtacggcac ctctgtgtac cacagccact actccaacca gtacggcaat 420
ggggctcgtg gcgcccctgat cgtgcgcggc ccggcatcag ccaactacga catcgacctc 480
ggtccctacc tgatcagtga ctactactac gaaaccgcag accgcctcca tctccgagcc 540
gagcttgtca gcaacggccc accgccagac agcgacaaca tcctgttccg cggcaaaaac 600
atcaacccca agcgtgcagg cagcggctcc tacgaccgcc tcgtcctaac ccccggaag 660
aagcatctaa tccggctcat caacgccagc gtggacaact ctttcgtcat ctccctcgtc 720
ggccacaact tcaccgtcat ctccaccgac atggtcccca tcaccctgt cgtgcgcagc 780
tccttattca tgggcgtcgg ccagcggtag gacgtcattg tcgaagccaa ccagcccgtg 840
ggcaactact ggctcaacgc gacgtcagc gccagaaca actgcgggca ttcggtcaac 900
cccttccccg cggctattgt ccagtacgag ggccgagct ccaccgccct gccgaccaac 960
cgcgggacgc ccctgacggc gacatgcaat ggcgagaaag ggttttcgcc cattgttaag 1020
cggacagttt caagtccct gttccagccg tctaccctgc cagtgcgcct tgaattcccc 1080
actacagacc gcgggcaggt gtttgagtgg cgggttaaga acacgccgat aagcgtcgaa 1140
tgaggagcatc ccgtgctgga gtacattttg caggggaaca cctccttccc ggcaaaggcc 1200
aatctcattg aagtcgccga ggcaaatgtc tggacgttct ggggtgattca gaacgggttt 1260
gggctgccgc acccgattca cctgcacggg cacga 1295

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<210> 35
 <211> 224
 <212> DNA
 <213> Chaetomium thermophilum ALKO 4265

<220>
 <221> misc_feature
 <222> (1)..(224)
 <223> Sequence of the PCR fragment obtained from Chaetomium thermophilum ALKO4265 using the primers POX8 and POX11

```

<400> 35
catctccatg ggcacgactt cctcattgtc ggccgctccc ccgacgtccc gccgggctcg 60
aaccagcgt acaacttcga ccccgccacc gacatctacc gcctgcgcgg tcagaaccgc 120
accgctcgtg acgtcgccat gtttcttccc ggccgctggc tgctgctcgc cttcctcacc 180
gacaaccccg gtgcctggct cttccactgc cacatgcctt ggca 224

```

<210> 36
 <211> 929
 <212> DNA
 <213> Chaetomium thermophilum ALKO4265

<220>
 <221> misc_feature
 <222> (1)..(929)
 <223> Sequence of the PCR fragment obtained from Chaetomium thermophilum ALKO4265 using the primers POX4 and POX9

```

<400> 36
tggtatcatt gccacttctc caaccagtac ggcaacggcg tgctcggcgc actggtggtc 60
aaaggcccg cttccgcaa ctacgacatc gacctggcc cgtacatcat tagcgactac 120
taccagaga cggccgacag attacatctc caggcagagc tactccgaa cgggccccct 180
ccagacagcg acaacatcct cttcaggggc aagaacatca accctgacgg ctcgggcccgc 240
ggctcttatg accgattaac cctcatccca ggcaagaaac acctgctgcg tctgatcaac 300
gcaagcgtgg ataactcctt cagggtctcc ctcgtgggac acaacttcac ggtcattgcg 360
accgatatgg tcccggccca gccaacgctc cgcaggagcc tgttcatggc cgtcggccag 420
cgttatgatg ttattgttac gcgcgaccag cccgtagata attattggct gaacgtcacc 480
ctggaagcaa acaacaactg cggccgctct cgcaaccctt acccagcagc catcatccac 540

```

roal2sequence listing.txt

tacgagggag ccagcccaac cgccctcccc accaaccgtg gcacccccct cgtagcaacc	600
tgcaccggcg agacaggctt ccccccgctc gtgccgcgga atatcccccc caacttcttc	660
cgcccttccg acatcgcttc caataccctg ccaatcggcc tcaacattgt caaccacacc	720
actaaaggcc aaatcttctc ctggcatgtg aagaacacgc ctatttccgt ggaatggggg	780
catccagtat tagagtacat cctcgaaggg aattactcct ttcccgcagc ggtaaatctg	840
attcaactca accaaaaaga cacctggaca ctctttttgg tgcatagttc gttatcatta	900
ccgcacccaa tccacctcca cgggcacga	929

<210> 37
 <211> 251
 <212> DNA
 <213> Chaetomium thermophilum ALK04265

<220>
 <221> misc_feature
 <222> (1)..(251)
 <223> Sequence of the PCR fragment obtained from Chaetomium thermophilum ALK0465 using the primers POX8 and POX11

<400> 37	
cacttccacg ggcacgactt ttacatcctc cagaggggcc ccggcgactg ggatggcacc	60
atggtccggc caagcaaccc ccacagacgg gacgtctacc tggtagcgcg gtttggtcat	120
cttgttctgc aattcgatgg tgaacctggg acgtgtgccg tagttctcag ccagactccg	180
tgtgcgcttg gaggaaggca atctaacaac ataataggag tctgggcctt ccactgtcac	240
atcgcggtggc a	251

<210> 38
 <211> 2680
 <212> DNA
 <213> Thielavia arenaria ALK04197

<220>
 <221> misc_feature
 <222> (178)..(2456)
 <223> Talcc1

<220>
 <221> Intron
 <222> (406)..(456)

<220>
 <221> Intron
 <222> (536)..(597)

<220>
 <221> Intron
 <222> (610)..(700)

<220>
 <221> Intron
 <222> (771)..(853)

<220>
 <221> Intron
 <222> (1824)..(1902)

<220>
 <221> Intron
 <222> (1975)..(2033)

<400> 38	
ggatccccgg gtcagtctat ataaggggct gagtgtccag ctcttccatg cctttgattc	60
tcttgaatca ccaggacact cgggcggcct cagtcttgca taactcgggt cttcccttcc	120
tctcactgct tttcttcgct cagatatatt tcaggcgacc tcaaacagct cgccatcatg	180
aagtcttggg ccgccgccgt ggcgctcatg gtgggcattc tcagccctca tgctgccgcc	240
gcacctcctg caaacccggg ccagagagac atgctccagg tccttgaggc gagacagtct	300
ggcccgactt gcaacacccc gtccaatcgt gcgtgctgga ccaatggttt cgacatcaac	360

roal2sequence listing.txt

```

accgactatg aagtcagcac tcctaatacc ggacgtactg tggccgtaag cttccctcc 420
ctttaaggag gcagagctag gactaacaag caccagtacc aacttacct cactgagaaa 480
gagaactgga tcggtcccga tggcgttctc aagaatgtgg tgatgttggg caatggtacg 540
ttgatgtcca attctgtata aagagaagaa acgtgctgat acgtccctt cgtctagaca 600
agattatagg tatgttgtca aaccgctgt aacccaacc gccaaacct ggaggctcct 660
cgcttgacg tgttgtacaa tatgtgacc tcgccgccag ggccaacct ccgcgcgaac 720
tggtgtgaca atatcgaagt cactgtcatc aacaatctca aaaccaatgg gtacgaccac 780
ttgaatcatc ccgggcctac ccctaacaca aaatctcaac gtgcatccga tctgacgtat 840
tatatccatc tagtacctcg atgactggc atggccttcg tcagctgggt aacgttttca 900
acgacgtgc caacggcgtg actgagtgc caatccgcc caaggaggg cgcaagacgt 960
acaagtccg tgcgacacag tatggacca gctggtatca ctcccactt tcggcccagt 1020
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cagccgcat cttccgctac cagggcgccc ccgctacgt cccgacggat cagggctac 1560
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cgcccgtaa caactttgtc aagcgtccg ccaacacgt gggcgctact ctcgatatcg 1680
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gagaacgacc cgaccaatcc cattgtcagc ttgccgacc cgatgcatct gcacgtacgt 1980
tcaaacctcc cccaccccc cacttcatac aaaatatact gacaaatcga cagggccacg 2040
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ttgacccggc caaggacctg ccccggtta agggcaacaa cccgtgagg cgggacgtga 2160
cgatgttcc ggcgggcggc tggctgtgc tggcgttcaa gacggacaac ccgggcgcat 2220
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acatggaggg gtctggggtt gggatataca ggcttctgc tccgttttta tcttttgct 2640
tgattaagac tttctgtct gatgtacgag tcaggccgcc 2680

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<210> 39
 <211> 617
 <212> PRT
 <213> Thielavia arenaria ALK04197

roal2sequence listing.txt

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<220>
<221> MISC_FEATURE
<222> (1)..(617)
<223> Deduced amino acid sequence of Thielavia arenaria ALK04197 TaLcc1
<400> 39
Met Lys Ser Trp Ala Ala Ala Val Ala Leu Met Val Gly Ile Leu Ser
1      5      10
Pro His Ala Ala Ala Ala Pro Pro Ala Asn Pro Val Gln Arg Asp Met
20      25      30
Leu Gln Val Leu Glu Ala Arg Gln Ser Gly Pro Thr Cys Asn Thr Pro
35      40      45
Ser Asn Arg Ala Cys Trp Thr Asn Gly Phe Asp Ile Asn Thr Asp Tyr
50      55      60
Glu Val Ser Thr Pro Asn Thr Gly Arg Thr Val Ala Tyr Gln Leu Thr
65      70      75
Leu Thr Glu Lys Glu Asn Trp Ile Gly Pro Asp Gly Val Leu Lys Asn
85      90      95
Val Val Met Leu Val Asn Asp Lys Ile Ile Gly Pro Thr Ile Arg Ala
100     105     110
Asn Trp Gly Asp Asn Ile Glu Val Thr Val Ile Asn Asn Leu Lys Thr
115     120     125
Asn Gly Thr Ser Met His Trp His Gly Leu Arg Gln Leu Gly Asn Val
130     135     140
Phe Asn Asp Gly Ala Asn Gly Val Thr Glu Cys Pro Ile Pro Pro Lys
145     150     155     160
Gly Gly Arg Lys Thr Tyr Lys Phe Arg Ala Thr Gln Tyr Gly Thr Ser
165     170     175
Trp Tyr His Ser His Phe Ser Ala Gln Tyr Gly Asn Gly Val Val Gly
180     185     190
Thr Ile Gln Ile Asp Gly Pro Ala Ser Leu Pro Tyr Asp Ile Asp Leu
195     200     205
Gly Val Phe Pro Leu Met Asp Tyr Tyr Tyr Arg Ser Ala Asp Glu Leu
210     215     220
Val His Phe Thr Gln Ser Asn Gly Ala Pro Pro Ser Asp Asn Val Leu
225     230     235     240
Phe Asn Gly Thr Ala Arg His Pro Glu Thr Gly Ala Gly Gln Trp Tyr
245     250     255
Asn Val Thr Leu Thr Pro Gly Lys Arg His Arg Leu Arg Ile Ile Asn
260     265     270
Thr Ser Thr Asp Asn His Phe Gln Val Ser Leu Val Gly His Asn Met
275     280     285
Thr Val Ile Ala Thr Asp Met Val Pro Val Asn Ala Phe Thr Val Ser
290     295     300
Ser Leu Phe Leu Ala Val Gly Gln Arg Tyr Asp Val Thr Ile Asp Ala
305     310     315     320

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roal2sequence listing.txt

Asn Ser Pro Val Gly Asn Tyr Trp Phe Asn Val Thr Phe Gly Asp Gly
 325 330 335
 Leu Cys Gly Ser Ser Asn Asn Lys Phe Pro Ala Ala Ile Phe Arg Tyr
 340 345 350
 Gln Gly Ala Pro Ala Thr Leu Pro Thr Asp Gln Gly Leu Pro Val Pro
 355 360 365
 Asn His Met Cys Leu Asp Asn Leu Asn Leu Thr Pro Val Val Thr Arg
 370 375 380
 Ser Ala Pro Val Asn Asn Phe Val Lys Arg Pro Ser Asn Thr Leu Gly
 385 390 395 400
 Val Thr Leu Asp Ile Gly Gly Thr Pro Leu Phe Val Trp Lys Val Asn
 405 410 415
 Gly Ser Ala Ile Asn Val Asp Trp Gly Lys Pro Ile Leu Asp Tyr Val
 420 425 430
 Met Ser Gly Asn Thr Ser Tyr Pro Val Ser Asp Asn Ile Val Gln Val
 435 440 445
 Asp Ala Val Asp Gln Trp Thr Tyr Trp Leu Ile Glu Asn Asp Pro Thr
 450 455 460
 Asn Pro Ile Val Ser Leu Pro His Pro Met His Leu His Gly His Asp
 465 470 475 480
 Phe Leu Val Leu Gly Arg Ser Pro Asp Glu Leu Pro Ser Ala Gly Val
 485 490 495
 Arg His Ile Phe Asp Pro Ala Lys Asp Leu Pro Arg Leu Lys Gly Asn
 500 505 510
 Asn Pro Val Arg Arg Asp Val Thr Met Leu Pro Ala Gly Gly Trp Leu
 515 520 525
 Leu Leu Ala Phe Lys Thr Asp Asn Pro Gly Ala Trp Leu Phe His Cys
 530 535 540
 His Ile Ala Trp His Val Ser Gly Gly Leu Ser Val Asp Phe Leu Glu
 545 550 555 560
 Arg Pro Asn Asp Leu Arg Thr Gln Leu Asn Ser Asn Ala Lys Arg Ala
 565 570 575
 Asp Arg Asp Asp Phe Asn Arg Val Cys Arg Glu Trp Asn Ala Tyr Trp
 580 585 590
 Pro Thr Asn Pro Phe Pro Lys Ile Asp Ser Gly Leu Arg His Arg Phe
 595 600 605
 Val Glu Glu Ser Glu Trp Met Val Arg
 610 615

<210> 40
 <211> 3084
 <212> DNA
 <213> Thielavia arenaria ALK04197

<220>
 <221> misc_feature
 <222> (1)..(2600)

roal2sequence_listing.txt

<223> Nucleotide sequence of Thielavia arenaria ALK04197 Ta lcc2 gene.

<220>
 <221> misc_feature
 <222> (644)..(2600)
 <223> Ta lcc2

<220>
 <221> misc_feature
 <222> (644)..(646)
 <223> putative start ,ATG

<220>
 <221> misc_feature
 <222> (674)..(676)
 <223> putative start ,ATG

<220>
 <221> Intron
 <222> (2003)..(2082)

<220>
 <221> Intron
 <222> (2142)..(2248)

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<400> 40
ggatcccgtg gtcaacaaga cccagcaccc agggtaacca tcctatacgg ggcaagatct    60
accacttgca gcgatatatt gaagtccaat acatcgctac tgtagtagtc atggtgttct    120
gtaagttccg gcagggactg tagtactcga cgggttcctc tccttcagtc tagccctcgt    180
agagcatgtt gacgttacga taggtttccc cccccccatt tggagctgtg atatactttc    240
aagttgttga aggccttttag ctgagcataa cgatcggtgc tgacgaaagc ctccgtgcgg    300
cacacctaaa attacgattc cgaatgtctc tccaaggagt tgtcatatga tactattgat    360
gtgagcttca ggggagcagg ccctattacg aaggtagacc agggacaact ccttctcggc    420
ttaaccgggc cggatcggtg aatgcctcgc caaagactca ttgagaggcg acatggagcc    480
aagcccacct tctcttgagg ctctgatatt gatgctgtac gagtataaag tcgatgatct    540
cctgcaatgt cccagagtct atccaagcag ctcttgacc aaggaaagcca tcggtcactc    600
aacaggcgcg gccacattcg ttcaactttg aattggtagc accatgttaa ccgagacact    660
caagtcaaga aacatgttgc aatcaatcct tgctcttctt gccaccgccc tgggctcatc    720
tgcttctgcc atccctgaac tcccagaggt ctgcgttcag ccccggaag cttgcgagaa    780
cactgcaacg tctcgccaat gctggggtga cctttcaatc gacaccaact actatgaagt    840
tcttcccagc accgggcgcc cgccgcagga gtattggctg agtgctcgtg aaggcccttg    900
tgccccagac ggatataaca ggacctgtat gacctttaac ggcaccgtgc cggggccaac    960
cctcttcgcc gattggggcg acgagctcgt tatccatgtc acaaacaaca tggtaacaa    1020
tggcaccgag atccactggc acgggattcg catgctgaat aacacgctca acgacggcgt    1080
tcccggagtg acgcagtgcg ccctcgcccc gggcgagtcc atgacatacc ggttcaacgt    1140
caccagtagc ggctcgactt ggtaccacag ccacttcagc ctccagtacg ccgagggctc    1200
cttcgggggc atgattctcc gggggcccag caccggccaac tgggacgagg acctcggagt    1260
cttgtttctg caggactggg cacacgtcga agccttcact aggtggcatg aagcgaaagc    1320
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cacactgtcg ccgacggacc ccaagtgcac cggcaatggc aagaagtctg agaccgtctt    1440
cgaaccgggc aagaagtatc ttatccgcct catcaacgtg gcaatcgacg gagtgttcca    1500
gttcagcatc gacggacaca gcctcaccgt catagcgacg gatttgggtc cgattgtccc    1560
ctacacgacc gacagcgctc agatcaccat cggccagcgt tatgatatca ttgtcgaggc    1620
aaacgcaacg ccgggaaact actggatgcg ggccgactgg gtcacagcct gcgtgaccaa    1680
cgatcacccc gagcacatga cgggcatcgt tcgctatgac gcgagcagca tcgaccccc    1740
gacgtccgaa agcaacgtca caaaaaccag cagctgcctc ggcgagccga atgagaaaac    1800

```

roal2sequence listing.txt

```

gatcccgcat ttgtcgctcg atgtcaccaa cattggcggc accaacgtcg aggaactgtc 1860
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cgattggggg aacccgacga tggcccgaat cttcaacggg gatgccatct tccccaccga 1980
gtacaacgtc gttgccgtca acgtgagtct ccccatgttt gcagtaaccc cgaacccta 2040
gagagtgcgg cgatttcgct aatcatggtt ccctatccgc agaaaaccgg cactggaccg 2100
gaatggacag tcctagtgat tcaagatcaa agcaatttgc cgtaagcctc ccaaattccc 2160
aatccacttg tccctgccct tgcaagtcag gtgcgctgcg tgggaatggg atcccagctg 2220
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acttttgggt gctggcggcc gaggaggcg tcttcaatgg caacattagc agcttcaaca 2340
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ccttcagat cgacaacccg ggcacctggc tgacctactg ccacattgcc tggcacgcca 2460
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<210> 41
 <211> 589
 <212> PRT
 <213> Thielavia arenaria ALK04197

<220>
 <221> MISC_FEATURE
 <222> (1)..(589)
 <223> Deduced amino acid sequence of Thielavia arenaria ALK04197 Ta
 Lacc2

<400> 41

Met Leu Thr Glu Thr Leu Lys Ser Arg Asn Met Leu Gln Ser Ile Leu
1 5 10 15

Ala Leu Leu Ala Thr Ala Leu Gly Ser Ser Ala Phe Ala Ile Pro Glu
20 25 30

Leu Pro Glu Val Ser Leu Gln Pro Arg Gln Ala Cys Glu Asn Thr Ala
35 40 45

Thr Ser Arg Gln Cys Trp Gly Asp Leu Ser Ile Asp Thr Asn Tyr Tyr
50 55 60

Glu Val Leu Pro Ser Thr Gly Arg Pro Pro Gln Glu Tyr Trp Leu Ser
65 70 75 80

Val Val Glu Gly Pro Cys Ala Pro Asp Gly Tyr Asn Arg Thr Cys Met
85 90 95

Thr Phe Asn Gly Thr Val Pro Gly Pro Thr Leu Phe Ala Asp Trp Gly
100 105 110

roal2sequence listing.txt

Asp Glu Leu Val Ile His Val Thr Asn Asn Met Val Asn Asn Gly Thr
 115 120 125
 Ala Ile His Trp His Gly Ile Arg Met Leu Asn Asn Thr Leu Asn Asp
 130 135 140
 Gly Val Pro Gly Val Thr Gln Cys Ala Ile Ala Pro Gly Glu Ser Met
 145 150 155 160
 Thr Tyr Arg Phe Asn Val Thr Gln Tyr Gly Ser Thr Trp Tyr His Ser
 165 170 175
 His Phe Ser Leu Gln Tyr Ala Glu Gly Leu Phe Gly Gly Met Ile Leu
 180 185 190
 Arg Gly Pro Ser Thr Ala Asn Trp Asp Glu Asp Leu Gly Val Leu Phe
 195 200 205
 Leu Gln Asp Trp Ser His Val Glu Ala Phe Thr Arg Trp His Glu Ala
 210 215 220
 Lys Ala Gly Phe Pro Pro Ser Leu Asp Gly Gly Leu Ile Asn Gly Thr
 225 230 235 240
 Asn Thr Phe Asp Cys Ser Thr Leu Ser Pro Thr Asp Pro Lys Cys Thr
 245 250 255
 Gly Asn Gly Lys Lys Phe Glu Thr Val Phe Glu Pro Gly Lys Lys Tyr
 260 265 270
 Leu Ile Arg Leu Ile Asn Val Ala Ile Asp Gly Val Phe Gln Phe Ser
 275 280 285
 Ile Asp Gly His Ser Leu Thr Val Ile Ala Thr Asp Leu Val Pro Ile
 290 295 300
 Val Pro Tyr Thr Thr Asp Ser Val Gln Ile Thr Ile Gly Gln Arg Tyr
 305 310 315 320
 Asp Ile Ile Val Glu Ala Asn Ala Thr Pro Gly Asn Tyr Trp Met Arg
 325 330 335
 Ala Asp Trp Val Thr Ala Cys Val Thr Asn Asp His Pro Glu His Met
 340 345 350
 Thr Gly Ile Val Arg Tyr Asp Ala Ser Ser Ile Asp Pro Pro Thr Ser
 355 360 365
 Glu Ser Asn Val Thr Lys Thr Ser Ser Cys Leu Gly Glu Pro Asn Glu
 370 375 380
 Lys Thr Ile Pro His Leu Ser Leu Asp Val Thr Asn Ile Gly Gly Thr
 385 390 395 400
 Asn Val Glu Glu Leu Ser Phe Asp Thr Thr Ser Gly Asp Tyr Phe Gln
 405 410 415
 Trp Thr Leu Asn Thr Ser Ser Leu Val Leu Asp Trp Gly Asn Pro Thr
 420 425 430
 Met Ala Arg Ile Phe Asn Gly Asp Ala Ile Phe Pro Thr Glu Tyr Asn
 435 440 445
 Val Val Ala Val Asn Lys Thr Gly Thr Gly Pro Glu Trp Thr Val Leu

roal2sequence listing.txt

450 455 460

Val Ile Gln Asp Gln Ser Asn Leu Pro Ile Ala His Pro Ile His Leu
465 470 475 480

His Gly His Asp Phe Trp Val Leu Ala Ala Glu Glu Gly Val Phe Asn
 485 490 495

Gly Asn Ile Ser Ser Phe Asn Thr Arg Asn Pro Ala Arg Arg Asp Val
 500 505 510

Ala Thr Leu Pro Gly Arg Gly Tyr Leu Ala Ile Ala Phe Gln Ile Asp
 515 520 525

Asn Pro Gly Thr Trp Leu Thr His Cys His Ile Ala Trp His Ala Ser
 530 535 540

Gln Gly Leu Ser Leu Glu Phe Val Glu Ser Gln Ser Glu Ile Val Thr
545 550 555 560

Asp Glu Val Ser Arg Gly Val Phe Asn Asp Val Cys Ala Ser Trp Arg
 565 570 575

Ala His Asp Pro Leu Trp Glu Gln Glu Asp Ser Gly Ile
 580 585

<210> 42
 <211> 2971
 <212> DNA
 <213> Thielavia arenaria ALK04197

<220>
 <221> misc_feature
 <222> (1)..(2971)
 <223> Nucleotide sequence of Thielavia arenaria AK04197 Talcc3 gene

<220>
 <221> misc_feature
 <222> (275)..(2289)
 <223> Talcc3

<220>
 <221> Intron
 <222> (521)..(585)

<220>
 <221> Intron
 <222> (662)..(715)

<220>
 <221> Intron
 <222> (798)..(857)

<400> 42
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 ttcggtcaag ggacggtgct ggggaataga tgcccgtcta ggaattccgg tagtggcggt 120
 ctttcggaaa tataaccgag caggacgttc agcccatcgt ccctgtgtat ataaagcatg 180
 aagttattct atgtagaatc ctagactatc ttagaagcga acctttcgtc gcagatgctt 240
 cgtgttttcc atcattactt cccgtagtac tagtatggtc tgccttcagt atcagctcac 300
 agcagcctgc tttgctgtgt tagcggtcgt caccacccca gttcctgct ctccgtgtcca 360
 gccgagcccc gttcacgata tcttaccaag acaacaata atccccggcg gtaagccatg 420
 tgggtcaaat aatgccacga ataggggggtg ctggaaaaac aactggaaca tcaccaccga 480
 ctatgaagtc gacacgcccc ctgcgttcaa caccagagtg gtatgtcctt gctcataata 540
 agtggctagc ctccaagatc aggcacaaga ctgataggag ctgagtatga cttcacatc 600
 accaatgtca ccaactggct cggccctgat ggggttcgga agcctgccat gcttataaac 660

roal2sequence listing.txt

```

ggtgtgttgc cagagcccac aagatcctcc aaggtcactt ctgacaacaa ggcaggctca 720
ttccctggtc ccacaatctc agccgactgg ggcgactaca ttattgtcaa tgtccacaat 780
gacatgcaag ataacgggta agaaatgcag cacggtaccc ccggcatcct caaacctgct 840
tgctaacaca taggtagaac gtcaatccat tggcatggaa tccgtcagct tggcgagagc 900
aaccaggacg gcgcaaacgg cgtaacggaa tgccccattc ctcccgatc cagcaaaacc 960
tacgacttcc atgtgacgca gtacggcacc tcgtggtacc acagccacta ctccaaccag 1020
tacggcaatg gggtcgtggg cgccctgatc gtgcgcggcc cggcatcagc caactacgac 1080
atcgacctg gtccctacct gatcagtgac tactactacg aaaccgcaga ccgcctccat 1140
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ggcaaaaaca tcaaccccaa gcgtgcaggc agcggctcct acgaccgcct cgtcctaacc 1260
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tcggtcaacc ctttccccgc ggctattgtc cagtacgagg gcgccagctc caccgccctg 1560
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gtcggcaacg ggacttttga tgtcgcgtct atgagggggc tgttgaaatt taataatcct 1980
gttcgtaggg atgtcgagca gatgcctggc aatgggtggc tcgtgattgc gttcaagacg 2040
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gtgcccatt gtgaggcttg gagggcctat gcaaggacga gtccatatct gccaaagctt 2220
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attgaagatg tttggacaca tgcaatgcgt actttgagtt gctatcaaaa agcatgaaac 2760
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ggagataata accgtcaact cacgcagggc ttcaacgtg gagtggtgac gacaggcccc 2880
tgacgtcttg gttgctggag ggatcgtcag gggtaaaaa taagggttag ggttcaagtc 2940
ttccgtcaac tccctaagga ggcaaggatc c 2971

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<210> 43
 <211> 611
 <212> PRT
 <213> Thielavia arenaria ALK04197

<220>

roal2sequence listing.txt

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<221> MISC_FEATURE
<222> (1)..(611)
<223> Deduced amino acid sequence of Thielavia arenaria LAK04197 TaLcc3

<220>
<221> MISC_FEATURE
<222> (1)..(611)
<223> Deduced amino acid sequence of Thielavia arenaria ALK04197 TaLcc3

<400> 43
Met Val Cys Leu Gln Tyr Gln Leu Thr Ala Ala Cys Phe Ala Val Leu
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Ala Val Val Thr Thr Pro Ala Ser Cys Ser Pro Val Gln Pro Ser Pro
20     25     30
Val His Asp Leu Leu Pro Arg Gln Thr Ile Ile Pro Gly Gly Lys Pro
35     40     45
Cys Gly Gln Asn Asn Ala Thr Asn Arg Gly Cys Trp Lys Asn Asn Trp
50     55     60
Asn Ile Thr Thr Asp Tyr Glu Val Asp Thr Pro Pro Ala Phe Asn Thr
65     70     75     80
Arg Val Tyr Asp Leu His Ile Thr Asn Val Thr Asn Trp Leu Gly Pro
85     90     95
Asp Gly Val Arg Lys Pro Ala Met Leu Ile Asn Gly Ser Phe Pro Gly
100    105    110
Pro Thr Ile Ser Ala Asp Trp Gly Asp Tyr Ile Ile Val Asn Val His
115    120    125
Asn Asp Met Gln Asp Asn Gly Thr Ser Ile His Trp His Gly Ile Arg
130    135    140
Gln Leu Gly Glu Ser Asn Gln Asp Gly Ala Asn Gly Val Thr Glu Cys
145    150    155    160
Pro Ile Pro Pro Gly Ser Ser Lys Thr Tyr Asp Phe His Val Thr Gln
165    170    175
Tyr Gly Thr Ser Trp Tyr His Ser His Tyr Ser Asn Gln Tyr Gly Asn
180    185    190
Gly Val Val Gly Ala Leu Ile Val Arg Gly Pro Ala Ser Ala Asn Tyr
195    200    205
Asp Ile Asp Leu Gly Pro Tyr Leu Ile Ser Asp Tyr Tyr Tyr Glu Thr
210    215    220
Ala Asp Arg Leu His Leu Arg Ala Glu Leu Val Ser Asn Gly Pro Pro
225    230    235    240
Pro Asp Ser Asp Asn Ile Leu Phe Arg Gly Lys Asn Ile Asn Pro Lys
245    250    255
Arg Ala Gly Ser Gly Ser Tyr Asp Arg Leu Val Leu Thr Pro Gly Lys
260    265    270
Lys His Leu Ile Arg Leu Ile Asn Ala Ser Val Asp Asn Ser Phe Val
275    280    285
Ile Ser Leu Val Gly His Asn Phe Thr Val Ile Ser Thr Asp Met Val
290    295    300

```

roal2sequence listing.txt

Pro Ile Thr Pro Val Val Arg Ser Ser Leu Phe Met Gly Val Gly Gln
 305 310 315 320
 Arg Tyr Asp Val Ile Val Glu Ala Asn Gln Pro Val Gly Asn Tyr Trp
 325 330 335
 Leu Asn Ala Thr Leu Glu Ala Gln Asn Asn Cys Gly His Ser Val Asn
 340 345 350
 Pro Phe Pro Ala Ala Ile Val Gln Tyr Glu Gly Ala Ser Ser Thr Ala
 355 360
 Leu Pro Thr Asn Arg Gly Thr Pro Leu Thr Ala Thr Cys Asn Gly Glu
 370 375 380
 Lys Gly Phe Ser Pro Ile Val Lys Arg Thr Val Ser Ser Ser Leu Phe
 385 390 395 400
 Gln Pro Ser Thr Leu Pro Val Ser Leu Glu Phe Pro Thr Thr Asp Arg
 405 410 415
 Gly Gln Val Phe Glu Trp Arg Val Lys Asn Thr Pro Ile Ser Val Glu
 420 425 430
 Trp Glu His Pro Val Leu Glu Tyr Ile Leu Gln Gly Asn Thr Ser Phe
 435 440 445
 Pro Ala Lys Ala Asn Leu Ile Glu Val Pro Gln Ala Asn Val Trp Thr
 450 455 460
 Phe Trp Val Ile Gln Asn Gly Phe Gly Leu Pro His Pro Ile His Leu
 465 470 475 480
 His Gly His Asp Phe Leu Val Leu Gly Val Gly Asn Gly Thr Phe Asp
 485 490 495
 Ala Ala Ser Met Arg Gly Leu Leu Asn Phe Asn Asn Pro Val Arg Arg
 500 505 510
 Asp Val Glu Gln Met Pro Gly Asn Gly Trp Leu Val Ile Ala Phe Lys
 515 520 525
 Thr Asp Asn Pro Gly Cys Trp Leu Met His Cys His Ile Gly Trp His
 530 535 540
 Val Ala Met Gly Leu Gly Ile Gln Phe Leu Glu Arg Arg Ser Asp Ile
 545 550 555 560
 Leu Ala Leu Met Lys Leu Asp Gln Met Val Pro Asn Cys Glu Ala Trp
 565 570 575
 Arg Ala Tyr Ala Arg Thr Ser Pro Tyr Leu Pro Lys Leu Asp Ser Gly
 580 585 590
 Leu Lys Arg Gly Val Glu Met Arg Glu Gly Met Glu Pro Ala Val Arg
 595 600 605
 Arg Ile Gly
 610

<210> 44
 <211> 2731
 <212> DNA
 <213> Thielavia arenaria ALK04197

roal2sequence listing.txt

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<220>
<221> misc_feature
<222> (1)..(2731)
<223> Nucleotide sequence of Thielavia arenaria ALK04197 Talcc4 gene

<220>
<221> misc_feature
<222> (455)..(2247)

<220>
<221> Intron
<222> (1371)..(1442)

<400> 44
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tggcgttctc tggctcgtga tggtcggatt tgacgacggc ggtgattggc aacgactttg 120
cgggacgttg gatttgaca ctttgggggt ttgacaatgg cgagagcgag acaagtaggt 180
actaaccgct ttcctccagg atttctgtgc tgggagggcg cagggaggcg ggctgacgat 240
ggacttggtt acaagggagt gaagatgtgg cttacaggga cgtacatcta ctatataagc 300
tgaacactgt ggcacttctc tgagaaaagc aatgggcagg tatctcagta catgttcagg 360
accccttaag ttgaccattt caacttacat gcttcttcca actcgtttct ccctcgtcgg 420
cccttggaag tttagaccgg gctcttacat gacaatgatt tcgctgtctt ttttcacaag 480
cagtttact gctacactgg cttctgctct gccaggctta ttatacgccc ctcatccaag 540
cgactactcc atcgacaccg actatacgac agagcccgtt gacaccgggg ttactcgaga 600
gtactggctt gagttaacgg acgtaaccgt gtcgccagac ggggtgtcgc ggtcagcgat 720
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catccgcaa aactacacca accagaacga cgggtgtgcc tccattacgc agtgtcctct 900
ggcggttggc gaatcaacca cctacacctg gaaagcaaca caatatggct catcttggtg 960
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ccaaactgtc gatgagctct attccagtgc tgaaacctct ggccaccca ctttgccaa 1140
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aagattggct gtctcattca cgtctggcac ctcataccgc atgcggctgg tgaatgccgc 1260
cgtcgacacg cattggaat tctccatcgg caaccacaca atgactgtga tggccgctga 1320
tctcgttccg attgagccat atgaaacgac ggtcttaaca attgggatgg gtaagtacct 1380
acatcatcag accacctact tattcggttt cttaggacac ccgagactga gttccacata 1440
ggacaaagat acgacatagt cgtcacggca gatcaagctg atgtcgcgga taacttctgg 1500
atgagggcta tcccacaatc agcatgctct gataacgaca gcgccgaca tatcagaggg 1560
atcgtctact atggtgatg tcccggcacc cttcaacaa ctggctacga cttcgaggac 1620
gcctgcgatg atgagacggc caacatcacc cttacatct ccaagacggt ttcttcagca 1680
gaatggaatg acttggaac tgcctcagtc tctaggaact cggccggcct tttcaaatgg 1740
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tgggtgtaca tgggtgttca aacaacgctc caggttccac acccaatcca tctgcacggt 1920
cacgatttct tcattctcgc ccaaggttca gggacatacg attcgtcgac agtaacattg 1980
aagacggata acccaccag acgcgacacg gccatgctgc catcgaggg atacttggtc 2040
atggcctggg agacggacaa ccctggcggt tggctgatgc actgtcacat cggttgcat 2100
acctcagagg gcttcgccct ccagttcatt gagcgaaga gcgagatcgc gtcgatcgtc 2160

```

roal2sequence listing.txt

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agtacgtcga ctatgaaaga tatttgcacc aagtgggagg agtttcagga ggaatattca 2220
attgaacaag aagactctgg tgtatgagat ggatgtgcgt tgaccatttt gcgtcaaaga 2280
catcctgcct gtcggttaact ctgatttgcc gtgtgaactt ggtttactag gtatttacag 2340
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atggaaccta gcacattagc aacagtttgc aaacgagtat cggcatgggc ctaagagtca 2520
tgattagggg gcagcgagg ttcgacttgt tgtttataca gttaatcgtg cgccccggg 2580
tacatattat gtaccctgct tacacatcca tatatactaa ggtgcttgag gtcgtgtgac 2640
cgctgttgag ctcctagcat tcattccagg gagcgtggca atgactggta tgtctgtggg 2700
cgcgggcgta gatggtgggg tgggagatat c 2731

```

```

<210> 45
<211> 573
<212> PRT
<213> Thielavia arenaria ALK04197

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```

<220>
<221> MISC_FEATURE
<222> (1)..(573)
<223> Deduced amino acid sequence of Thielavia arenaria ALK04197 TaLcc4

```

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<400> 45
```

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Met Ile Ser Arg Leu Leu Phe Thr Ser Ser Phe Thr Ala Thr Leu Ala
1 5 10 15
```

```
Ser Ala Leu Pro Gly Leu Leu Tyr Ala Pro His Ser Ser Ala Leu Leu
20 25 30
```

```
Ala Arg Ser Ser Cys Ser Gly Asn Thr Ala Ser Thr Arg Ser Gln Trp
35 40 45
```

```
Cys Asp Tyr Ser Ile Asp Thr Asp Tyr Thr Thr Glu Pro Val Asp Thr
50 55 60
```

```
Gly Val Thr Arg Glu Tyr Trp Leu Glu Leu Thr Asp Val Thr Val Ser
65 70 75 80
```

```
Pro Asp Gly Val Ser Arg Ser Ala Met Ala Val Asn Gly Ser Ile Pro
85 90 95
```

```
Gly Pro Thr Ile Phe Ala Asp Trp Gly Asp Thr Val Val Val His Val
100 105 110
```

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Thr Asn Ser Leu Ser Thr Ser Leu Asn Gly Thr Ser Ile His Trp His
115 120 125
```

```
Gly Ile Arg Gln Asn Tyr Thr Asn Gln Asn Asp Gly Val Ala Ser Ile
130 135 140
```

```
Thr Gln Cys Pro Leu Ala Val Gly Glu Ser Thr Thr Tyr Thr Trp Lys
145 150 155 160
```

```
Ala Thr Gln Tyr Gly Ser Ser Trp Tyr His Ser His Phe Ser Leu Gln
165 170 175
```

```
Ala Trp Glu Gly Val Phe Gly Gly Ile Ile Ile Asn Gly Pro Ser Thr
180 185 190
```

```
Ala Asn Tyr Asp Glu Asp Leu Gly Met Leu Phe Leu Asn Asp Trp Asp
195 200
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roal2sequence listing.txt

His Gln Thr Val Asp Glu Leu Tyr Ser Ser Ala Glu Thr Ser Gly Pro
 210 215 220
 Pro Thr Leu Ala Asn Gly Leu Ile Asn Gly Thr Asn Val Tyr Gly Glu
 225 230 235 240
 Asp Gly Asp Ser Ser Gln Thr Gly Thr Arg Leu Ala Val Ser Phe Thr
 245 250 255
 Ser Gly Thr Ser Tyr Arg Met Arg Leu Val Asn Ala Ala Val Asp Thr
 260 265 270
 His Trp Lys Phe Ser Ile Gly Asn His Thr Met Thr Val Met Ala Ala
 275 280 285
 Asp Leu Val Pro Ile Glu Pro Tyr Glu Thr Thr Val Leu Thr Ile Gly
 290 295 300
 Met Gly Gln Arg Tyr Asp Ile Val Val Thr Ala Asp Gln Ala Asp Val
 305 310 315 320
 Ala Asp Asn Phe Trp Met Arg Ala Ile Pro Gln Ser Ala Cys Ser Asp
 325 330 335
 Asn Asp Ser Ala Asp Asn Ile Arg Gly Ile Val Tyr Tyr Gly Asp Ser
 340 345 350
 Pro Gly Thr Pro Ser Thr Thr Gly Tyr Asp Phe Glu Asp Ala Cys Asp
 355 360 365
 Asp Glu Thr Ala Asn Ile Thr Pro Tyr Ile Ser Lys Thr Val Ser Ser
 370 375 380
 Ala Glu Trp Asn Asp Leu Glu Thr Ala Ser Val Ser Arg Asn Ser Ala
 385 390 395 400
 Gly Leu Phe Lys Trp Tyr Leu Asn Ser Thr Thr Met Leu Val Asp Trp
 405 410 415
 Ala Lys Pro Thr Leu Glu Met Val Thr Asp Asn Val Thr Glu Tyr Asp
 420 425 430
 Ser Asp Asp Ala Ile Ile Glu Leu Asn Glu Ala Asn Gln Trp Val Tyr
 435 440 445
 Met Val Val Gln Thr Thr Leu Gln Val Pro His Pro Ile His Leu His
 450 455 460
 Gly His Asp Phe Phe Ile Leu Ala Gln Gly Ser Gly Thr Tyr Asp Ser
 465 470 475 480
 Ser Thr Val Thr Leu Lys Thr Asp Asn Pro Pro Arg Arg Asp Thr Ala
 485 490 495
 Met Leu Pro Ser Gln Gly Tyr Leu Val Met Ala Trp Glu Thr Asp Asn
 500 505 510
 Pro Gly Val Trp Leu Met His Cys His Ile Gly Trp His Thr Ser Glu
 515 520 525
 Gly Phe Ala Leu Gln Phe Ile Glu Arg Lys Ser Glu Ile Ala Ser Ile
 530 535 540
 Val Ser Thr Ser Thr Met Lys Asp Ile Cys Thr Lys Trp Glu Glu Phe

545 roal2sequence listing.txt 555 560

Gln Glu Glu Tyr Ser Ile Glu Gln Glu Asp Ser Gly Val
565 570

<210> 46
<211> 2954
<212> DNA
<213> Chaetomium thermophilum ALK04265

<220>
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<222> (1)..(2954)
<223> Nucleotide sequence of Chaetomim thermophilum ALKK04265 Ctlcc1 gene

<220>
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<223> Nucleotide sequence of Chaetomium thermophilum ALK04265 Ctlcc1 gene

<220>
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<222> (371)..(2497)
<223> Ctlcc1

<220>
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<222> (581)..(630)

<220>
<221> Intron
<222> (710)..(762)

<220>
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<222> (775)..(824)

<220>
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<220>
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<222> (1992)..(2086)

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ggaactaaga cggcatcacc gtcgatatat tggataggat ccgagttttc gcgatctttg 180
ttgccttcac tgaggagaag gtgcactctg ggtgttgagt ctgtcgtcta tataacgacc 240
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actggcacgg cattcaccag aagggcagta accttcacga cggtgccaac ggcgttactg 1020
aatgccccat cctcccaac ggtggccagc gcacctatcg cttccgtgcc cagcagtatg 1080

roal2sequence listing.txt

```

gcaccagctg gtaccactct cactttctctg ctcatgtatgg caatggcatc gtcgggtccca 1140
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<210> 47
<211> 607
<212> PRT
<213> Chaetomium thermophilum ALK04265

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<220>
<221> MISC_FEATURE
<222> (1)..(607)
<223> Deduced amino acid sequence of Chaetomium thermophilum ALK04265
      CtlLcc1

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<400> 47
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Val Val Gly Ala Ile Pro Thr Arg Ser Gly Thr Lys Lys Lys Tyr Ile
      20             25             30
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roal2sequence listing.txt

Arg Asp Gly Pro Gly Pro Cys His Thr Pro Ser Asn Arg Ala Cys Trp
 35 40 45
 Ala Pro Gly Phe Asp Ile Asn Thr Asp Tyr Glu Val Asn Thr Pro Asn
 50 55 60
 Thr Gly Val Thr Arg Asn Tyr Thr Leu Thr Leu Thr Glu Glu Asp Asn
 65 70 75 80
 Trp Thr Gly Pro Asp Gly Val Val Lys Glu Lys Ile Met Leu Val Asn
 85 90 95
 Gly Lys Thr Leu Gly Pro Thr Ile Glu Ala Asn Trp Gly Asp Trp Ile
 100 105 110
 Glu Val Lys Val Ile Asn Asn Leu Leu Thr Asn Gly Thr Ser Ile His
 115 120 125
 Trp His Gly Ile His Gln Lys Gly Ser Asn Leu His Asp Gly Ala Asn
 130 135 140
 Gly Val Thr Glu Cys Pro Ile Pro Pro Asn Gly Gly Gln Arg Thr Tyr
 145 150 155 160
 Arg Phe Arg Ala Gln Gln Tyr Gly Thr Ser Trp Tyr His Ser His Phe
 165 170 175
 Ser Ala Gln Tyr Gly Asn Gly Ile Val Gly Pro Ile Val Ile His Gly
 180 185 190
 Pro Ala Ser Leu Pro Tyr Asp Ile Asp Leu Gly Pro Phe Pro Leu Thr
 195 200 205
 Asp Tyr Tyr Tyr Lys Ser Ala Asp Glu Leu Val Arg His Thr Gln Asn
 210 215 220
 Asn Gly Pro Pro Phe Ser Asp Asn Val Leu Phe Asn Gly Thr Gly Val
 225 230 235 240
 His Pro Gln Thr Gly His Gly Gln Tyr Ala Lys Val Thr Leu Thr Pro
 245 250 255
 Gly Lys Arg His Arg Leu Arg Ile Ile Asn Met Ser Thr Glu Asn His
 260 265 270
 Phe Gln Val Ser Leu Val Gly His Gln Phe Thr Val Ile Ala Ala Asp
 275 280 285
 Met Val Pro Val His Ser Tyr Thr Thr Asp Ser Leu Phe Leu Ala Val
 290 295 300
 Gly Gln Arg Tyr Asp Val Thr Ile Asp Ala Ser Gln Thr Pro Gly Asn
 305 310 315 320
 Tyr Trp Phe Asn Val Thr Phe Gly Gly Gly Phe Ala Cys Gly Gly Ser
 325 330 335
 Phe Asn Pro Asn Pro Ala Ala Ile Phe His Tyr Glu Gly Ala Pro Asp
 340 345 350
 Ala Leu Pro Thr Asp Pro Gly Val Pro Pro Arg Asp His Asn Cys Leu
 355 360 365

roal2sequence listing.txt

Asp Thr Leu Asp Leu Val Pro Val Val Pro Arg Asn Val Gln Val Asn
 370 375 380

Gln Phe Val Lys Lys Pro Glu Asn Thr Leu Pro Val Glu Leu Ser Leu
 385 390 395 400

Gly Gly Thr Pro Leu Phe Val Trp Lys Val Asn Gly Ser Ala Ile Asp
 405 410 415

Val Asp Trp Gly Asn Pro Val Leu Gln Tyr Val Met Asp Arg Asn Thr
 420 425 430

Ser Tyr Arg Gln Ala Asp Asn Ile Val Glu Val Asn Gly Val Asn Gln
 435 440 445

Trp Thr Tyr Trp Leu Ile Glu Asn Asp Pro Asn Gly Ala Phe Ser Leu
 450 455 460

Pro His Pro Met His Leu His Gly His Asp Phe Leu Ile Val Gly Arg
 465 470 475 480

Ser Pro Asp Val Pro Pro Gly Ser Asn Gln Arg Tyr Asn Phe Asp Pro
 485 490 495

Ala Thr Asp Ile Tyr Arg Leu Arg Gly Gln Asn Pro Thr Arg Arg Asp
 500 505 510

Val Ala Met Leu Pro Ala Gly Gly Trp Leu Leu Leu Ala Phe Leu Thr
 515 520 525

Asp Asn Pro Gly Ala Trp Leu Phe His Cys His Ile Ala Trp His Val
 530 535 540

Ser Gly Gly Leu Ser Val Asp Phe Leu Glu Arg Pro Asn Asp Leu Arg
 545 550 555 560

Asn Ser Ile Leu Gln His Asp Lys Asp Glu Phe Asn Arg Val Cys Asn
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Glu Trp Arg Thr Tyr Trp Pro Asn Ser Pro His Pro Lys Ile Asp Ser
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Gly Leu Lys His Arg Trp Val Glu Glu Ser Glu Trp Leu Val Arg
 595 600 605

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 <211> 2516
 <212> DNA
 <213> Chaetomium thermophilum ALK04265

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 <222> (1)..(2516)
 <223> Nucleotide sequence of Chaetomium thermophilum ALK04265 Ctlcc2 gene

<220>
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 <222> (246)..(2231)
 <223> Ctlcc2

<220>
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 <222> (483)..(531)

<220>
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<220>

roal2sequence listing.txt

<221> Intron

<222> (751)..(829)

<400> 48

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roal2sequence listing.txt
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<210> 49
 <211> 598
 <212> PRT
 <213> Chaetomium thermophilum ALK04265

<220>
 <221> MISC_FEATURE
 <222> (1)..(598)
 <223> Deduced amino acid sequence of Chaetomium thermophilum ALK04265
 CtLcc2

<400> 49

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Leu Leu Ala Arg Gln Thr Ile Thr Pro Gly Gly Gln Pro Cys Gly Gln
 35 40 45

His Gly Pro Glu Asn Arg Leu Cys Trp Arg Asn Leu Trp Asn Ile Ser
 50 55 60

Thr Asp Pro Asp Val Ser Ile Pro Pro Ala Tyr Asn Asn Arg Tyr Tyr
 65 70 75 80

Asp Leu His Ile Thr Asn Glu Thr Asn Trp Leu Gly Pro Asp Gly Val
 85 90 95

Arg Lys His Ala Met Leu Ile Asn Asn Gln Phe Pro Gly Pro Thr Ile
 100 105 110

Glu Ala Glu Trp Gly Asp Tyr Ile Val Val Asn Val Tyr Asn Asp Leu
 115 120 125

Glu Asp Asn Gly Thr Ser Ile His Trp His Gly Ile Arg Gln Phe Gly
 130 135 140

Glu Ser Asn Gln Asp Gly Thr Asn Gly Val Thr Glu Cys Pro Ile Pro
 145 150 155 160

Pro Gly His Met Lys Thr Tyr Ser Phe His Val Thr Gln Tyr Gly Thr
 165 170 175

Ser Trp Tyr His Ser His Phe Ser Asn Gln Tyr Gly Asn Gly Val Leu
 180 185 190

Gly Ala Leu Val Val Lys Gly Pro Ala Ser Ala Asn Tyr Asp Ile Asp
 195 200 205

Leu Gly Pro Tyr Ile Ile Ser Asp Tyr Tyr His Glu Thr Ala Asp Arg
 210 215 220

Leu His Leu Gln Ala Glu Leu Leu Arg Asn Gly Pro Pro Pro Asp Ser
 225 230 235 240

Asp Asn Ile Leu Phe Arg Gly Lys Asn Ile Asn Pro Asp Gly Ser Gly
 245 250 255

Arg Gly Ser Tyr Asp Arg Leu Thr Leu Ile Pro Gly Lys Lys His Leu
 260 265 270

Leu Arg Leu Ile Asn Ala Ser Val Asp Asn Ser Phe Thr Val Ser Leu

<210>	50
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roal2sequence listing.txt

<213> Chaetomium thermophilum ALK04265

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 <221> (1)..(3025)
 <223> Nucleotide sequence of Chaetomim thermophilum ALK04265

<220> Intron
 <221> (711)..(768)
 <223> Intron 1

<220> Intron
 <221> (1326)..(1390)
 <223> Intron 2

<220> Intron
 <221> (2187)..(2255)
 <223> Intron 3

<400> 50
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roal2sequence listing.txt

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Gln Ala Leu Ser Thr Val Val Glu Lys Val Thr Ser Ala Leu Ser Gln
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Leu Asp Thr Asn Gly Tyr Ser Ile Trp Gly Thr Leu Leu Ala Ser Ser
 35 40 45

Leu Ala Pro Phe Leu Thr Asp Asn Pro Leu Pro Asp Gly Tyr Pro Trp
 50 55 60

Gly Asn Leu Thr Asp Tyr Gly Asn Asn Pro Tyr Arg Glu Cys Pro His
 65 70 75 80

Thr Gly Ile Thr Arg Ser Tyr His Phe Thr Ile Ser Arg Gly Val Ile
 85 90 95

Ala Pro Asp Gly Tyr Glu Arg Glu Val Leu Leu Val Asn Gly Ala Phe
 100 105 110

Pro Gly Pro Leu Ile Glu Ala Asn Trp Gly Asp Thr Ile Ile Val Lys
 115 120 125

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Val Phe Asn Asn Ile Ser Asn Pro Glu Glu Gly Thr Ser Ile His Trp
 130 135 140
 His Gly Phe Leu Gln His His Thr Pro Trp Glu Asp Gly Thr Pro Gly
 145 150 155 160
 Ile Thr Gln Cys Pro Ile Pro Ser Gly Lys Ala Tyr Thr Tyr Lys Phe
 165 170 175
 Asn Ala Ser Leu Tyr Gly Thr Thr Trp Tyr His Ala His Tyr Ser Ala
 180 185 190
 Gln Tyr Ala Gly Gly Ile Val Gly Pro Ile Val Ile His Gly Pro Thr
 195 200 205
 Lys Glu Gly Tyr Asp Ile Asp Val Gly Pro Val Met Leu Gly Asp Trp
 210 215 220
 Tyr His Gln Glu Tyr Tyr Asn Ile Val Lys Thr Met Leu Ser Pro Ser
 225 230 235 240
 Glu Ser Pro Leu Arg Val Tyr Ser Asp Asn Asn Leu Ile Asn Gly Lys
 245 250 255
 Met Asp Phe Asn Cys Ser Thr Val Ser Glu Asp Asp Pro His Arg Cys
 260 265 270
 Thr Pro Asn Ala Gly Ile Ser Lys Phe Arg Phe Gln Ala Gly Gln Val
 275 280 285
 His Arg Leu Arg Leu Ile Asn Leu Gly Gly Asp Gly Ile Gln Arg Phe
 290 295 300
 Ser Ile Asp Glu His Val Leu Thr Val Ile Ala Glu Asp Phe Val Pro
 305 310 315 320
 Val Lys Pro Tyr Asn Thr Thr Val Val Val Leu Gly Val Gly Gln Arg
 325 330 335
 Ala Asp Val Leu Val Thr Ala Asn Ala Gly Gly Pro Lys Ser Thr Phe
 340 345 350
 Trp Met Arg Ser Ser Leu Thr Thr Cys Ser Pro Ala Arg Gln Pro Asn
 355 360 365
 Ala Val Ala Val Val Leu Tyr Asp Glu Ala Asp Glu Asn Ala Val Pro
 370 375 380
 Asn Ser Lys Pro Trp Glu Ile Pro Asn Pro Asp Val Cys Ala Asn Leu
 385 390 395 400
 Pro Leu Glu Ile Thr Glu Pro Leu Tyr Pro Ile Pro Leu Pro Glu Pro
 405 410 415
 Thr Phe Thr Glu Arg Met Glu Ile Glu Ile Phe Lys Asn Glu Ser Lys
 420 425 430
 Ile Trp Leu Trp Lys Phe Asn Asp Ile Ser Met Arg Thr His Tyr Asn
 435 440 445
 Lys Pro Val Leu Leu Leu Ala Asn Gln Gly Glu Tyr Asp Tyr Pro Glu
 450 455 460
 Glu Trp Asn Val Val Asn Tyr Tyr Gln Asn Glu Ser Val Arg Ile Val

roal2sequence listing.txt
475 480