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PCR CLONING AND HETEROLOGOUS EXPRESSION OF SCYTALIDIUM THERMOPHILUM LACCASE GENE IN ASPERGILLUS SOJAE

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Approval of the Graduate School of Natural and Applied Sciences

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I certify that this thesis satisfies all the requirements as a thesis for the degree of Master of Science.

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This is to certify that we have read this thesis and that in our opinion it is fully adequate, in scope and quality, as a thesis for the degree of Master of Science.

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ABSTRACT

PCR CLONING AND HETEROLOGOUS EXPRESSION OF *SCYTALIDIUM THERMOPHILUM* LACCASE GENE IN *ASPERGILLUS SOJAE*

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In this study, *Scytalidium thermophilum* laccase gene was first cloned into *E. coli* and then heterologously expressed in *A. sojae. S. thermophilum* is a thermophilic fungus with an important role in determining selectivity of compost produced for growing *Agaricus bisporus*. *S. thermophilum* laccase gene was first cloned by Novo Nordisk Bio Tech, Inc. in 1998. This laccase gene *(lccS*) has an open reading frame of 2092bp. It is composed of five exons punctuated by four small introns. The coding region, excluding intervening sequences is very GC-rich (60.8% G+C) and encodes a preproenzyme of 616 amino acids: a 21 amino acid signal peptide and a 24 amino acid predicted propeptide. *lccS* gene was amplified using specific primers to exclude the signal and pro-peptide coding regions and ligated to expression vector pAN52-4. The recombinant plasmid was used to transform *Aspergillus sojae* ATCC11906 ($pyrG^-$). Heterologuos expression was observed in glucose-containing media, under

the control of the glyceraldehydes 3-phosphate dehydnogenese promoter and the secretion signal of glucoamylase gene. Laccase gene is an important step towards the high level expression of this enzyme in a GRAS eucaryotic host and for further biotransformation and enzyme engineering studies. In this study also bioinformatic analysis of N-terminal and C-terminal propeptide cleavage sites of fungal proteins including laccases were studied.

Keywords: laccase, gene cloning, *Scytalidium thermophilum*, propeptide

ÖZ

SCYTALIDIUM THERMOPHILUM LAKKAZ GENİNİN PCR TEKNİĞİ İLE KLONLANMASI VE *ASPERGILLUS SOJAE'DE* HETEROLOG EKSPRESYONU

Koçlar, Gülden
Yüksek Lisans, Biyoteknoloji Bölümü
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Bu çalışmada, *Scytalidium thermophilum* lakkaz geni önce *E. coli*'ye klonlandı ve daha sonra *A.sojae*'de heterolog üretimi gerçekleştirildi. *S. thermophilum* sıcaklığa dayanıklı bir küf olup, *Agaricus bisporus* organizmasının büyümesi için üretilen gübrenin seçiciliğinin belirlenmesinde önemli rol oynamaktadır. *Scytalidium thermophilum* lakkaz geni ilk olarak 1998 yılında Novo Nordisk Bio. Tech. tarafından klonlanmıştır. Bu gen (*lccS*) okuma çerçevesinde 2092 baz çiftinden oluşmaktadır. Gen, dört kısa intronun böldüğü beş ekzondan oluşmaktadır. Intronların dışında kalan kodlanan bölge sekansı G-C'lerce zengin olup (60.8% G+C), 21 amino asiti signal peptit ve 24 amino asiti propeptit olmak üzere 616 amino asitlik bir preproenzim sentezlemektedir. *lccS* geni, signal peptit ve pro-peptit kodlayan bölgeleri dışarıda bırakacak özgül primerle çoğaltılmış, pAN52-4 ekspresyon vektörüne takılmış ve *Aspergillus sojae* ATCC11906 ($pyrG^-$)

organizmasına klonlanmıştır. Glukoamilaz geninin signal peptit bölgesi altına takılan genin, gliseraldehit 3-fosfat dehidrojenaz promotorı kontrolünde, glukoz içeren ortamda heterolog ekpresyonu gözlenmiştir. Lakkaz geninin heterolog üretimi, enzimin ökaryotik GRAS bir organizmada yüksek düzeyde ekspresyonunu sağlamak ve daha sonraki biotransformasyon ve enzim mühendisliği çalışmalarında kullanılması yönünde atılmış önemli bir adımdır. Bu çalışmada ayrıca lakazları da içeren küf proteinlerin N ve C uçlarındaki propeptit kesim bölgeleri incelenmiştir.

Anahtar Sözcükler: lakkaz, gen klonlama, *Scytalidium thermophilum*, propeptit

To My Family

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INTRODUCTION

1.1 Phenol Oxidase

The general terms, phenoloxidase, phenolase or polyphenol oxidases are used to describe enzymes which catalyse the oxidation of aromatic compounds by molecular oxygen which is also substrate for phenol oxidases. Since a wide range of substrates are used by these enzymes, there is confusion with respect to terminology and identification of the substrates of particular enzymes (Griffith GW., 1994).

Phenol Oxidases represent a major group of enzymes involved in secondary metabolic activity, most commonly being associated with the production of melanins and other pigments.

1.2 Laccases

Laccases, (E.C. 1.10.3.2, *p*-benzenediol: oxygen oxidoreductase) are multi-copper enzymes, which catalyze the oxidation of a variety of organic and inorganic substrates coupled to the reduction of molecular oxygen to water with the one-electron oxidation mechanism (Figure 1.1). Laccase catalyze the oxidation of a broad range of substrates e.g. polyphenols, substituted phenols, diamines, but also some inorganic compounds (Thurson, 1994).

These enzymes are widely distributed in nature. Laccase or laccase-like activity has been demonstrated in higher plants; in some insects and sequence homology analyses suggest that laccases could also occur in bacteria such as *Mycobacterium tuberculosis* (Alexandre et al., 2000). However, the best-known

laccases are of fungal origin and have been reported from ascomycete, basidiomycete and deuteromycete fungi (Mayer 1987; Bollag and Leonowicz 1984).

Fungal laccases, being part of the lignin-degrading enzyme system, are present in most of the wood-rotting fungi. In addition, the fungal laccases are important in *regular* processes like pigment formation *(Aramayo et al., 1990; Cardenas et al*., 1981, Clutterbuck, 1972, 1990), spore formation (Kurtz *et al.,* 1981,1982), and plant pathogenesis (Choi *et al*., 1992; Leatham and Stahmann, 1981) In plants, laccases play a role in lignin polymerization (Ranocha *et al.,* 1999).

Both plant and fungal laccases are glycosylated enzymes with the degree of glycosylation varying between 22% and 45% (Kumar *et al*., 2003).

1.2.1 Structure of Laccase

1.2.1.1 The Copper Active Center of Laccase

Blue copper oxidases contain at least one type-1 (T1) copper, which is presumably the primary oxidation site. Blue multicopper oxidases typically employ at least three additional coppers: one type-2 (T2) and two type-3 (T3) coppers arranged in a trinuclear cluster (Piontek *et al*., 2002).

In general, laccases have four copper atoms in different binding sites, which play important role in the enzyme catalytic mechanism. Type I copper confers the typical blue color to multicopper absorption caused by the covalent copper-cysteine bond. Due to its high redox potential of ca. +790mV, type 1 copper is the site where substrate oxidation takes place. Type 2 copper shows no absorption in the visible spectrum and reveals paramagnetic properties in EPR studies. It is strategically positioned close to the type 3 copper, a binuclear center spectroscopically characterized by a electron absorption at 330 nm and by the absence of an EPR signal as the result of the anti-ferromagnetic coupling of the copper pair. The type 3 copper center is also common feature of another protein superfamily including the tyrosinases and haemocyanins (Decker and Terwilliger, 2000). Type 2 and type3 copper form a trinuclear cluster, where reduction of molecular oxygen and release of water takes place. Type2 copper is coordinated by two and type 3 copper atoms by

six histidines. The strong anti-ferromagnetical coupling, between the type 3 copper atoms is maintained by a hydroxyl bridge (Figure 1.2, 1.3) (Claus, 2003).

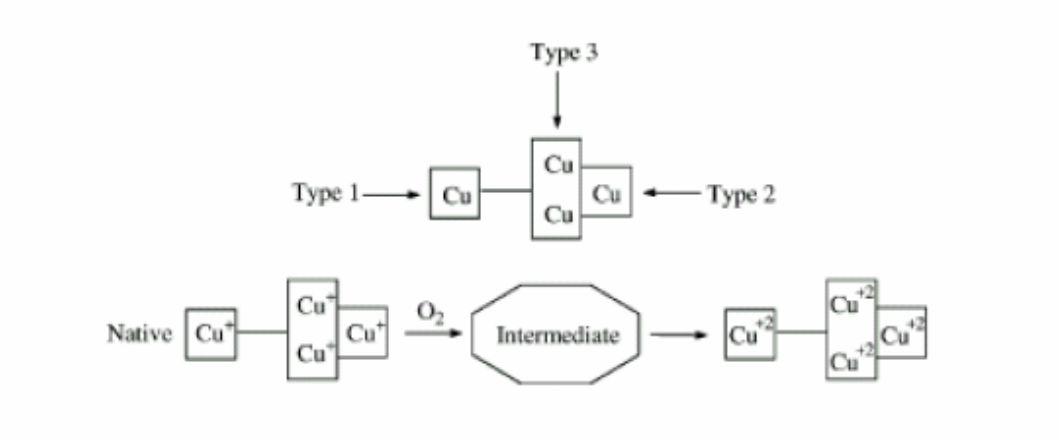

Figure 1.1 Reactivity of laccase derivatives with oxygen (Duran *et al*. 2002)

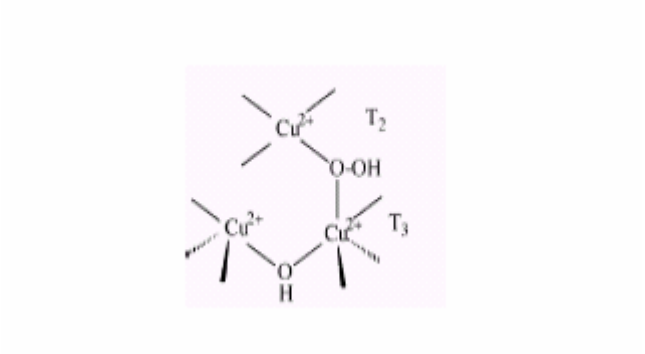

Figure 1.2 Bridging between Type 2 and one of Type 3 copper (Duran *et al*. 2002)

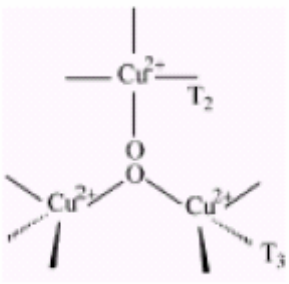

Figure 1.3 Bridging between all three copper (Duran *et al*. 2002)

Multiple sequence alignments of more than 100 laccases resulted in identification of four ungapped sequence regions, L1-L4, as the overall signature of laccases, distinguishing them within the broader class of multicopper oxidases (Kumar *et al.,* 2003). The 12 amino acid residues in the enzymes serving as the copper ligands are housed within these conserved regions (Figure 1.4). The amino acid ligands of the trinuclear cluster are the eight histidines, which occur in a highly conserved pattern of four HXH motifs. In one of these motifs, X is the cysteine bound to the T1 copper while each of the histidines is bound to one of the two type 3 coppers (Figure 1.5). Intraprotein homologies between signatures L1 and L3 between L2 and L4 suggest the occurrence of duplication events (Claus, 2003).

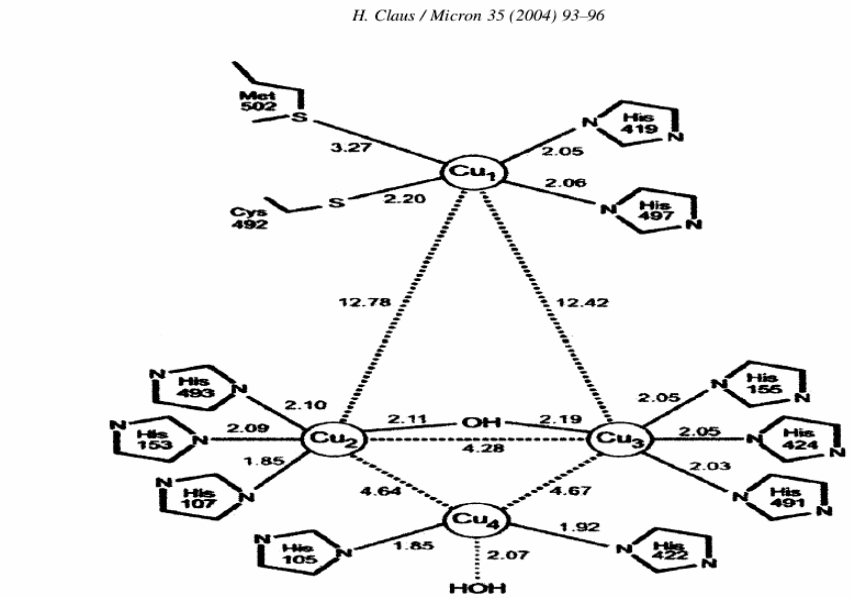

Figure 1.4 Copper centers of the laccase from *B. subtilitis* (adapted from Enguita *et al.,* 2003).

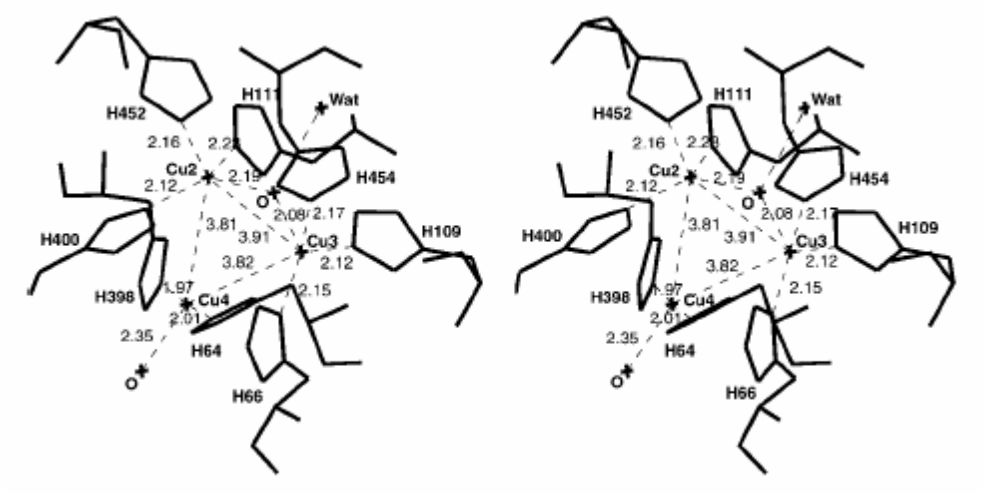

Figure 1.5 Stereo view of the T2/T3 coppers and their close environment in laccase of *Trametes versicolor*. Bonds are represented by thin, dashed lines, and lengths are given in Å (Piontek *et al*., 2002).

1.2.1.2 Three Dimensional Structure of Laccase

The first crystal structure of an active laccase containing a full complement of coppers from *Trametes versicolor* is shown in Figure 1.6. Domain 1 comprises two four-stranded β-sheets and four 3_{10}-helices. Three of the 3_{10}-helices are in connecting peptides between the β-strands, and one is in a segment between domain 1 and 2. The second domain has one six-stranded and one five stranded β-sheet, and like in domain 1, there are three 3_{10}-helixes in peptides connecting individual β-strands and domains 1 and 3, respectively. Finally, domain 3 consists of a β-barrel formed by two five-stranded β-sheets and a two-stranded β-sheet that, together with an α-helix and a β-turn, form the cavity in which the type-1 copper is located. The tri-nuclear copper cluster (T2/T3) is embedded between domains 1 and 3 with both domains providing residues for the coordination of the coppers. Finally, at the C-terminal end of domain 3, three sequentially arranged α -helixes complete the fold. A 13-aminoacid- long α -helix at the C-terminal portion is stabilized by a disulfide bridge to domain 1 (Cys-

85–Cys-488), and a second disulfide bridge (Cys-117–Cys-205) connects domains 1 and 2. Both N-terminal and C-terminal amino acids benefit from hydrogen bonding networks to the rest of the protein, providing sufficient rigidity so that excellent electron density can be observed for these regions in the crystal structure.

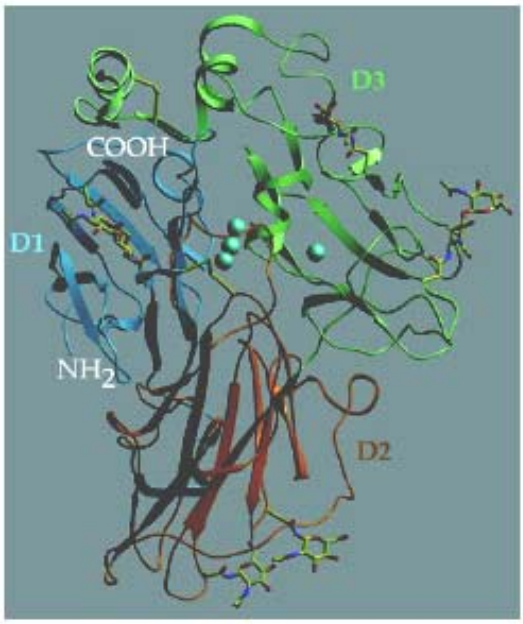

Figure 1.6 The 3-D structure and the active site of laccase from *Trametes versicolor*. The arrangement of the domain structure is depicted in different color-coding (D_1-D_3). Copper ions are drawn as blue spheres.

1.2.2 Substrates and Reaction Mechanisms of Laccase

The various copper centers of laccases drive electrons from a reducing substrate to molecular oxygen without releasing toxic peroxide intermediates. This is accomplished by four-monoelectronic oxidation of the substrate catalyzed by the type 1 copper. The electrons are further transferred to the trinuclear cluster, where reduction of molecular oxygen and release of water takes place. The oxidation of substrates creates reactive radicals that can undergo non-enzymatic reactions:

Cross-linking of monomers: The enzymatic oxidation of phenolic compounds and anilines by laccases generates radicals that react with each other to form dimers, oligomers or polymers covalently coupled by C-C, C-O and C-N bonds (Filip and Claus, 1995; Duran and Esposito, 2000).

Degradation of Polymers: Laccases are involved in the degradation of complex natural polymers, such as lignin or humic acids (Claus and Filip, 1998). The reactive radicals generated, lead to the cleavage of covalent bonds and to the release of monomers.

Ring cleavage of aromatics: In several cases a laccase-catalyzed ring-cleavage of aromatic compounds has been reported. This action is of biotechnological interest in view of the degradation of xenobiotics like nitroaromatics and synthetic dyes (Duran and Esposito, 2000; Claus et *et al*., 2002)

1.2.3 Fungal Laccases in Melanin Biosynthesis

For more than 40 years fungi have been known to produce pigments known as melanins. Melanins are a large group of diverse substances having some common properties (reviewed in Bell and Wheeler, 1986; Butler and Day, 1998; Butler *et al.,* 2001; Henson *et al.,* 1999; Jacobson, 2000; Wheeler and Bell, 1988).

In general, melanins are macromolecules formed by the oxidative polymerization of phenolic or indolic compounds. Often the resulting pigments are brown or black in color but many other colors have been observed. Melanins are also hydrophobic or negatively charged.

In fungi several different types of melanin have been identified to date. The two most important types are DHN melanin (named for one of the pathway intermediates, 1,8-dihydroxynaphthalene) (Figure 1.7) and DOPA-melanin (named for one of the precursors, L-3,4-dihydroxyphenylalanine). Both types of melanin have been implicated in pathogenesis (Hamilton and Gomez, 2002; Jacobson, 2000; Kwon-Chung *et al.,* 1982; Perfect *et al.,* 1998; Wheeler and Bell, 1988).

A number of possible functions have been postulated for fungal melanins in general, based on the properties of melanin. However, it is difficult to know which of these (or other) properties of fungal melanins are of survival value without extensive

knowledge of the ecology of the individual fungus (Butler and Day, 1998). The proposed functions of fungal melanins include protection against UV irradiation, enzymatic lysis, oxidants, and in some instances extremes of temperatures. Also, melanins have been shown to bind metals, function as a physiological redox buffer, thereby possibly acting as a sink for harmful unpaired electrons, provide structural rigidity to cell walls, and store water and ions, thus helping to prevent desiccation (reviewed in Butler and Day, 1998; Jacobson, 2000).

In L-3,4-Dihydroxyphenylalanine-melanin biosynthesis phenoloxidases form a second major group of enzymes commonly associated with the production of melanins. They fall into two subgroups, laccases (EC 1.10.3.2) and catechol oxidases (EC 1.14.18.1), more commonly known as tyrosinases. Both laccases and tyrosinases have copper ligands and require bound copper ions for activity, but they differ in their substrate specificities and overall structure. In general, laccases catalyse the one-step oxidation of dihydroxyphenols to quinones while tyrosinases catalyse a two-step oxidation of tyrosine.

The polymerization of 1,8-DHN is thought to be catalyzed by a laccase enzyme (see Table 1). Reactions 7 and 8 generate shunt products that can be identifed by thin layer chromatography (TLC) when specific steps in the pathway are inhibited (e.g., by adding tricyclazole, a reductase inhibitor). CJ-12,371 is the name given to a molecule which exhibits strong bacteriocidal effects on Gram-positive bacteria (Sakemi et al., 1995). This and other intermediate products could result from the DHN-melanin pathway.

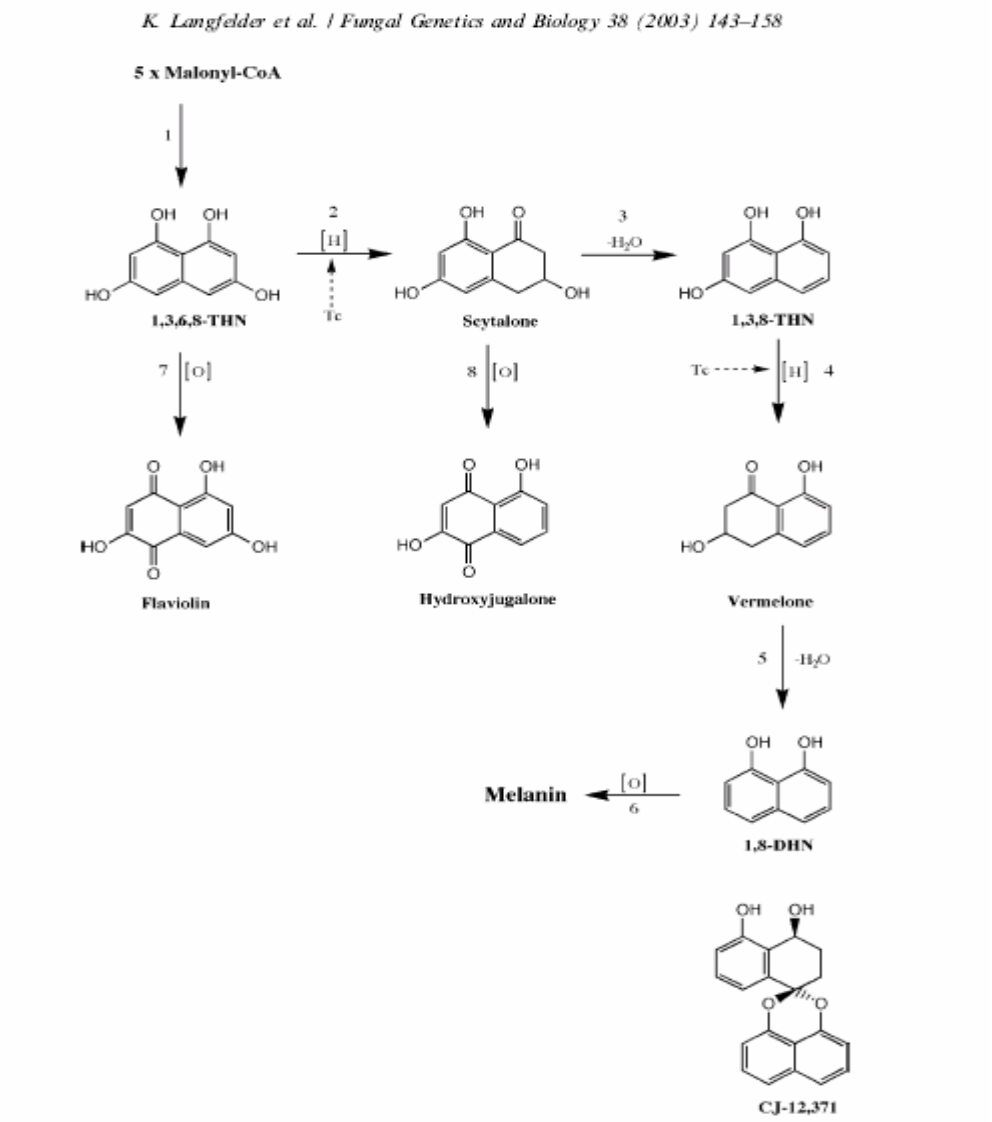

Figure 1.7 Fungal DHN-melanin biosynthetic pathway (Langfelder *et al*., 2002) Fungal dihydroxynaphthalene (DHN)-melanin biosynthesis pathway is shown. (adapted from Butler and Day, 1998; Tsai *et al*., 1999; Wheeler and Bell, 1988). Probable reaction types are indicated ([O], oxidation; [H], reduction; –H2O, dehydration). Step 1 is catalyzed by a polyketide synthase, steps 2 and 4 by a reductase and steps 3 and 5 by a dehydratase enzyme.

Fungal DOPA-melanin biosynthetic pathway is shown in Figure 1.8 Essentially, biosynthesis pathway for fungal DOPA-melanin strongly resembles the pathway found in mammalian cells, though some of the details may differ. In brief,

tyrosinase or laccase catalyses the hydroxylation of L-tyrosine to dopaquinone, or the L-DOPA oxidation to dopaquinone. For both these reactions L-DOPA is an essential co-factor (Pomerantz and Warner, 1967). Dopaquinone is a highly reactive intermediate. Reactions 1–3 can be catalyzed by tyrosinase enzyme, allele of which is oxidation reactions. In reaction 4 cyclysation of dopaquinone results in leucodopachrome which is oxidized by reaction with dopaquin one to produce dopachrome. Reaction 6 is an aromatisation reaction (also called tautomerisation). Simultaneous oxidation and polymerization are thought to produce DOPA-melanin.

Little is known about the localization of melanin in human pathogenic fungi. In general, some fungal melanin is found as part of the cell wall, often recognizable as a distinct and fairly sharply defined outside layer, and some melanin is found in association with the fibrillar matrix, which extends out from the cell wall of many fungi. These types of melanin are referred to as cell wall bound melanin. They are either enmeshed within the structure of the cell wall, or are incorporated as its outermost layer. By contrast, in humans melanin is located in specialized cells (the melanocytes) where the melanin is located internally in specialized vacuoles (the melanosomes) (reviewed in Butler and Day, 1998).

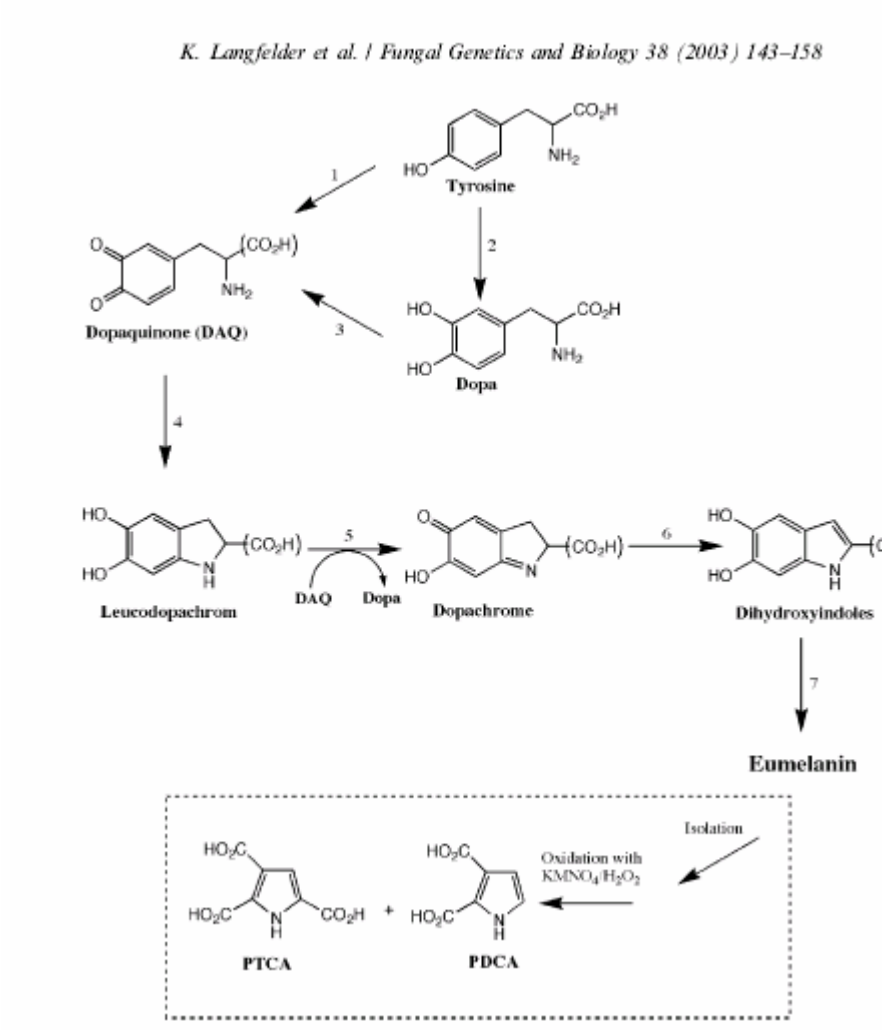

Figure 1.8 Dihydroxyphenylalanine (DOPA)-melanin biosynthetic pathway in fungi (Langfelder *et al*., 2002)

1.2.4 Application Areas of Laccase

Laccase is an important enzyme because of its potential use in several areas such as textile, paper and pulp industries. Laccase can be used in bioremediation, beverage (wine, fruit juice and beer) processing, ascorbic acid determination, baking, and as biosensor.

1- Bioremediation is a general concept that includes all those processes and action that take place in order to biotransform an environment, already altered by contaminants, to its original status (Thassitou and Arvanitoyannis, 2001). Aromatic compounds, including phenols and aromatic mines, constitute one of the major classes of pollutants (Karam and Nicell, 1997). Laccase is a well-known enzyme studied in bioremediation because its ability to degrade phenolic compounds (Duran and Esposito, 2000; Gianfreda *et al*., 1999).

2- In pulp and paper industry, removal of lignin from woody tissues is an important process. One of the approaches to delignification of wood fibers for preparation of pulp has been the use of laccases for this purpose (Argyropoulos, 2001).

3- In beverage industry, hazes or sediments in beer, wines and fruit juice results from a number of different causes, but the most frequent is protein-polyphenol interaction (Siebert, 1999). These beverages are typically stabilized to delay the onset of protein-polyphenol haze formation. Laccase treatments have been proposed for beverage stabilization by removing undesired phenolic compounds (Cantarelli and Giovanelli, 1990; Plank and Zent, 1993).

4- A biosensor is a device that detects, transmits and records information regarding a physiological or biochemical change. Technically, it is a probe that integrates a biological component with an electronic transducer thereby converting a biochemical signal into a quantifiable electrical response. The function of a biosensor depends on the biochemical specificity of the biologically active material (D' Souza, 2001). A number of biosensors containing laccase have been developed for immunoassays (Bauer *et al*., 1999; Ghindilis, 2000; Ghindilis, Makower, and Scheller, 1995; Huang *et al.,* 1999; Scheller, Wollerberger and Makower, 1994), glucose determination (Scheller *et al*., 1986; Wollenberger *et al.,* 1986), aromatic amines (Simkus and Laurinavicius, 1995) and phenolic compound determinations (Freire, Duran and Kubota, 2001; Yaropolov *et al*., 1995; Zouari *et al*., 1994).

Laccase has great potential application in several areas. The use of this enzyme could improve productivity, efficiency, and quality of food products without high investment cost and has the advantages of being a mild technology.

Table1.1 Industrial Applications of Laccase

Industry	Function	Reference
Pulp and paper industry	Removal of lignin from woody tissues	Duran *et al*., 2002
Ethanol production	Production of fuel ethanol from renewable raw materials	Duran *et al*., 2002
Drug analysis	To distinguish morphine from codeine	Duran *et al*., 2002
Wine industry	Removal of phenolic compound coming from grape	Croser, 2000
Fruit juice industry	Removal of phenolics and their oxidation products	Mayer *et al., 2002*
Beer industry	Removal of excess phenolic products	Mayer *et al., 2002*
Waste water treatment	Removal of toxic phenolic compounds	Atlow *et al*., 1984

1.3 Laccases of Thermophilic Fungi

Thermophilic and thermotolerant fungi are of central importance as a source of thermostable enzymes, which are important for industrial utilization because of the possible economic benefits of being able to degrade plant residues at elevated tempreture.

1.3.1 *Scytalidium thermophilum*

Scytalidium thermophilum is a thermophilic Deuteromycete, and a member of the *Torula-Humicola* complex, which are recognized as dominant species in mushroom compost. The effects of this fungus on the growth of the mushroom mycelium have been described at three distinct levels. First, this fungus decreases the concentration of ammonia in the compost, which otherwise would counteract the growth of the mushroom mycelium. Second, it immobilizes nutrients in a form that apparently is available to the mushroom mycelium, and third, it may have a growth-promoting effect on the mushroom mycelium, as has been demonstrated for *Scytalidium thermophilum* and for several other thermophilic fungi (Wiegant, W. M.).

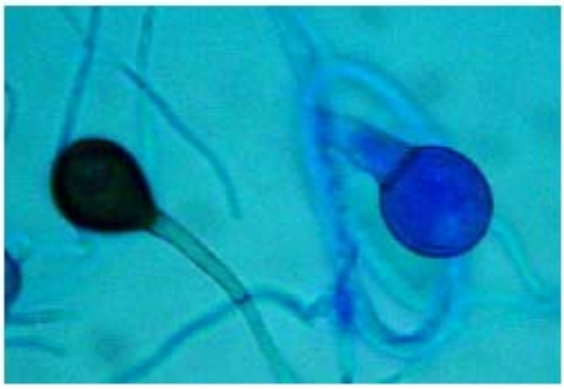

Figure 1.9 *Humicola insolens* spores (type 1) under light microscope (Hamilton, 2002) .

1.3.2 *Scytalidium thermophilum* Laccase gene

The *Scytalidium thermophilum* laccase gene was first cloned by Novo Nordisk Bio Tech, Inc in 1998. To identify the presence of a laccase gene *Scytalidium*, a 5' portion of the *Neurospora crassa* laccase gene (*lccS*) was used as a probe, in screening genomic libraries of different fungal species. An approximately 3 kb laccase specific sequence was detected in the *Scytalidium* DNA. Once the

sequence was determined, the positions of introns and exons within the gene were assigned based on alignment of the deduced amino acid sequence to the corresponding *N. crassa* laccase gene product. From this comparison, it was found that the gene (*lccS*) of *S. thermophilum* is 2476 bp. It is composed of five exons (243, 91, 70, 1054 and 390 nucleotides) punctuated by four small introns (63, 58, 55 and 65 nucleotides). The coding region encodes a preproenzyme of 616 amino acids: a 21 amino acid signal peptide and a 24 amino acid predicted propeptide. The sequence of the *S. thermophilum* laccase gene and the predicted amino acid sequence is shown in Appendix C.

1.4 Heterologous Protein Production by Filamentous fungi

A variety of proteins have been isolated from filamentous fungi. An important group of enzymes isolated from fungi are the pectic enzymes (Alkorta *et al.,* 1998). A more recent application of filamentous fungi is their use as cell factories for heterologous protein production (Archer, 2000). The advantage of the use of these organisms in comparison to prokaryotes is their capability to produce eukaryotic proteins needing post-translational modifications like glycosylation and disulfide bridge formation (Maras *et al.,* 1999).

Nevertheless, heterologous non-fungal proteins are still produced at far lesser quantities by filamentous fungi than fungal proteins (Gouka et al., 1997). Obviously non-fungal proteins have not been optimized during evolution to be secreted efficiently by the fungal secretory pathway. As a result the production efficiency of these proteins can be compromised at any stage of the secretion process. Problems were found to occur at the levels of: mRNA stability, codon-usage, translocation, folding, sorting, and protease susceptibility (Maras *et al.,* 1999).

Especially proteases are a major problem for heterologous protein production, they are present at every stage of the secretion process and their action on the foreign protein has a catastrophic influence on the yield (van den Hombergh, 1997). Also intracellular proteases are important for protein secretion. They play an important role in protein modification and regulation.

Only a relatively small number of fungal species is presently used as a protein production hosts (Punt et al., 2002). Among these, *Aspergillus* species play a dominant role. An important reason for this focus stems from the fact that *Aspergillus* species are highly represented among the strains used for traditional production process. By the fact that several *Aspergillus*-derived food additive products had already obtained a Generally Recognized as Safe (GRAS) status from the regulatory authorities thereby easing its recognition as a safe and reliable expression platform. As a consequence and due to the obvious commercial attraction of fungal expression hosts, several parties set out of to identify alternative species and to assess the possibilities of employing the newly identified fungal expression hosts for heterologous gene expression (Punt et al.,2002). As a result of this search for new hosts *Aspergillus sojae* was identified.

1.4.1 Aspergillus sojae

Aspergillus sojae, which is believed to be a domesticated strain of *Aspergillus parasiticus*, contains all of the aflatoxin biosynthetic genes but is unable to produce aflatoxins and is generally recognized as safe (GRAS) for producing fermented foods (Perng-Kuang Chang, 2003).

The non-aflotoxigenic species have been widely used in industry for food fermentation or for the production of enzymes. Like *A. oryzae*, *A. sojae* is a species of industrial importance. It is widely used in food fermentation, such as soy sauce and bean paste production (Perng-Kuang Chang, 2003).

Section *Flavi* is commonly referred to as the *Aspergillus flavus* group, which also includes the aflatoxin-producing species *A. flavus*, *A. parasiticus* and *A. nomius*, and other species used in food fermentation, such as *A. oryzae* and *A. tamarii* (Samson, 1992). In many ways, *A. sojae* is morphologically similar to *A. parasiticus* and, to a lesser extent, to *A. oryzae* and *A. flavus*. (Kurtzman et al. 1986) based on the high degrees of DNA complementarily of these aspergilli proposed that *A. sojae*, *A. oryzae* and *A. parasiticus* be reduced to varietal status of *A. flavus*. (Geiser et al. 1998) analyzing genes of primary metabolism in strains of *A. oryzae* and *A. flavus* further concluded that *A. oryzae* is evolved by domestication from an *A. flavus*

group. Although the molecular evidence concerning the origin of *A. sojae* has been scant, it is generally agreed upon that *A. sojae*, which has never been isolated from the field, is a domesticated strain of *A. parasiticus* (Wicklow, 1984). In support of this hypothesis, aflatoxin biosynthetic genes from *A. sojae* share approximately 99% of identity to those of *A. parasiticus* (Watson et al., 1999; Yu et al., 2000). While *A. parasiticus* and 40% of *A. flavus* isolates (Tran-Dinh et al., 1999) produce the potent natural hepatocarcinogenic aflatoxins, the reason(s) why *A. sojae* is not aflatoxigenic has only recently begun to be understood. More than 20 clustered genes are involved in aflatoxin biosynthesis (Payne and Brown, 1998). The gene *aflR*, required for transcriptional activation of the aflatoxin biosynthetic genes, encodes a protein with Gal4-type zinc-finger DNA-binding domain (Chang *et al.*, 1995; Ehrlich *et al*., 1999) and a transcriptional activation near its carboxyl terminus (Chang *et al*., 1999). In *A. sojae aflR* a nucleotide change results in a pretermination defect, which truncates the last 62 amino acids of the predicted protein (Watson et al., 1999). This defect apparently is associated with non-aflatoxigenicity of *A. sojae* (Matsushima et al., 2001; Takahashi et al., 2002).

A new expression host from *A. sojae* ATCC strain was developed by Margreet Heerikhuisen, Cees van den Hondel and Peter Punt, TNO Nutrition and Food Reseach Deparment Microbiology. In this study, first strain selection was performed. *A. sojae* ATTCC11906 strain which showed the lowest proteolytic activity was selected for further research. For transformation experiments NaCl method which revealed the better protoplasting effiencies for *A. sojae* ATCC11906 strains was selected. In selection marker research as the transformation first amdS marker was tested. As the transformation frequency was found to be low when using the *amdS* marker for selection, an auxotrophic marker system was developed for *A. sojae*. The usage of *pyrG* as a selection marker was provided a higher transformation frequency. In this study, it has been achieved to transform *A. sojae* and to get high levels of the fungal phytase and glucoamylase proteins using pFytF3 and pGLA6S plasmids which were included *A. nidulans* gpdA promoter and trpC terminator regions (Punt et al; 1991).

1.5 The Fungal Secretory Pathway

Proteins are synthesized by ribosomes in the cytosol. If their final destination is the ER, the Golgi, the vacuole, the plasma membrane or extracellular environment they will reach their target after a journey through the secretory pathway (Figure 1.9). The endoplasmic reticulum is the entry point into the secretory pathway. The synthesized precursor proteins contain a hydrophobic prepeptide, which mediates their translocation into the ER. This prepeptide is removed from most proteins by signal peptidase upon entry. Some proteins keep their prepeptide as membrane anchor.

As the precursor proteins progress on their journey through the secretory pathway they are gradually altered until they obtain their mature form (Shinde and Inouye, 2000). These post-translational alterations include site-specific proteolysis and amino acid modifications (such as phosphorylation, glycosylation, deamination, and acetylation). Via the ER the proteins travel towards the Golgi, which is in effect a branching point of the secretory pathway and the vacuolar pathway (Nakano, 2004). There are at least two protein sorting pathways leading from the Golgi to the vacuole, and there are indications that more than one pathway exists for protein sorting to the plasma membrane. Protein transport from one organelle to another is mediated by vesicles. Membrane proteins without sorting signals are transported via the endosomes to the vacuole. Soluble proteins are transported by default towards the plasma membrane, where they are released into the extracellular environment.

Until recently not much was known about proteases present in the secretory pathway of filamentous fungi. Several proteases present in the secretory pathway have a proteolytic processing function. In contrast to their extracellular degrading partners, their function is needed to ensure that the proteins of interest are correctly produced. The proteolysis of the precursor protein can result in cleavage of different subunits, cleavage of identical copies, removal of a peptide fragment, or removal of amino acid residues at either one of the termini.

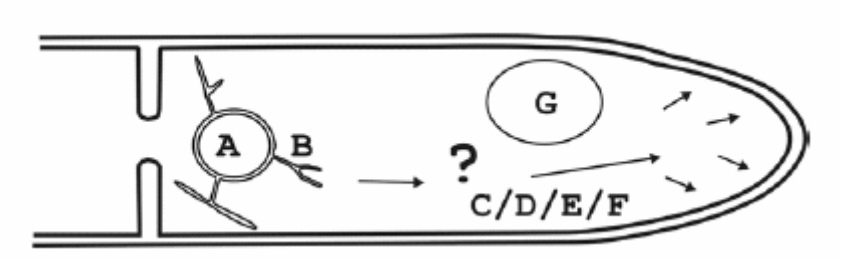

Figure 1.10 The secretory pathway of filamentous fungi. The indicated organelles are: A) nucleus; B) endoplasmic reticulum; C) Golgi; D) post Golgi endosome (PGE) / early endosome; E) prevacular endosome (PVE) / late endosome; F) multivesicular body; G) vacuole. The question mark indicates organelles that have not been visualised in filamentous fungi. The arrows represent the protein transport pathways between the organelles (Ruud Jalving).

1.5.1 Post–translational Processing

Many secreted eukaryotic proteins contain a signal peptide and an adjacent propeptide at the amino terminus. The signal peptide specifies a sequence for translocation over the endoplasmic reticulum membrane and is normally removed in the lumen during translocation by a signal peptidase. Propeptides have been implicated in correct folding and in subcellular sorting of proteases. They also often function as (auto)inhibitors. Many proteases remove their propeptide in an autocatalytic manner. The propeptide is removed upon or before departure from the secretory pathway by maturases (Baker et al., 1993). This group of endoproteases resides in either the late stage of the Golgi, the secretory vesicles or is extracellularly anchored to the cytoplasmic membrane with a GPI-anchor.

One or two basic amino acid residues on the target protein comprise the common recognition site for a maturase to remove the propeptide. The processing of most of these propeptides occurs at either a monobasic or a dibasic cleavage site (Baker *et al*., 1993).

Dibasic recognition sites are processed by the kexin family of endoproteases. Some propeptides have a monobasic amino acid cleavage site. These monobasic

cleavage sites are recognized in yeast by the yapsin family of endoproteases. The function of the N-terminal propeptide is very diverse. It can play a role in folding, sorting, hierarchical organization, and regulation of activity (Shinde and Inouye, 2000).

1.5.1.1 Dibasic Processing

Propeptides, which are cleaved after a Lys-Arg or Arg-Arg basic doublet at the P2 and P1 positions (the nomenclature used is according to Schechter and Berger, 1967) (Figure 1.11), are specifically recognized and processed in the trans-Golgi network by the kexin family of proteases, a subfamily of the subtilase family of proteases (Siezen and Leunissen, 1997). In the majority of dibasic processing sites, cleavage takes place following a ‘ Lys-Arg ’ pair whereas ‘ Lys-Lys’ and ‘Arg-Arg’ pairs are less encountered.

The kexin family consists of the yeast Kex2-like proteases (E.C. 3.4.21.61), the mammalian prohormone convertases (PCs) (E.C. 3.4.21.93 and E.C. 3.4.21.94), and the furins (E.C. 3.4.21.75). All members of the kexin subfamily are calcium-dependent, neutral, serine proteases that are activated by the removal of the amino-terminal propeptide at a kexin-specific (auto) processing site. The active proteases all contain two additional domains, a subtilisin-like domain containing the catalytic triad and a conserved P or Homo B domain of approximately 150 residues. The P domain, which is absent in other subtilases, is essential for the catalytic activity (Nakayama**,** 1997) and the stability of the protein (Lipkind et al., 1998). Kex2-like yeast proteases; furins and some of the PCs also have a single transmembrane domain (Molloy et al., 1992; Nakayama, 1997; Seidah et al., 1996). In its cytoplasmic tail, yeast Kex2 contains a Golgi retrieval signal, necessary to remain in the trans-Golgi network (Wilcox et al., 1992).

1.5.1.2 Monobasic Processing

A considerable number of filamentous fungal extracellular proteins possess a monobasic cleavage site at their leader/mature protein junction. An aspartic protease, designated yapsin 1, was found to be responsible for this activity.

Yapsin-like proteases have specificity for monobasic processing side (X-R). The yapsin-like family, of which four members have been characterised so far, processes the majority of monobasic cleavage sites (Olsen et al., 1999). Members of this family are GPI anchored cell surface aspartyl proteases. Three of these four characterised yapsin-like maturases yapsin 1 (yps1), yapsin 2 (yps2), and yapsin 3 (yps3) are from *Saccharomyces cerevisiae*.

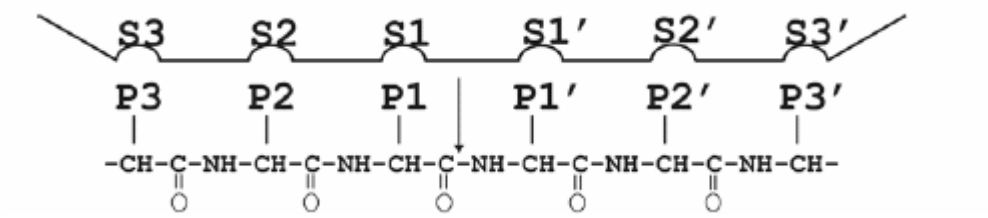

Figure 1.11 Proteolytic cleavage site definition. The protein sequence below represents the substrate; the upper structure represents the protease. The amino acid bond cleaved by the protease is called the scissile bond and is indicated in the figure by the arrow. The pre-scissile amino acid residues are numbered from the scissile bond towards the N-terminus in the form of P1, P2, P3, and so on. The first amino acid following the scissile bond is designated P1'. The remaining amino acid residues are numbered in a similar fashion P2', P3', and so on. The protease subsites that interact with these amino acid residues are numbered in a similar fashion S1- S3, and S1' - S3'.

1.5 Scope and Aim of the Study

In the first part of this study it was aimed to clone and express the *Scytalidium thermophilum* laccase gene in *Aspergillus sojae*. Two cloning procedures were carried out. In the first approach, *S. thermophilum* laccase gene was cloned into *E.coli* XL1 Blue MRF' on vector pUC19 and sequenced. Next, laccase gene was ligated on to pAN52-4 vector and by using protoplast transformation method, recombinant plasmids were transformed in to *Aspergillus sojae* which is a auxotrophic mutant lacking the ability to synthesize uridine. The marker plasmid pAMDSPYRG containing *amds* and *pyrG* genes was co-transformed. After cloning, this region was characterized; sequenced and heterologous expression was studied. The aim of the heterologous expression was to overexpress the enzyme for future use in biotransformatives and to be able to perform site-directed mutagenesis.

In the second part of the study, a bioinformatic approach was used to collect fungal propeptide sequences and to classify them according to their cleavage sites. This information was used to have an overview of the amino acid requirements for cleavage during secretion of fungal proteins. A separate comprise was made between laccase propeptide regions and the overall results were evaluated to draw a number of conclusions that might be of value in further understanding the importance of propeptides in the fungal secretion process.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Fungal and Bacterial Strains

The industrial strain of *Humicola insolens* was provided by Dr. Mehmet Batum from ORBA Inc.

Scytalidium thermophilum type cultures were inoculated onto YpSs agar slants (Appendix B) and incubated at 45 ^{o}C for 4-5 days until sporulation and stored at room temperature 20 ^{o}C for maximum 2 months to use as stock cultures.

Spores from the stock culture were inoculated into liquid preculture, which consisted of YpSs medium broth and containing 10% glucose instead of starch as the carbon source. The preculture was incubated 24 hours at 45 ^{o}C, and was transferred into the main culture, which could be one of different types of modified YpSs broth. Preculture volume was 2 % of the main culture volume. All the cultures were incubated in a shaker incubator at 45 ^{o}C and 155 rpm.

Eschericia coli XL1 Blue MRF' was provided in the purchased ZAP Express® cDNA Gigapack® III Gold Cloning Kit (Stratagene). Stock cultures of XL1 Blue MRF' were grown on LB Tetracycline agar plates 25 mg/ml (Appendix B) at 37 ^{o}C and maintained at 4 ^{o}C. The cells were refreshed onto a new plate every 3 weeks.

The *Aspergillus sojae* (ATCC11906) strain was kindly supplied by TNO Nutrition and Food Research, Department of Microbiology Holland.

Stock cultures of *A. sojae* were grown on complete medium agar (Appendix B). For transformation, *A. sojae* was cultivated in 250 ml complete medium broth (Appendix B) in 500 ml Erlenmeyer flasks at 30 ^{o}C and 150 rpm for 18 hours.

2.1.2 Chemicals, Enzymes and Kits

The list of chemicals, enzymes and kits used and their suppliers are given in Appendix A.

2.1.3 Growth Media, Buffers and Solutions

The preparation of the growth media, buffers and solutions used are given in Appendix B.

2.1.4 Plasmids, Molecular Size Markers

Plasmids used throughout the study and Molecular size markers are given in Appendix C.

2.2 Methods

2.2.1 Nucleic Acid Isolation

2.2.1.1 Genomic DNA Isolation

Mycelia were filtered through Whatman 3MM filter paper and frozen in liquid nitrogen, ground into powder with a mortar and pestle. Powdered mycelia were transferred to a 50 ml falcon tube. Then 15 ml TTE buffer (Appendix B) was added to ground mycelia and centrifuged at 3000 rpm for 10 minutes. The supernatant was discarded and resuspended in 10 ml lysis buffer (Appendix B). It was incubated at 37 ^{o}C for 15 minutes in water bath. Equal volume of phenol: chloroform:isoamylalcohol (25:24:1) was added and centrifuged at 5500 rpm for 8

minutes, then upper phase was transferred to a new falcon tube. Phenol:choloform:isoamylalcohol extraction was repeated until the interphase was clear. After it was clear, 3 ml supernatant was transferred to a Sorvall tube. 0.1 volume sodium acetate (3 M, pH = 5.2) (Appendix B), and 2.5 volume cold absolute ethanol was added to the collected upper phase. DNA was precipitated at –20 ^{o}C for 1 hour-overnight. After that, the tubes were centrifuged at 12 000 rpm for 10 minutes at 4 ^{o}C. The supernatant was discarded and the pellet was washed with 3 ml 70 % ethanol (Appendix B). The tubes were centrifuged at 6000 rpm for 3 minutes. The supernatant was discarded. The pellet was air-dried for 15 minutes or dried at 45 ^{o}C for 10 minutes. After drying, the pellet was dissolved in 100-200 µl sterile double distilled water at 4 ^{o}C overnight. The upper phase was transferred to sterile appendorf tube and 15 µl , 10 mg/ml Dnase free Rnase (Appendix A) was added and incubated at 37 ^{o}C for 30 minutes. DNA was maintained at –20 ^{o}C.

The concentration of resulting DNA was determined by running on an agarose gel (Appendix B) with a marker of known concentration (λ. DNA / *Eco*RI *Hin*d III) and comparing the intensity of the DNA bands with those of the marker bands.

The concentration was calculated according to the following formula:

$$\frac{L_{FRAGMENT}}{TL_{MARKER}} \times C_{MARKER} \times V_{MARKER} \times I_{BAND} \times \frac{1}{V_{DNA}}$$

$L_{FRAGMENT}$: length of the fragment
I_{BAND}: intensity of the band
V_{DNA}: volume of loaded DNA
V_{MARKER}: volume of marker
C_{MARKER}: concentration of marker
TL_{MARKER} : total length of the marker

2.2.1.2 Isolation of Plasmid DNA

LB Broth each of which contained 5 ml including ampicilin at a concentration of 75 µg/ml was prepared in falcon tubes. *E.coli* XL1 Blue MRF' cells containing plasmid were cultivated in these LB Broth overnight at 37^{o}C. The cells were centrifuged at 6000 rpm for 6 minutes. The supernatant was discarded. The pellet was resuspended in 200 µl of solution 1 (Appendix B) room temperature for 15 minutes. Then, 200 µl of solution 2 (Appendix B) was added and mixed gently for 7-8 times, incubated 5 minutes on ice. After that, solution 3 was added gently mixed for 7-8 times and inoculated on ice for 15 minutes. The tubes were centrifugated at 13 000 rpm at 4 ^{o}C for 10 minutes. The supernatant was transferred to sterile appendorf . 2 volume of cold absolute ethanol was added to each tube and incubated at –20 ^{o}C for 1 hour. The tubes were centrifuged at 13000 rpm at 4 ^{o}C for 10 minutes and supernatant was discharged. The pellet was resuspended in 200 µl NE buffer (Appendix B) on ice for 1 hour. After the tubes were centrifuged at 4 ^{o}C for 15 minutes the supernatant was transferred to another appendorf tube. 400-µl absolute cold ethanol was added and incubated at –20 ^{o}C for 30 minutes. Supernatant was discarded after centrifugation at 4 ^{o}C for 10 minutes.

The DNA pellet was air dried at room temperature or in vacuum concentrater for 5 minutes, and redissolved in 15 µl of sterile double distilled water.

2.2.1.3 Recovering DNA from Agarose Gels

For recovering DNA from agarose gels Fermentas DNA Extraction Kit was used. DNA containing solution was loaded on agarose gel and the gel was run to separate reaction products. The gel slice containing the DNA band was then excised. The approximate volume of the gel slice was determined and the gel was then placed the slice into an eppendorf tube. 3 volumes of Binding Solution (Appendix B) was added. The tube was incubated for 5 min. at 55 ^{o}C to dissolve agarose. Per 1 µl of DNA 2 µl of silica powder (Appendix B) was added and incubated for 5 min. at 55 ^{o}C. The tube was vortexed every 2 min. to keep silica powder in suspension. The silica powder/DNA complex was spun for 5 second to form a pellet and remove

supernatant. 500 µl of ice-cold wash buffer (Appendix B) was added, vortexed and spun for 5 sec. The supernatant was discarded. This procedure was repeated three times. After the supernatant from the last wash was removed, the tube was spun again and the remaining liquid was removed together with the pellet. The pellet was air-dried for 10-15 min. The DNA was dissolved into 10-15 µl sterile deionized water. The tube was spun and the supernatant was removed into a new tube avoiding mixing with the pellet. The procedure was repeated once more for the removal of small amounts of remaining silica powder.

2.2.1.4 Purification of Plasmid DNA for Sequencing

QIAGEN Plasmid Mini Kit is used for purification of plasmids for sequencing. First QIAGEN-tip was placed into a tip holder. 1 ml Buffer QBT (Appendix B) was aiiow to flow through the tip by reduction in surface tension due to the presence of detergent in the equilibration buffer. QIAGEN-tip was allowed to drain completely. The plasmid solution was loaded onto the QIAGEN-tip promptly. 4x1 ml Buffer QC (Appendix B) was allowed to move through the QIAGEN-tip by gravity flow. The first 2 ml was sufficient to remove all contaminants in the majority of plasmid DNA preparations. The second 2 ml ensures complete removal of contaminants. DNA was eluted with 0.8 ml Buffer QF (Appendix B). Plasmid DNA is collected in 1.5 ml appendorf tube. DNA was precipitated with 0.7 volumes of isopropanol at room temperature. The tube was centrifuged at lower than 10,000 rpm for 30 min and the supernatant was decanted. DNA was washed with 1 ml of 70% ethanol, air dried for 5 min, and redissolved in 20 µl sterile double distilled water.

2.2.2 The Polymerase Chain Reaction (PCR)

A 50 µl reaction mixture contained sterile double distilled water to give a final volume of 50 µl, 10X reaction buffer to give final concentration of 1X, 200µM dNTP mix, 1 unit Taq DNA polymerase (Fermentas), 50 pmoles from each specific primer, 0.25 µg genomic DNA, were mixed and 30 µl mineral oil was added to prevent evaporation.

Amplifications were performed according to the following cycle:

95 °C	3 min	
95 °C	1 min	X 35
Ta °C	1 min	
72 °C	1 min / kb	
72 °C	3 min	

where Ta is the annealing temperature, ranging from 48 °C to 60 °C, according to the degeneracy and melting temperature of the primers.

If PCR amplification was carried out with Pfx polimerase (Invitrogene) for (50µl) reaction mixture 10X reaction buffer to give final concentration of 1XPCR buffer 50 mM $MgSO_4$, approximately 25ng of the template DNA, 50 p mole each of primers and 1 unit pfx polymerase were used.

Amplifications were performed according to the following cycle.

94°C	2 min	
94°C	30 sn	X 35
Ta °C	1 min	
72 °C	1 min / kb	
72 °C	5min	

PCR products (20µl) were analyzed by agarose gel electrophoresis.

2.2.3 Agarose Gel Electrophoresis

In order to visualize DNA samples and PCR products, 0.8 %(w/v) agarose gel was prepared. Agarose gel electrophoresis is based on the relative speeds of motion of the DNA molecules in an electrical field as a function on their fragment length. To prepare 150 ml gel solution, 1.2 g of agarose was melted in 1X TAE buffer by boiling in a microwave oven. 0.5-µg/ml ethidium bromide solution was added and mixed with the gel solution when it cooled down to 55 ^{o}C. Gel was then poured into a gel tray with o comb. After the polymerization of the gel, the comb was removed to form the wells and the tray was placed into the electrophoresis tank containing 1X TAE buffer (Appendix B).

The DNA samples, which were mixed with 1/6 final concentration of loading buffer (Appendix A), were loaded into the wells. *Eco*RI *Hin*d III λ DNA was used as a size marker. The gel was run for 45 min to several hours depending on the length of the gel tray at 5-10 V/cm. The gel was visualized under UV light and photographed by a digital camera (Nikon Coolpix 4500).

2.2.4 Transformation of *E. coli* XL1 Blue MRF'

2.2.4.1 Preparation of *E coli.* Competent Cells

E coli. XL1 Blue MRF' cells were cultivated in 5 ml LB medium (Appendix B) overnight at 37 ^{o}C, 200 rpm. 100 ml of LB medium was inoculated with 1 ml of an overnight culture of *E coli*. XL1 Blue MRF' and incubated at 37 ^{o}C until the optical density at 550 nm reached 0.4-0.5.

The culture was dispensed into 2 falcon tubes. The tubes were chilled on ice for 10 min. and centrifuged at 6000 rpm for 5 min at 4 ^{o}C. Then, the supernatant was discarded and the cells were resuspended in a total volume of 50 ml ice-cold solution (25 ml for each tube) containing 50mM $CaCl_2$ and 10 mM Tris-HCl (pH 8.0), kept on ice for 15 min. and centrifuged at 6000 rpm for 5 min. at 4 ^{o}C. Again, the supernatant was discarded, the cells were resuspended in a total volume of 7 ml (3.5 ml for each tube) ice- cold 50 mM $CaCl_2$ and 10 mM Tris-HCl (pH 8.0) solution and

100 % sterile glycerol was added to a final concentration of 20 % (700 µl for each tube).

Finally, 300 µl aliquots were dispensed into appendorf tubes and stored at – 80 ^{o}C until use.

2.2.4.2 Transformation of Competent Cells

1-10 µl plasmid (1µg) was mixed with sterile double distilled water to a final volume of 50 µl. 300 µl competent cells were mixed with diluted plasmid and kept on ice for 30 min. The mixture was transferred into a 42 ^{o}C water bath for 90 second followed by 2 minutes on ice. Then, 1ml LB medium (Appendix B) preheated before to 42 ^{o}C was added and the mixture was incubated in water bath at 37 ^{o}C. 100-150 µl aliquots were spread on LB agar plates containing 75 µg/µl ampicilin and incubated overnight at 37 ^{o}C.

2.2.5 Restriction Enzyme Digestion of the DNA

Restriction enzymes used in this study and their 10 x reaction buffers were supplied by Fermentas. For restriction enzyme digestion 0.1- 1 µg DNA and 1 to 30 U restriction enzyme were used. Appropriate 10 x restriction enzyme buffer was used to give a final concentration of 1x and sterile double distilled water to give a final volume of 10-30 µl. The reaction mixture was incubated at 37 ^{o}C overnight.

Before digestion, PCR products were cleaned by Fermentas DNA Extraction Kit (Appendix B) or Phenol:choloform:isoamylalcohol (25:24:1) extraction and after phenol:choloform:isoamylalcohol extraction DNA was concentrated by isopropanol precipitation to get rid off interfering oligonucleotides and dNTPs before restriction digest for ligation.

2.2.6 Ligation

After restriction digestion, PCR fragments and the plasmid DNA were directly treated with Alkaline Phosphatase (Fermentas) in order to prevent religation

of the linearized vector DNA. For dephosporylation of plasmid DNA 10-30 µl DNA solution, appropriate 10 x calf intestine alkaline phosphatase buffer to give a final concentration of 1x, 2 U alkaline phosphatase, and sterile double distilled water to give a final volume of 50 µl were used. The reaction mixture was incubated for 4 hours up to overnight at 37 ^{o}C.

After that phenol:choloform:isoamylalcohol (25:24:1) extaction was used to separate DNA from other components. An equal volume (50 µl) of phenol: choloform: isoamylalcohol (25:24:1) was added to the DNA mixture in the eppendorf tube and after gentle mixing the tube was centrifuged at 14 000 rpm 5 min. 40-45 µl of upper phase was transferred to a new tube. 0.1 volume of Na Ac (3 M PH =5.2) (Appendix B) and 2 volume of cold absolute ethanol were added to precipitate the DNA. The mixture was stored at – 20 ^{o}C for 4 hours. After that the tube was centrifuged at 14 000 rpm for 15 min. at 4 ^{o}C and supernatant was poured off. The pellet was washed of with 150 µl 70% ethanol and then DNA was dried and suspended in appropriate buffer.

A standard ligation mixture was prepared with 50-100 ng vector DNA, 100-500 ng insert DNA, 10x ligation buffer to give a final concentration of 1x, 1 U T4 DNA ligase (Roche), sterile double distilled water to give a final volume of 20 µl and 1 mM ATP (Fermentas) the ligation mixture was incubated at 16 ^{o}C overnight. Following incubation ligation mixture was transformed into *E. coli* as described.

2.2.7 Identification of Recombinant Clones

1-10 µl ligation mixture was transformed to a suitable strain of competent E. coli as explained before. Transformed *E. coli* cells were inoculated on agar plates containing LB medium with ampillin. For blue-white colony selection, 10 µl 100 mM IPTG (Appendix B) and 50µl 2% X-gal were spread on LB plates and incubated at 37 ^{o}C for 4 hours before inoculation of transformed cells.

The following incubation white colonies were picked and plasmid isolation was done to see the presence of insert. Potential recombinant plasmids were then digested with suitable restriction enzymes before loading on an agarose gel to analyze the presence and size of insert.

If the vector used in ligation did not allow the use of the blue-white colony selection, all of the colonies after the transformation, were picked and plasmid isolations were done.

2.2.8 Tranformation of *Aspergillus sojae*

2.2.8.1 Strains

Aspergillus sojae ATCC11906 ($pyrG^-$) is strain has a low proteolytic activity and it is a uridine auxotrophic mutant which is unable to produce the enzyme orotidine-5 monophosphate decarboxylase, involved in the biosynhesis of uridine was used for heterologous expression. Absence of orotidine-5 monophosphate decarboxylase synthesis is caused by a mutation within the *pryG* locus. In general such a system provides a higher transformation frequency. A commonly used auxotrophic selection method for fungal transformants is based on the usage of orotidine-5 monophosphate decarboxylase (*pyG*) mutants.

2.2.8.2 Selective Plasmids

In the cotransformation studies pAMDSPYG plasmid was used as a selective marker. This plasmid include *amdS* gene of *A. nidulans* and *pyrG* gene from *A. niger*. The amds gene encodes an enzyme that confers the ability to use acetamide and acrylamide as nitrogen and carbon sources and the *pyrG* gene encodes orotidine-5 monophosphate decarboxylase.

2.2.8.3 Expression Vectors

Vector pAN52-4 was used as an expression system, includes *A. nidulans* glyceraldehydes-3-phoshate dehydrogenase (*gpd*) as a strong and constitutive promoter and trpC terminator. The 21 amino acids of the laccase signal peptide were replaced by the 24 amino-acid glucoamylase (GLA) preprosequence from *A. niger.*

2.2.8.4 Transformation Method

Fungal co-transformations was basically carried out as described by Punt & van den Hondel and L.H de Graaff using pAN52-4 fungal expression vector containing laccase gene and pAMDSPYRG containing the *pyrG* selection marker, in a 1:10 ratio. Transformants were selected for uridine prototrophy.

In the first step of co-transformation, 250 ml of transformation medium (Appendix B) with 1x 10^6 spores per ml grow for 16-18 hours at 30 ^oC and 250rpm yielding 1-5 mycellium (wet weight) per 100 ml. Mycelium was harvest on nylon gauze using a Büchner funnel and mild suction, washed once with 0.27 M $CaCl_2$, 0.6 M NaCl buffer (1g mycelium/20 ml). 100 mg Novozyme 234 was dissolved in 10 ml STC (Appendix B) and filter sterilized (0.2 µm membrane). 1 g mycelium was resuspended in the Novozyme solution by gently pipetting up and down a 10 ml pipette. Protoplasts were prepared at 30 ^oC with slow agitation (50-100 rpm). At 30-min intervals protoplast formation is checked on a microscope. When many free protoplasts were observed (more then 1* 10^8) the mycelial debris was removed by filtration over a sterile glass wool plug. The filtrate was collected in a 10 ml glass tube. The protoplasts were centrifuged 10 minutes at 2000 rpm for 10 min at 4 ^oC in a bench centrifuge, carefully resuspended in STC. Wash step was repeated twice and the protoplasts concentration determined before the last centrifugation step, using a heamocytometer. The protoplasts were resupended in STC at a density of 1* 10^8 per ml. 200 µl of the protoplasts suspension were pipetted in a universal tube, 1 µg of selection gene DNA and 10 µg of co-transforming DNA (dissolved in a 10-20 µl TE). 50 µl of PEG buffer was added, mixed gently by shaking and incubated at RT for 20 minutes. 1 µg of selection gene DNA was used as a positive control and 20 µl of TE as a negative control. The rest of the protoplasts suspension is kept on ice. 2 ml of PEG buffer was added, mixed gently, incubated at RT for another 5 minutes and subsequently 4 ml STC was added, and mixed gently. Selective MMS-top agar (Appendix B) was added to the transformation mixture so the tube is almost completely filled. The tube was mixed by inverting several times and the mixture was poured onto two 15 cm selective MMS-plates (Appendix B). The plates were incubated for 3 days at 30 ^oC.

2.2.8.5 Preparation Spores Suspension and Inoculation Cultures

10 ml saline-Tween solution was pipetted on the spore-mat. Spores were scraped off using a bend inoculation needle. The spore suspension was transferred to a sterile bottle with a sterile plastic Pasteur Pipette. 20-fold dilution (50 µl + 950 µl saline-Tween) and the concentration of spores was determined by counting he diluted sample in a heamocytometer. The concentration of spores was calculated according to the following equation:

$$\frac{\text{\# of spores counted x 1000 mm}}{\text{\# of squares counted x width}^2 \text{ x dept}} \text{ x dilution} = \text{\# / ml}$$

width ^{2} : 0.05 mm

dept: 0.02 mm^2

2.2.8.6 Enzymatic Assays

Extracellular laccase activity was determined by a spectrophotometrical method. The change in the A_{420} per minute was calculated with respect to appearance of quinones from diphenols at 60 ^{o}C. The substrate solution was prepared freshly just before use, due to rapid autooxidation of phenolic substrate ABTS with molecular oxygen in the air. Therefore, buffer solution, used to dissolve ABTS was preincubated instead of preincubation of ABTS solution. Both buffer and enzyme solutions were preincubated for 5 minutes at 60 ^{o}C before the activity measurement.

Laccase activity was determined on an aliquot of culture supernatant, by monitoring the oxidation time course of 0.5 mM ABTS at 420 nm, in the presence of 0.1 mM acetate buffer, pH 5.0 at 60 ^{o}C, 0.5 ml of culture supernatant and 1 ml of buffer solution. The substrate blank cuvette contained 500 µl buffer solution.

One unit of enzyme activity was defined as a change in optical density at 420 nm of 0,01 per minute under the stated assay conditions. The enzyme activity was expressed in units defined as 1 U = 1 µmole of substrate oxidized (or 1 µmole product formed) in one minute by 1 ml of culture supernatant. The experimentally determined extinction coefficient of laccase at 420 nm was taken as ε_{420} = 3600 M^{-1} cm^{-1}. Laccase activities were calculated as following equation.

Enzyme Activity (U/ml) = (ΔOD/Δt) (1/ε) (1000) (4)

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Experimental Strategies for *Scytalidium thermophilum* Laccase Gene Cloning

The experimental strategy for cloning *Scytalidium thermophilum* laccase (*lccS*) gene is shown in Figure 3.1.

In this study, it was aimed to re-clone *Scytalidium thermophilum* laccase (*lccS*) gene that was formerly cloned by Novo Nordisk into *A. oryzae*. For this purpose first, specific primers homologous to the beginning and end of the gene were designed and used for the amplification. The amplified gene fragment was ligated onto vector pUC19 and cloned into *E. coli* XL1 Blue MRF'. After plasmid purification, the products were sequenced to be sure about the specificity of the process. However, this gene had a number of unwanted mutations. In the second part of the study, again specific primers were designed that corresponded to the new expression vector pAN52-4. The laccase gene (*lccS*) excluding the signal and propeptide region (*A. niger*) was amplified using Pfx polymerase and ligated onto vector pAN52-4 to fuse the gene to the signal and propeptide region of the glucoamylase gene followed by transformation into to *E. coli* XL1 Blue MRF'. The efficiency of the ligation was tested by restriction enzyme digestion and amplification of the insert using specific primers. After plasmid purification the insert was sequenced. Plasmids including laccase gene were transformed to an eucaryotic organism *Aspergillus sojae* by protoplast transformation method. 5 number of transformants were obtained and were analyzed for laccase production.

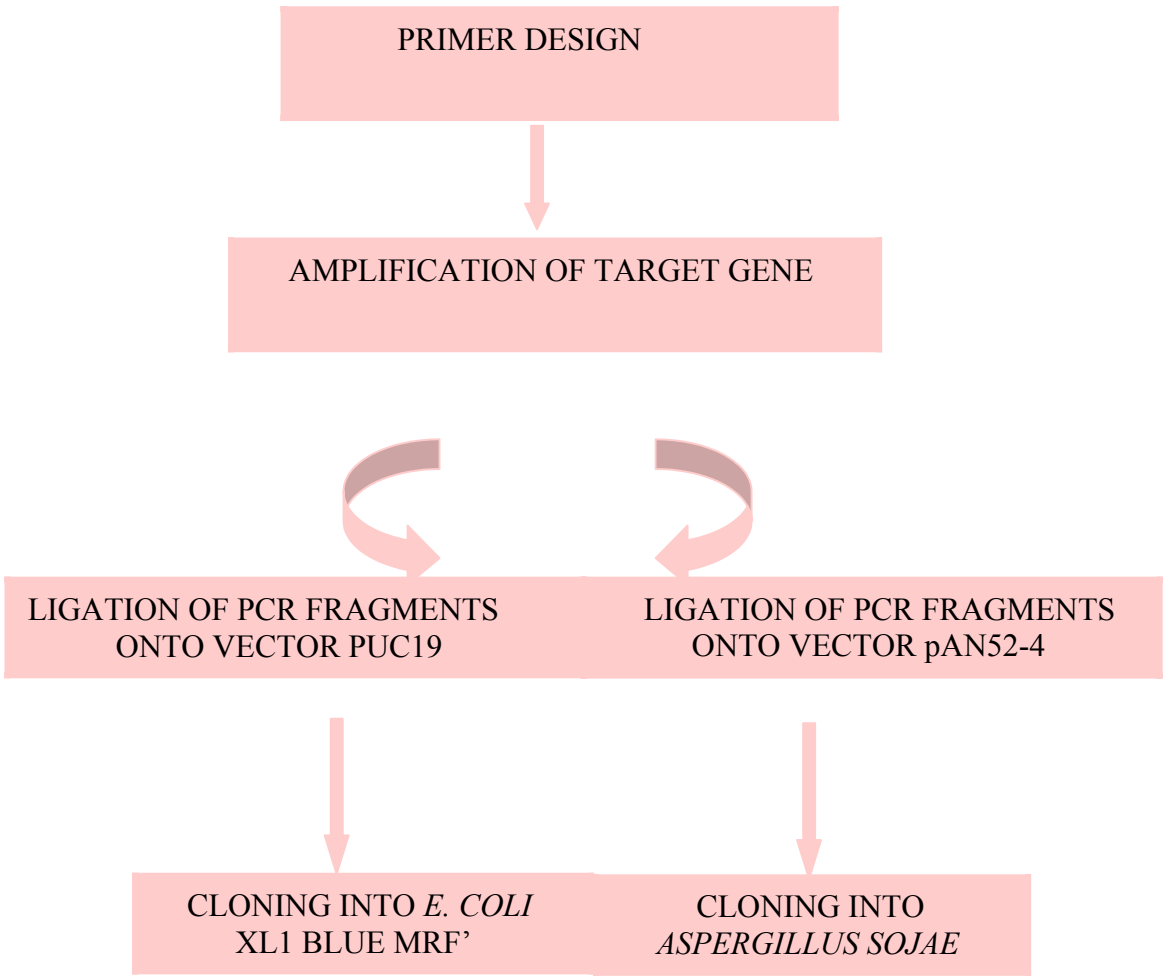

Figure 3.1 Flowchart of the experimental strategies.

3.2 PCR Cloning onto pUC19

3.2.1 Primer Design

For the specific detection of the laccase gene *lccS*, three different primers (lacS1, lacS2, lacS3) were designed using the sequences of the *S. thermophilum* laccase gene. The amplified gene fragments were then used in the ligation step onto vector pUC19. lacS1 corresponded to the upstream of the ATG including signal peptide region. lacS2 corresponded to the downstream of the start codon and when it was cleaved by restriction enzyme *Eco*RI, signal peptide region of *lccS* was discarded, and primer lacS3 corresponded to the downstream of the 3' region stop codon.

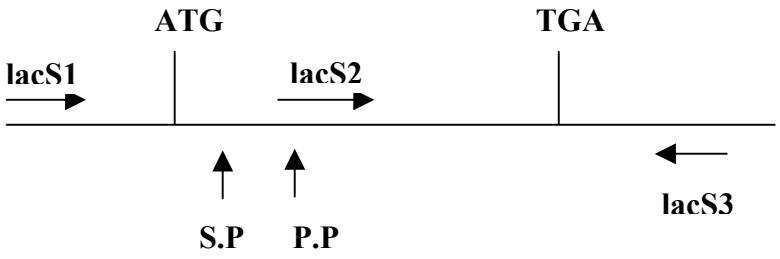

Figure 3.2 The schematic illustration of the primers lacS1, lacS2, lacS3, signal peptide cleavage site (S.P), propeptide cleavage site (P.P).

3.2.2 Isolation and Purification of *Scytalidium thermophilum* Genomic DNA

Scytalidium thermophilum total genomic DNA was isolated as described in the Materials and Methods section 2.2.1.1. Purified DNA samples were visualized on agarose gel after electrophoresis. Figure 3.3 shows the genomic DNA samples after electrophoresis. Purified DNA samples concentration was calculated according to the formula described in the section 2.2.1.1.

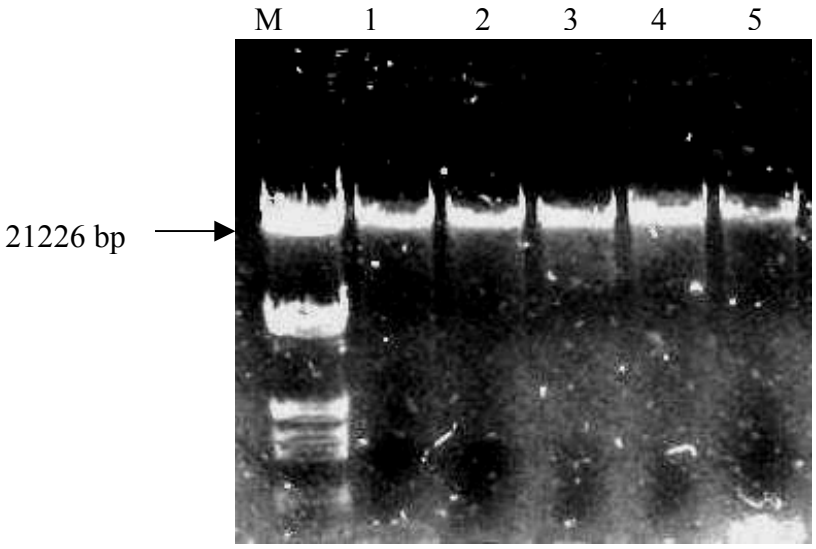

Figure 3.3 Total genomic isolation DNA *of Scytalidium thermophilum.* M. Molecular size marker λ DNA/*Eco*RI+*Hin*dIII (0.5µg/µl); lane1, 0.1 µg/µl DNA

3.2.3 Amplification of *S. thermophilum* Laccase Gene

As indicated before in section 1.3.2, the *lccS* gene of *S. thermophilum* has been previously cloned by Novo Nordisk and expressed in *A. oryzae*. In this study, it was aimed to re-clone the same gene to obtain a better expression system and to perform further biotransformation and enzyme engineering studies. In this respect, the *S. thermpohilum* laccase gene was amplified from the genomic DNA using three specific primers, namely lacS1, lacS2, lacS3 (Table 3.1), which included *EcoRI* restriction cut sites. To optimize PCR reactions, different annealing temperatures were tested. Amplification with lacS2 and lacS3 primers yielded a PCR product of an expected size of about 2155 bp (Figure 3.5). This fragment corresponded to the 2092 bp open reading frame of *S. thermophilum* laccase gene and 42 bp upstream and 21 bp downstream-untranslated regions. Amplification with lacS1 and lacS3 primers yielded a PCR product of an expected size of about 2067 bp (Figure 3.4). lacS1-lacS3 amplified fragment did not include signal peptide region of the laccase gene.

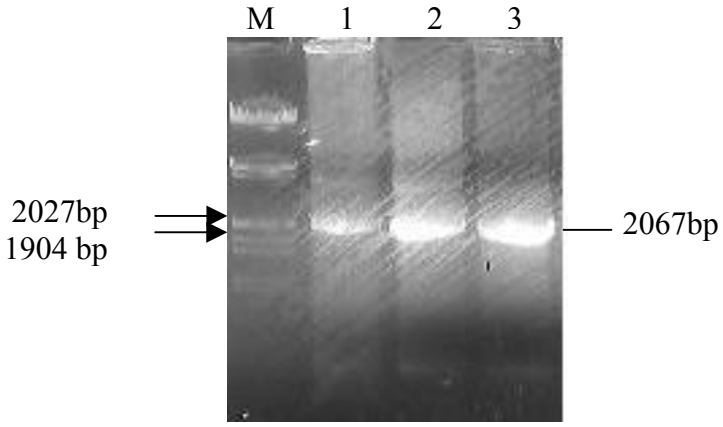

Figure 3.4 PCR amplification with lacS1-lacS3 primers results at different annealing temperatures: lane 1, at 57^{o}C; 2, at 58^{o}C; 3, at 59^{o}C. Amplifications yielded a major band of 2067 size. M: Molecular size marker λ DNA/*Eco*RI+*Hin*dIII .

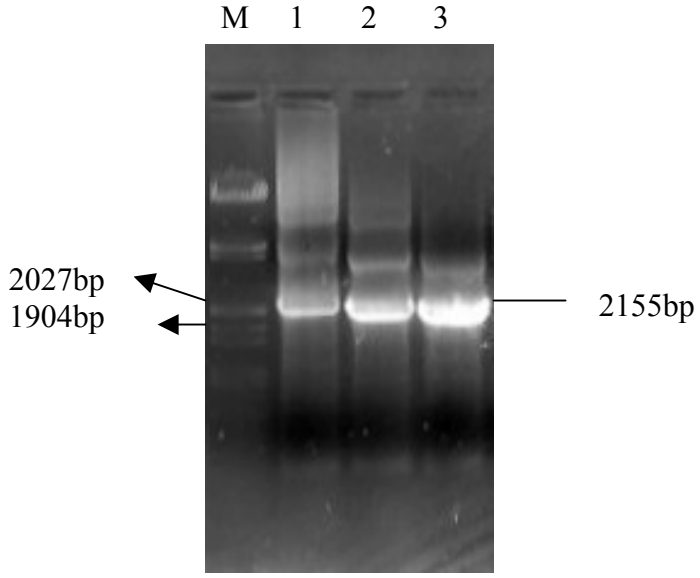

Figure 3.5 PCR amplification with lacS2-lacS3 primers results at different annealing temperatures: lane1, 60^{o}C 2, 59^{o}C; 3, 58^{o}C; Amplifications yielded a major bands of 2155bp size. M: Molecular size marker: λ DNA/*Eco*RI+*Hin*dIII.

3.2.4 Restriction Digestion of Laccase PCR Fragments

Restriction digestion analysis was done before the sequencing of the fragments. *Bam*HI, *Eco*RI and *Hin*dIII enzyme digestion supported that the PCR fragment correspond to the laccase gene. There were no restriction region on the laccase open reading frame for *Hin*dIII and *Eco*RI, so digestion yielded single bands and there was one cut site for *Bam*HI, so digestion yielded two bands of expected size namely, 1277 bp. and 815 bp.

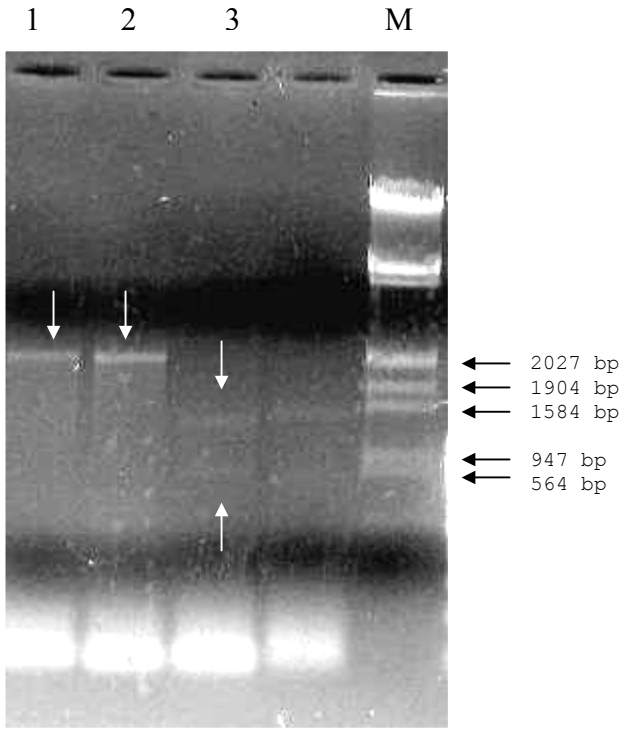

Figure 3.6 Restriction digestion with *Eco*RI, *Hind*III and *Bam*HI enzymes
a-lane 1, *Eco*RI digested PCR fragment amplified by primers lacS2 and lacS3;
b-lane 2, *Hind*III digested PCR fragment amplified by primers lacS2 and lacS3;
c-lane 3, *Bam*HI digested PCR fragment amplified by primers lacS2 and lacS3
d- lane M, Molecular size marker: λ DNA/*Eco*RI+*Hin*dIII

3.2.5 Ligation of the Laccase Gene (*lccS*) onto Vector pUC19 and Transformation into *E. coli* Blue MRF'

The PCR amplified laccase gene fragment (2155bp) was cut with *Eco*RI and ligated onto pUC19 cloning vector from the *Eco*RI cut site on the vector. The vector was dephosphorylated to prevent religation as explained in section 2.2.8.

After the completion of ligation, the plasmids were transformed into *E.coli* XL1 Blue MRF'. The used vector allows ampicillin and blue and white screening of recombinant colonies. The recombinant colonies were selected through blue/white selection. Some of white colonies were chosen at random. From these chosen white colonies, plasmid DNAs were isolated and analyzed by agarose gel electrophoresis (Figure 3.7).

3.2.6 Isolation of Recombinant Plasmids Containing the Laccase Gene

The presence of insert in the putative recombinant plasmids, which were isolated white colonies, were analyzed by restriction digestion analysis and PCR amplifications.

Out of 9 plasmids analyzed by single digestions by *Eco*RI, 6 plasmids were identified as true recombinants (Figure3.8). Among these recombinants, pUC12C (Figure 3.9) was selected to be further characterized by restriction analysis. The recombinant plasmids were linearized with *EcoRI* digestion, which yielded two fragment one corresponding to pUC19 vector (2686bp) and other to the insert (2155bp) (Figure3.8). PCR amplification with primers lacS2-lacS3 of pUC12C also yielded the expected 2155 bp fragment (Figure 3.7). According to the restriction analysis and amplification of the expected fragment from recombinant plasmid, the presence of laccase gene in pUC12C was confirmed.

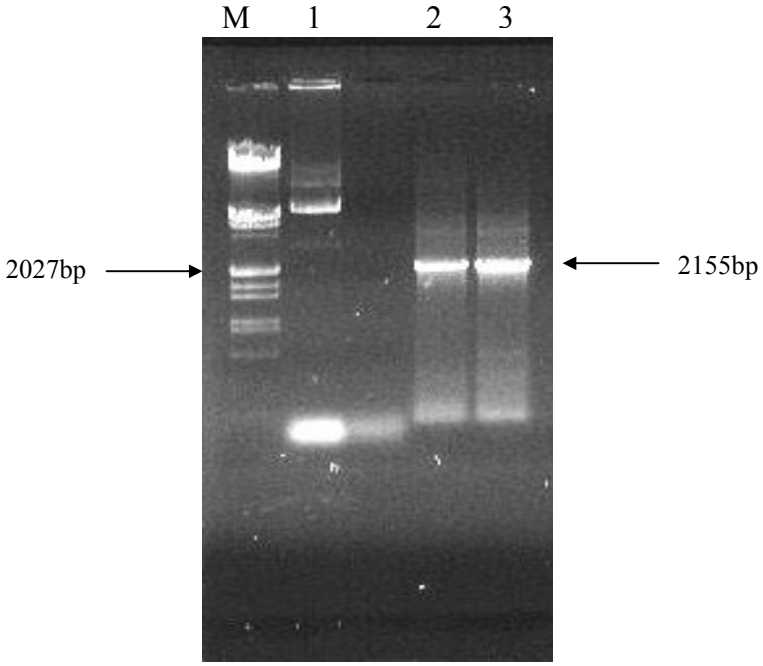

Figure 3.7 PCR amplification with primers lac2-lac3 of pUC12C.
lane1, recombinant plasmid pUC12C; lane2 and 3 PCR amplification of pUC6C plasmid by lacS1 and lacS3 primers; lane M, Molecular size marker: λ DNA/*Eco*RI+*Hin*dIII

At the beginning of the study it was aimed to clone both of the two fragments that were amplified with lacS1-lacS3 and lacS2-lacS3. However, these fragments were amplified with Taq polymerase which cannot correct errors. Indeed, a number of unwanted mutations were later detected by sequencing. Nevertheless, these clones can be used in the future in directed evolution studies.

Table 1. Sequence of primers designed based on nucleotide sequence of the laccase gene**

Name of Primer	Sense or Antisense	Number of Bases	Sequence of Primer (5'to 3')
lacS1	Sense	37	T C A A C T C A G G T G C C G A A T T C G C T C C G T C T A C A C A T C C *Eco*RI cut site
lacS2	Sense	37	C C G T C G C A C A C C T G G G A A T T C T G T T A A T A A C G G C C T G *Eco*RI cut site
lacS3	Antisense	37	T A A A C A A T C C G C C C A G A A T T C T C A C G C A T C C C A T C G C *Eco*RI cut site
lacP1	Sense	32	C G T C T C G G C A A G C T T C C T G T C A T T C T C C A A G C *Hind*III cut site
lacP2	Antisense	32	A A T C C G C C C A A A G C T T T C A C C G A T C C C A T C G C *Hind*III cut site
lacP3	Sense	32	C G C A C A C C T G C C C A T G G T G T T A A T A A C G G C C T *Nco*I cut site
lacP4	Antisense	32	A A T C C G C C C A C C A T G G T C A C C G A T C C C A T C G C *Nco*I cut site

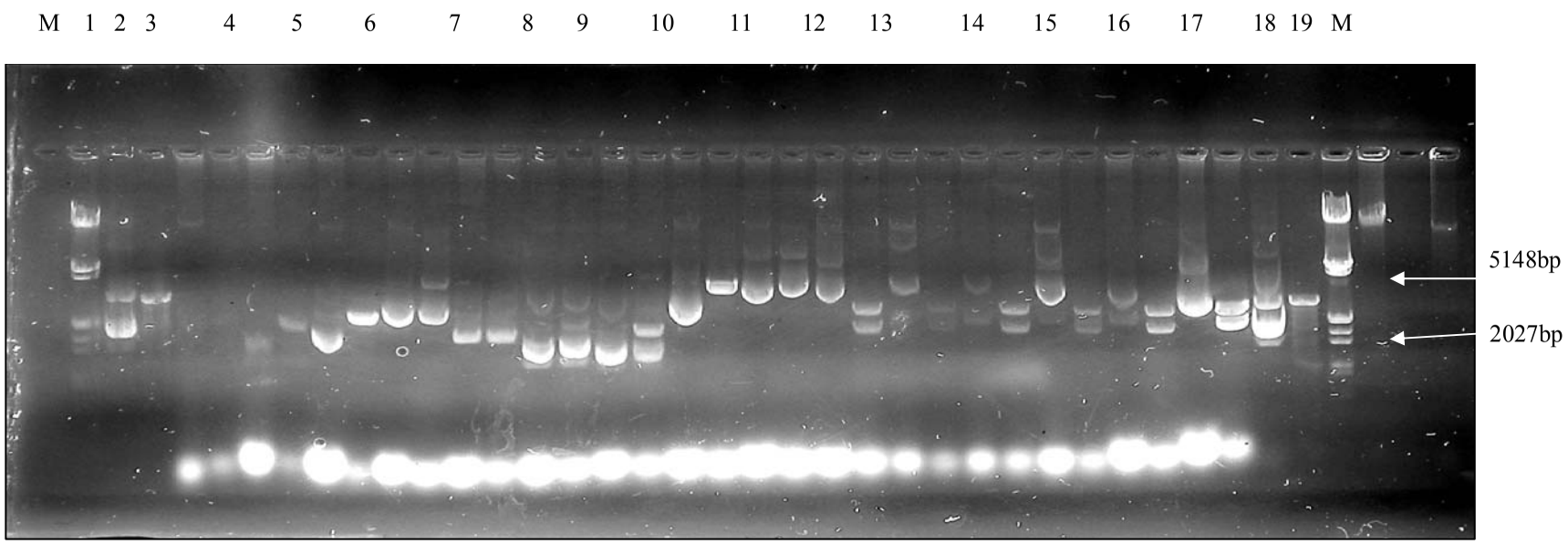

Figure 3.8 Isolated and *Eco*RI digested plasmids after ligation of lccS onto pUC19 lane1, *Eco*RI digested pUC19; lane2, plasmid pUC19; lane 3 , pUC1C; lane4, pUC2C ; lane 5, pUC3C ; lane6, pUC4C; lane 7, pUC5C; lane 8, pUC6C; lane 9, pUC7C; lane 10, pUC8C ; lane11 pUC9C; lane12, pUC10C; lane 13, pUC11C lane 14, pUC12C; lane 15, pUC13C, lane 16, pUC14C; lane 17, pUC15C; and one side of them are *Eco*RI digested of these plasmids. A linearized vector would be 4831bp long including 2067 bp amplified laccase gene and 2686bp pUC19 vector. Thus ; pUC12, pUC13, pUC14, pUC15i pUC16 and pUC17 proved to be true recombinants. lane 18 plasmid pUC19; line 19, *Eco*RI digested pUC19; line M, Molecular size marker. λ DNA/*Eco*RI + *Hin*dIII.

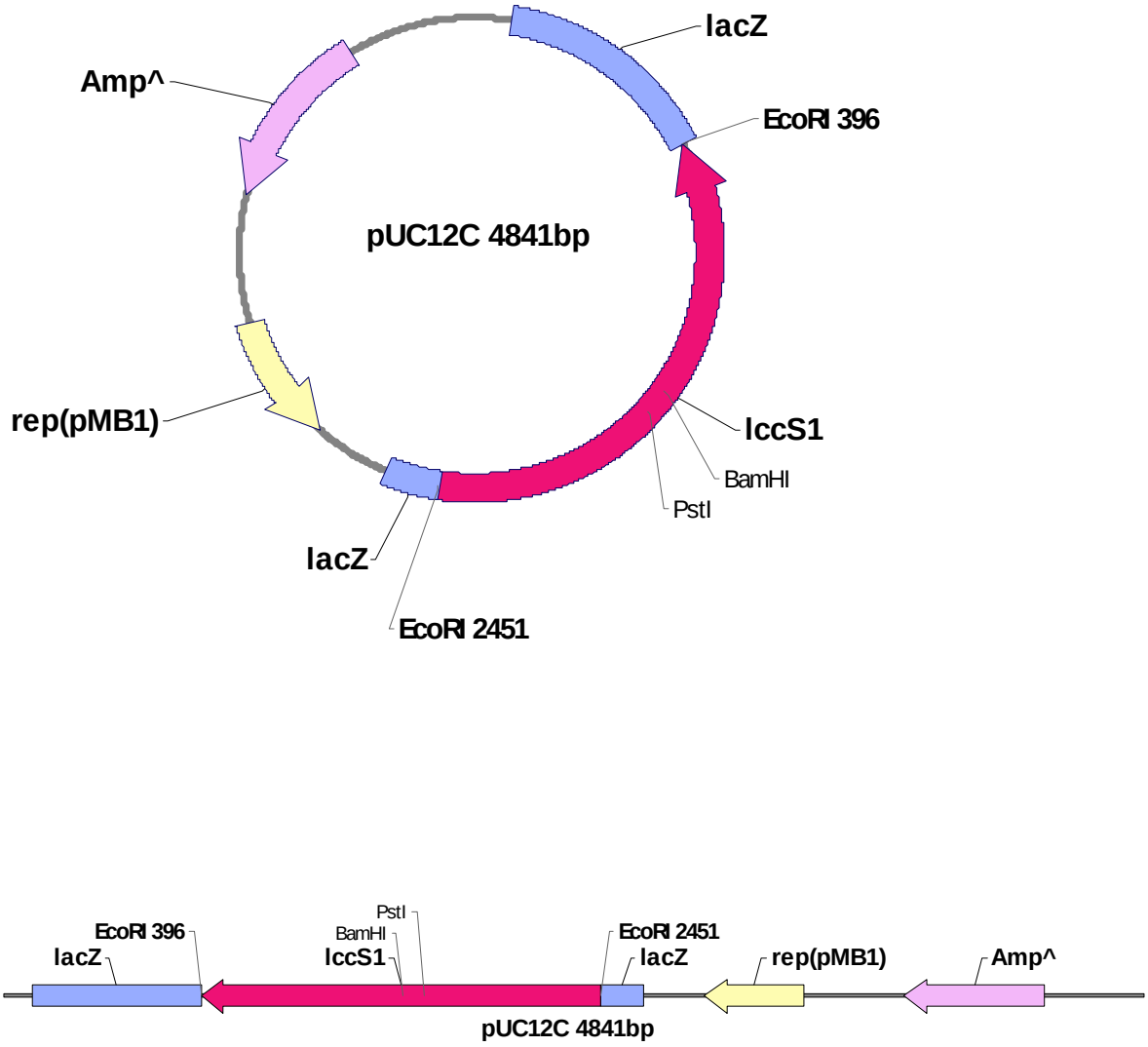

Figure 3.9 Recombinant pUC12C vector

3.3 PCR Cloning onto pAN52-4

3.3.1 Primer Design

In order to clone *lccS* gene onto vector pAN52-4, a new set of primers were designed. These primers were lacP1, lacP2 lacP3 and lacP4 (Table 3.1). lacP1-lacP2 primer pair include *Hin*dIII restriction enzyme recognition regions to provide cloning the amplified fragment to vector pAN52-4, lacP3-lacP4 primer pair include *Nco*I restriction enzyme recognition regions to vector pAN52-1. Because the vector pAN52-4 includes glucoamylase gene of *Aspergillus niger* signal peptide and pro-peptide regions, the primer lacP1 was designed such that the amplified *lccS* gene lacks the signal peptide and propeptide. Amplification with lacP1-lacP2 primers was yielded a PCR product of an expected size of about 1983bp and 2151bp with lacP3-lacP4 primers.

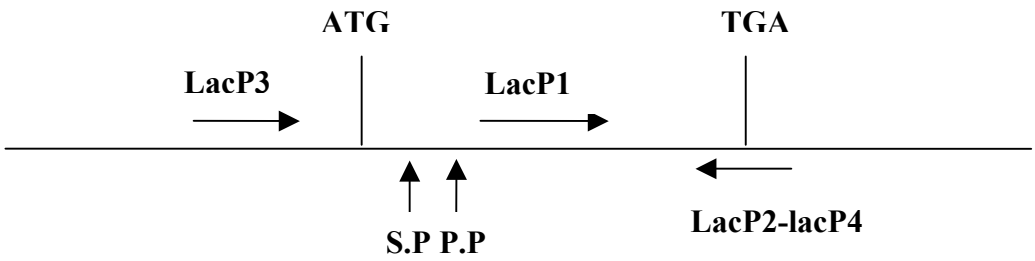

Figure 3.10 The schematic illustration of the primers lacP1, lacP2, lacP3 and lacP4, signal peptide cleavage site (S.P), propeptide cleavage site (P.P).

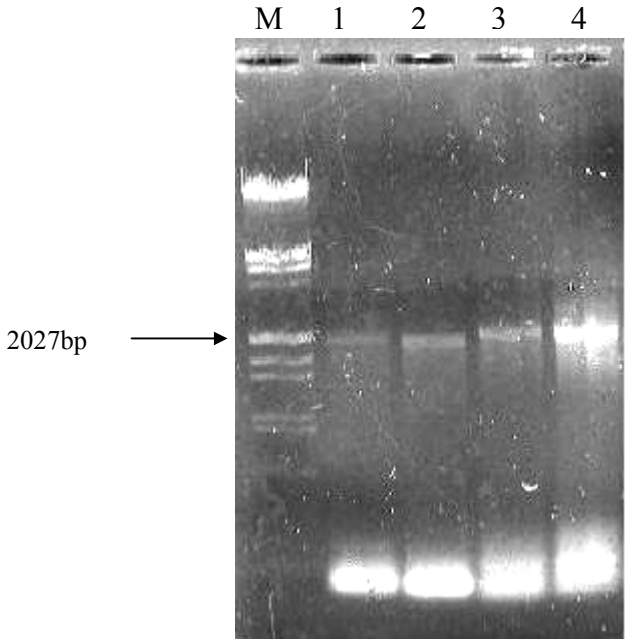

Figure 3.11 PCR with lacP1, lacP2, lacP3 and lacP4 primers. lane 1,2, PCR amplified fragments by lacP1 and lacP2 (1983 bp); lane 3,4 PCR amplified fragments by lacP3 and lacP4 (2151bp); lane M, Molecular size marker: λ DNA/*Eco*RI+*Hin*dIII

3.3.2 Ligation of Laccase Gene onto Vector pAN 52-4 and Transformation into *E. coli* Blue MRF'

pAN52-4 is a fungal expression and secretion vector. It includes *A. niger* glucoamylase gene preprosequence, but pAN52-1 lacks these regions. It was aimed to ligate the lacP1-lacP2 amplified fragment onto the pAN52-4 vector, and lacP3-lacP4 amplified fragment, which include *lccS* gene preprosequence, onto pAN52-1 vector. Because the glucoamylase preprosequence *of A. niger* allowed an 80-fold increase in laccase production in previous studies carried out by Record et al., 2002., it was found acceptable to start the experiments by using pAN52-4 secretion vector.

The laccase gene fragment was cut with *Hin*dIII and was ligated onto expression vector pAN52-4. The vector was dephosphorylated to prevent religation as explained in section 2.2.8.

After ligation of the laccase gene, the plasmid was transformed into *E.coli* XL1 Blue MRF'. Only white colonies were formed because of the lack of a screening marker. The recombinant clones were selected randomly. A total of 20 white colonies were picked up and plasmid DNAs were isolated from all of these colonies as described in the Materials and Methods (section 2.2.12). These isolated plasmids were analyzed by agarose gel electrophoresis (Figure 3.12). The picked colonies were named as G1-G20. The only recombinant plasmid was from G2, as can be seen from the gel.

3.3.3 Isolation of Recombinant Plasmids Containing the Laccase Gene

The plasmid, carrying the laccase gene on vector pAN52-4 was named as pAN524LS (Figure3.15). To check the orientation of the insert PCR amplification was done by primer 315 (reverse primer) and lacP1 (forward primer). If the insert was in the correct orientation this should yield a PCR fragment of 2745 bp (Figure 3.13). Indeed, after the amplification, it was seen that the laccase gene on the vector pAN52-4 was in the correct orientation.

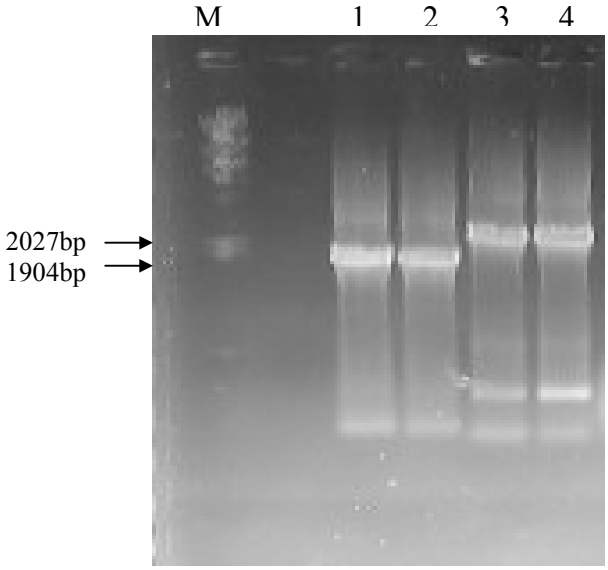

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Figure 3.13 PCR with recombinant plasmid pAN524LS. lane 1, 2, Isolated 1983 bp PCR fragment amplified by primers lacP1 and lacP2 from recombinant plasmid pAN524LS; lane 2, 3, Isolated 2745 bp PCR fragment amplified by 315 and lacP1 primers; lane M, Molecular size marker: λ DNA/*Eco*RI+*Hin*dIII

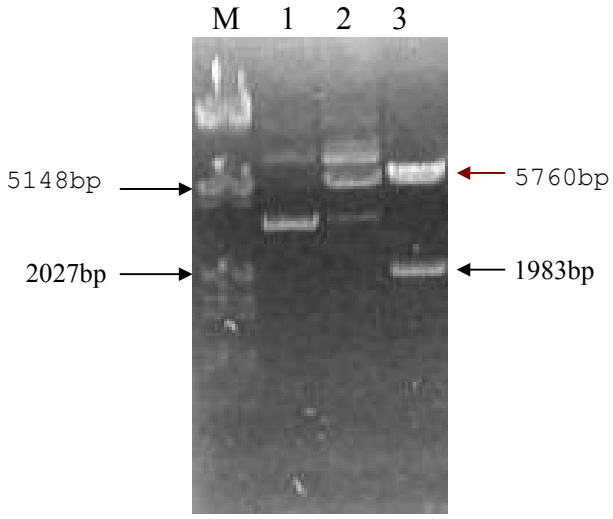

Figure 3.14 Restriction digestion of recombinant plasmid pAN524LS. lane1, *Hin*d III digested pAN52-4; lane2, undigested pAN52-4, lane 3, *Hin*d III digested pAN524LS, 1983 bp insert and 5760bp plazmid pAN52-4

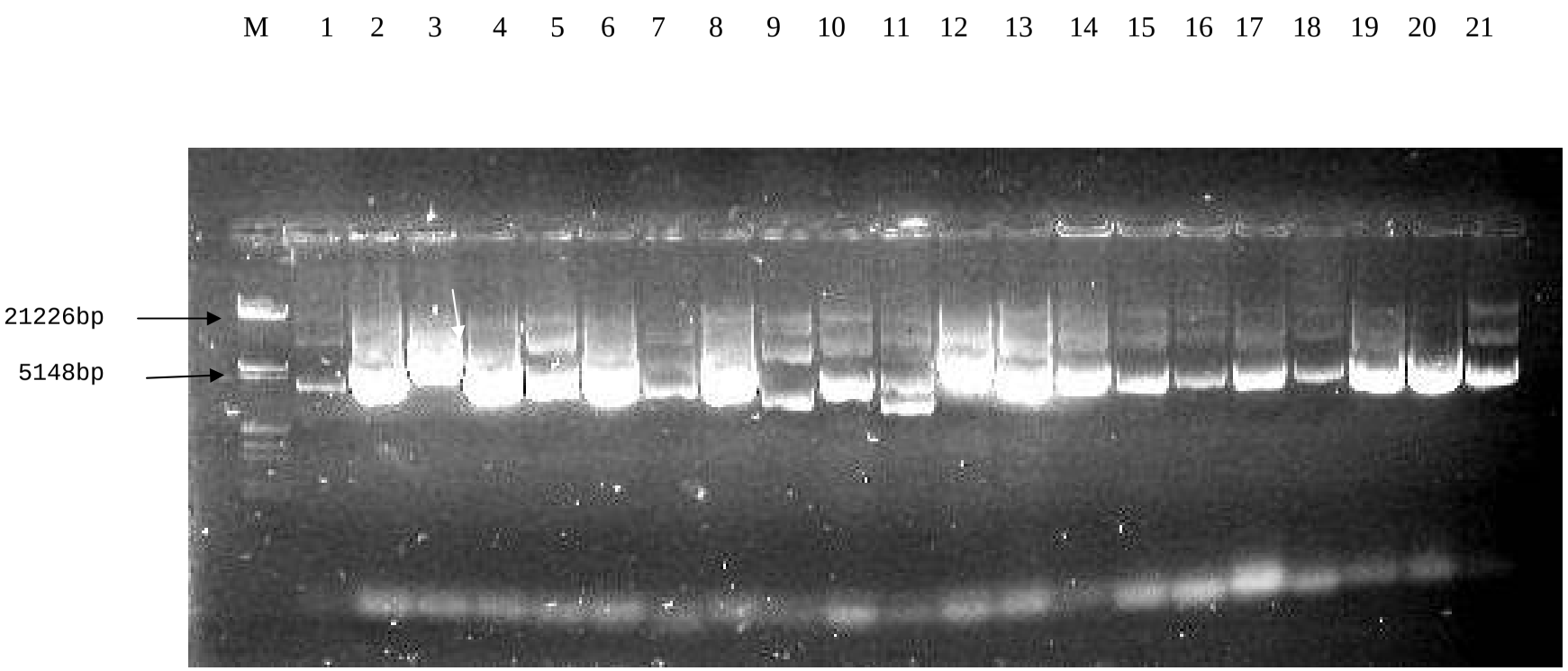

Figure 3.12 Isolated plasmids after ligation lane1, pAN52-4; lane 2 , lane 4-line 21 pAN52-4 plasmids not included insert; lane, 3 pAN524-32. The recombinant plasmid including insert gene would be 7900 bp so it was expected that pAN52-32 to be true recombinants.

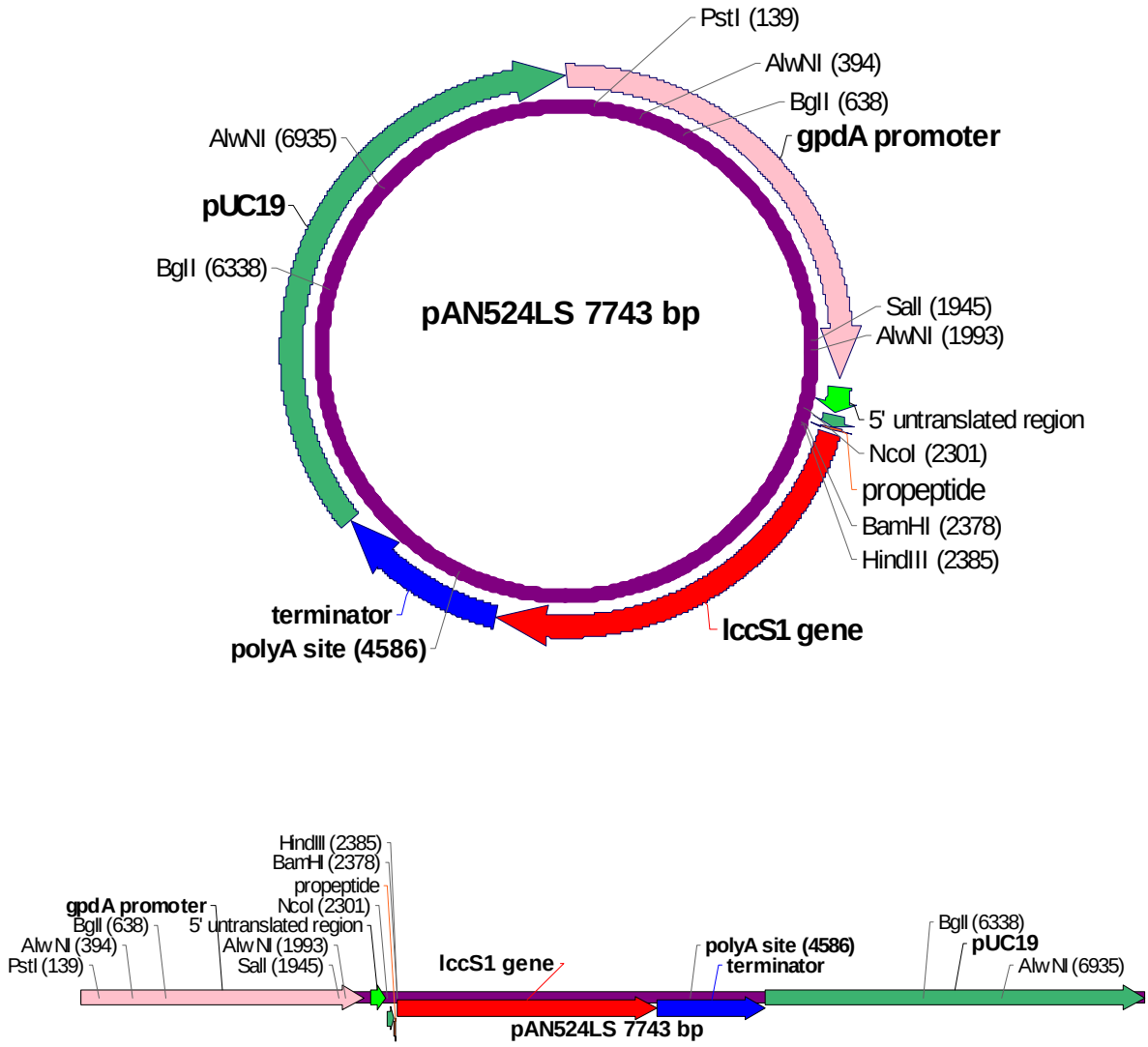

Figure 3.15 Recombinant pAN524LS vector

3.4 Transformation into *Aspergillus sojae* and Screening

3.4.1 Protoplast Transformation

In a co-transformation experiment *A. sojae* ATCC11906 protoplats were transformed with a mixture of selective plasmid pAMDSPYRG, and expression vector pAN524LS containing laccase gene from *S. thermophilum* in a ratio of 1:10 described in section 2.2.10.4. Transformants were selected for their abilities to grow on minimal medium without uridine and uracil. As a result, a total of 5 uridine prototrophic transformants were obtained. In a productive experiment approximately 100 uridine prototrophic transformants were obtained per microgram of expression vector (Record et al., 2002). Comparison of the number of transformants shows the requirement of optimization. To investigate the recombinant transformants that include laccase gene *lccS*, screening of laccase activity and PCR studies were performed.

(a) **(b)**

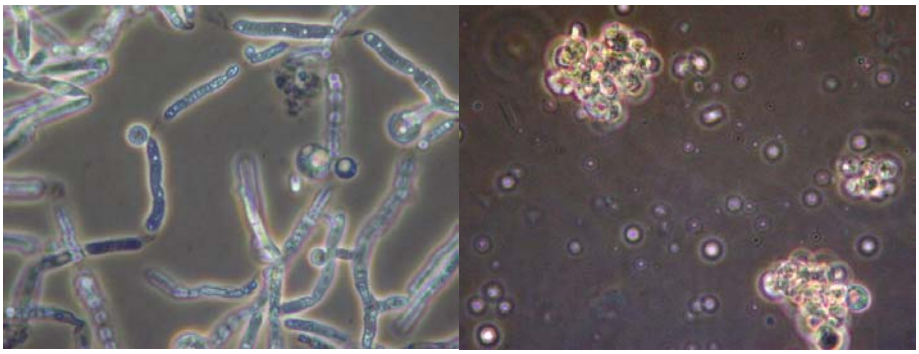

Figure 3.16 Protoplast formation from *A. sojae* mycelium. Photographs were taken at 2 hours after mycelium was resuspended in the Novozyme solution. (a), and at 4 hours after, when the protoplast formation was completed (b).

Table 3.2 Expression and Selection vectors used in *Aspergillus sojae* co- transformation

Expression vectors	Expression cassette	Selection marker	Reference
pAN52-4	*A.nidulans gpd A* promoter and *trpC* terminator	None	Punt et al; 1991
pAN52-1	*A.nidulans gpd A* promoter and *trpC* terminator	None	Punt et al; 1991

Selection Vector	Origin	Selection Marker	Reference
pAMDSPYRG	*A. nidulans* *A. niger*	*amdS* *pyrG*	Verdoes et al., 1993

3.4.2 PCR Studies of Transformants Genomic DNAs

3.4.2.1 Genomic DNA Isolation of Transformants

Aspergillus sojae transformants total genomic DNA was isolated as described in the Materials and Methods section 2.2.1.1. Isolated genomic DNA samples were visualized on agarose gel after electrophoresis (Figure 3.17). Purified DNA samples concentration was calculated according to the formula described in the section 2.2.1.1.

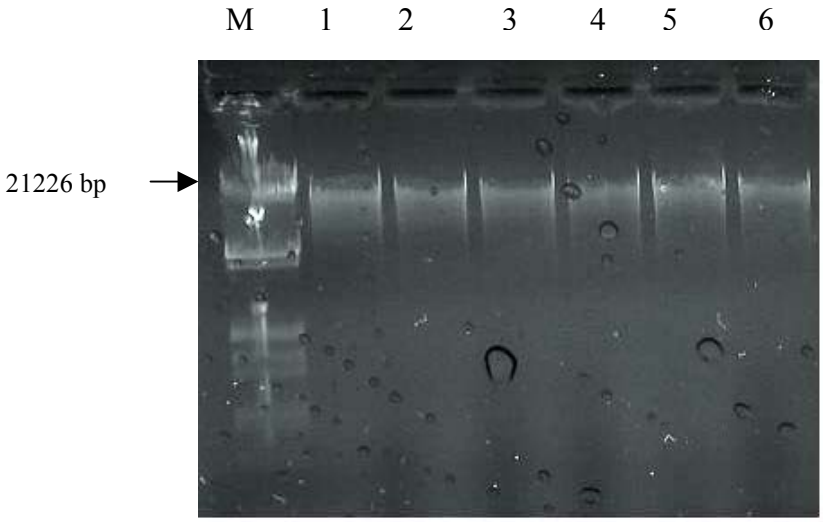

Figure 3.17 Genomic DNA isolation from *Aspergillus sojae* transformants. Genomic DNA of transformants (1, 2, 3, 4, 5), genomic DNA of *Aspergillus sojae* (6). lane M, Molecular size marker: λ DNA/*Eco*RI+*Hin*dIII

3.4.2.2 Ampification of *S. thermophilum* Laccase Gene *lccS* from the Genomic DNA of Transformants

PCR studies were done by using transformants genomic DNAs as a template to analyse the laccase gene *lccS*. For this purpose the appropriate primers lacP1 and lacP2 were used for amplification. *Aspergillus sojae* strain ATCC11906 genomic DNA was used as a negative control and *Scytalidium thermophilum* genomic DNA was used as a positive control. The *lccS* gene was clearly seen in one of the transformants.

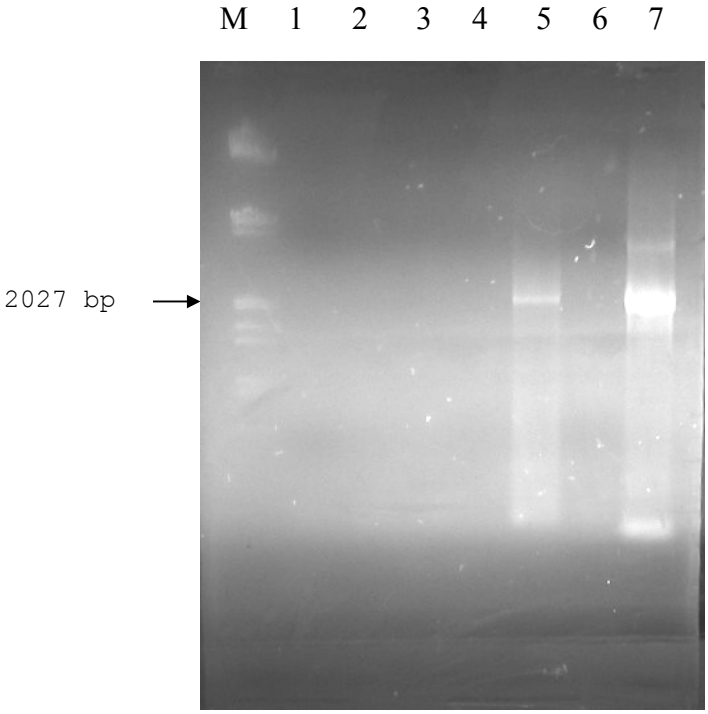

Figure 3.18 PCR analysis of transformants. (1) PCR with *Scytalidium thermophilum* genomic DNA, (2- 5) PCR with genomic DNAs of *Aspergillus sojae* transformants, lane M, Molecular size marker: λ DNA/*Eco*RI+*Hin*dIII

3.4.3 Laccase Activities of the Transformants

Transformant A5, containing the laccase gene *lccS* was tested for laccase activity by growing complete 1% glucose medium without uridine and uracil (Appendix B). The control strain *A. sojae* ATCC11906 (uridine auxotroph) was grown in complete medium including uracil and uridine. Since laccase gene was inserted onto integrative vector pAN52-4 based on a strong constitutive promoter and secretion signal, addition of inducer does not required. 1L Erlenmeyer flasks containing 250 ml culture medium, were inoculated using 5 ml overnight preculture, and incubated at 30 ^{o}C in a shaker incubator (160rpm). Laccase activity was determined by monitoring the oxidation time course of 0.5 mM ABTS at 420 nm (section 2.2.8.6). Extracellular enzyme activity was assayed every 24 hours starting

from the third day of the cultivation. Figure 3.19 shows the laccase activity transformant A5. The maximum laccase activity was 70 U/L at 96 hours. The laccase activity of host strain *A. sojae* ATCC11906 was not be detected (Figure 3.19).

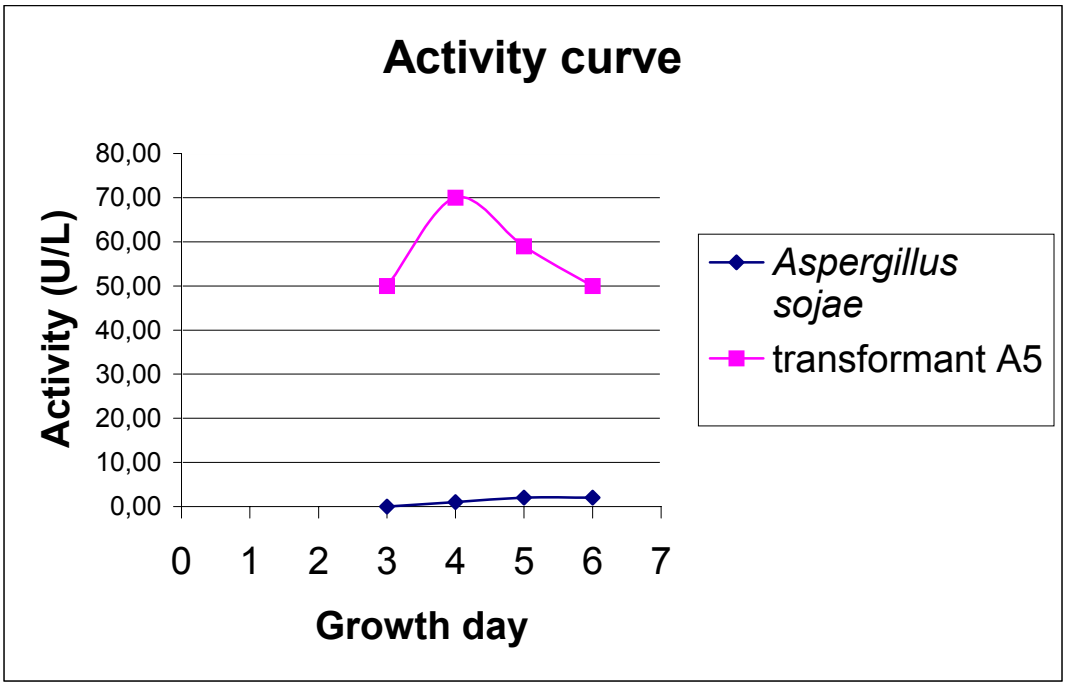

Figure 3.19 Extracellular laccase production by *Aspergillus sojae* transformant A5

According to Z. B. Ögel et al; 2005, *Scytalidium thermophilum* does not produce extracellular laccase. Thus this study, it was possible to produce *Scytalidium thermophilum* laccase gene extracellularly in *Aspergillus sojae*.

In the previous study carried out by Novo Nordish Bio Tech. activity of *Scytalidium thermophilum* laccase gene cloned into *Aspergillus oryzae* was tested by using either syringaldazine or ABTS as substrates and found 2.2 or 4.2 U/ml respectively. This value is higher than the activity of the *S. thermophilum* laccase expressed in *A. sojae*. The optimum conditions can be studied to improve the

stability and extracellular activity of heterologous laccase enzyme by altering pH, tempreture and culture medium contents.

3.5 Bioinformatic Studies on Fungal Propeptides

3.5.1 Multiple Sequence Aligment of Laccase

Comparisons with other laccase proteins were performed in order to detect the sequence conservation patterns. For this aim, multiple sequence alignments were undertaken with the help of software ClustalW and NCBI web sites. The results of the alignment analysis are summarized in Figure 3.19. The fungal laccases contain several highly conserved ungapped regions, distributed almost throughout the length of the proteins (Kumar et al., 1998). The copper-ligating residues in the *S. thermophilum* laccase are housed in regions that are conserved across all laccases. A total of 10 histidines and 1 cysteine are completely conserved as they serve as the copper ligands. These residues are housed in the four conserved regions L1-L4. Specifically, the motif HWHG in the region L1 is equivalent to the motif HLHG in the region L3. Similarly, between the region L2 and L4, Gly is the common conserved residue at positions 1 and 16, while His is the common conserved residue at positions 6 and 8 (Kumar et al., 2003). At the C-terminal some laccases from ascomycetes *N. crassa* (Germann et al., 1988), *P. anserine* (Fernandez-Larrea, J., and U. Stahl, 1996), *M. thermophila* (Bulter et al.,) are processed at the processing site Asp-Ser-Gly-Leu, which is the conserved region of these laccases. Interestingly, the same kind of sequence is found at the C-terminal ends in contrast to some other ascomycetes laccases with no C-terminal extensions. In the laccases of *Cryphonectria parasitica* (Choi et al. 1992), *Botrytis cinerea* (Cantone et al., 1993), *Glomerella lagenarium* (Tsuji et al., 2001), and *Gaeumannomyces graminis var. tritici* (Litvintseva et al., 2002), the last four C- terminal amino acids are Asp, Ser, Gly, and Leu/Ile/Val. It is believed that this C- terminus end is conserved because it

is related to the catalytic activity of ascomycete laccases (Kiiskinen and Saloheimo, 2003). When we look at the C- terminus of the *S. thermophilum* laccase after the alignment of amino acid sequence with some other fungal sequence, we see that it may also require the removal of a C-terminal peptide in addition to the posttranslational removal of a signal sequence and propeptide cleavage at the N-terminus (Figure 3.20) like other ascomycete laccases.

```
1.  ----MRLSNALVLVAACIS---------------------------------------- 15
2.  ---MSRFHSLLAFVVASLA---------------------------------------- 16
3.  --MK--FLGIAALVAGLLAPSLVLGAPAPGTEGVNLLTPVDKRQDSQAERYGGGGGGGCN 56
4.  --MKRFFINSLLLLAGLLN-SGALAAPSTHPR-SNPDILLERDDHSLTSRQ-----GSCH 51
5.  MDVTKSLLCFISFVAFLLFS---------------------------------------- 20
                :     ::.  :

1.  ----------------------SVVAKTR--TFDFDLVNTRLAPDGFERDTVVIN-GEFP 50
2.  ----------------------AVAHAGIGPVADLTITNAAVSPDGFSRQAVVVN-GGTP 53
3.  SPTNRQCWSPGFNINTDYELGTPNTGKTRRYKLTLTETDNWLGPDGVIKDKVMMVNDNII 116
4.  SPSNRACWCSGFDINTDYETKTPNTGVVRRYTFDITEVDNRPGPDGVIKEKLMLINDKLL 111
5.  ----------------------SVAEANKAHHHEFIIQATKVKRLCETHNSITVN-GMFP 57
                          . .         :             :: : :  .

1.  GTLIQVNKGDSVRIPLHNKLTSPTMRRSVSIHWHGFFQARTSGQDGPSFVNQCPQPPNTT 110
2.  GPLITGNMGDRFQLNVIDNLTDHTMLKSTSIHWHGFFQKGTNWADGPAFINQCPISSGHS 113
3.  GPTIQADWGDYIEITVINKLKS----NGTSIHWHGMHQRNSNIQDGVNGVTECPIPPRGG 172
4.  GPTVFANWGDTIEVTVNNHLRT----NGTSIHWHGLHQKGTNYHDGANGVTECPIPP-GG 166
5.  GPMLVVNNGDTLVVKVINRARY-----NITIHWHGVRQMRTGWADGPEFVTQCPIRPGSS 112
    *. :  : ** . : : :.    :   . :*****. *  :.  **   :.:**  .

1.  FTYEFSVAEQSGTFWYHSHLSTQYCDGLRGAFIVYDPRDPLRHLYDVDDESTVITLAEWY 170
2.  FLYDFQVPDQAGTFWYHSHLSTQYCDGLRGPFVVYDPNDPAADLYDVDNDDTVITLADWY 173
3.  SKVYRWRATQYGTSWYHSHFSAQYGNGIVGPIVING---PASANYDVDLG--PFPLTDYY 227
4.  SRVYSFRARQYGTSWYHSHFSAQYGNGVSGAIQING---PASLPYDIDLG--VLPLQDWY 221
5.  YTYRFTIQGQEGTLWWHAHSSWLRAT-VYGSLLVFPP-AGSSYPFTKPHRNVPLLLGEWW 170
             * ** *:*:* *     .: *.: :  .       :     .  : * :::

1.  HILAPDATNEFFSSGIIPV-QDSGLINGK-GRFNGGPLTPFAVVNVRRGKRYRLRVIAIS 228
2.  HVAAKLGP--AFPLG-----ADATLINGK-GRSPSTTTADLTVISVTPGKRYRFRLVSLS 225
3.  YDTADRLVLLTQHAG-PPP-SNNVLFNGF-AKHPTTGAGQYATVSLTKGKKHRLRLINTS 284
4.  YKSADQLVIETLAKGNAPF-SDNVLINGT-AKHPTTGEGEYAIVKLTPDKRHRLRLINMS 279
5.  DANPVDVLRESIRTGGAPNNSDAYTINGQPGDLYKCSSQDTTVVPINVGETILLRVINSA 230
       .          *  .   :   :**  .          : : :  .:   :*::  :

1.  CRPFFTFSVDNHSLVFMEADGVEHDPVEVQNVDIYAAQRVSVILHANQPIDNYWIRAPMT 288
2.  CDPNHTFSIDGHNMTIIETDSINTAPLVVDSIQIFAAQRYSFVLEANQAVDNYWIRANPS 285
3.  VENHFQLSLVNHSMTIISADLVPVQPYKVDSLLLGIGQRYDVIIDANQAVGNYWFN--VT 342
4.  VENHFQVSLAKHTMTVIAADMVPVNAMTVDSLFMAVGQRYDVTIDASQAVGNYWFN--IT 337
5.  LNQPLFFTVANHKLTVVGADASYLKPFTTNVIVLGPGQTTDVLITGDQPPNRYYMAARAY 290
          .::  *.:..: :*     .  .: : :  .*  .. : ..*. ..*:: :

1.  GGNPDRNPNLNISLTLAILRYHGARHVEPTTVNVP--GHKLLDQE-MHPIR--------- 336
2.  FGNVGFTGGINS----AILRYDGAAAIEPTTTQTT--STEPLNEVNLHPLV--------- 330
3.  FGGNDLCGTSDNKYPAAIFRYQGAPKALPTNKGVAPPDHQCLDLNDLKPVL--------- 393
4.  FGGQQKCGFSHNPAPAAIFRYEGAPDALPTDPGAAPKDHQCLDTLDLSPVV--------- 388
5.  QSAQN--APFGNTTTTAILQYKSAPCCGVGGGSGTKKGNSFKPIMPILPAYNDTNTVTRF 348
     .            . **::*..*          .  . .      : *
```

```
1.  --------------------------------QEG-PGKLGDGPPDKHITLNIAQPN---- 360
2.  --------------------------------ATAVPGSPAAGGVDLAINMAFNFNG---- 355
3.  --------------------------------QRSLNTNSIALNTGNTIPITLDG------ 416
4.  --------------------------------QKNVPVDGFVKEPGNTLPVTLHVDQAAAP 417
5.  SQSFRSLRRAEVPTEIDENLFVTIGLGLNNCPKNFRSRRCQGPNGTRFTASMNNVSFALP 408
                                                    .  :   :

1.  --------APFFDINGISYISPTVPVLLQILSGAKRPEDVLP---SEQIFFVPKNSLIEV 409
2.  --------TNFF-INGASFTPPTVPVLLQIISGAQNAQDLLP---SGSVYSLPSNADIEI 403
3.  --------FVWRVNGTAININWNKPVLEYVMTGNTN-YSQSD---NIVQVEGVNQWKYWL 464
4.  H------VFTWKINGSAADVDWDRPVLEYVMNNDLSSIPVKN---NIVRVDGVNEWTYWL 468
5.  SNYSLLQAHHHGIPGVFTTDFPAKPPVKFDYTGNNISRSLYQPDRGTKLYKLKYGSRVQI 468
                 .         * :    ..   .        .            :

1.  NIPG-----EGAHPFHLHGHNFDVVLASNDDTFNFKNPPRRDVY----PINGGN------ 454
2.  SFPATAAAPGAPHPFHLHGHAFAVVRSAGSTVYNYDNPIFRDVVSTGTPAAGDN------ 457
3.  IENDPDGAFSLPHPIHLHGHDFLILGRSPDVTAISQTRYVFDPAVDMARLNGNNPTRRDT 524
4.  VENDPEGRLSLPHPMHLHGHDFFVLGRSPDVSPDSETRFVFDPAVDLPRLRGHNPVRRDV 528
5.  VLQDTGIVTPENHPIHLHGYDFYIIAEGFGNFNPKKDTAKFNLEDPPLRNTVGVPVN--- 525
               **:****: * ::  . .     .     :

1.  --------TTFRFFTDNPGAWFLHCHIDWHLEAGLAIVFAEAPEDNVSGPQSQITPQDWL 506
2.  --------VTIRFRTDNPGPWFLHCHIDFHLEAGFAVVFAEDIPDVAS---ANPVPQAWS 506
3.  AMLPAKGWLLIAFRTDNPGSWLMHCHIAWHVSGGLSNQFLERAQDLRN-SISPADKKAFN 583
4.  TMLPARGWLLLAFRTDNPGAWLFHCHIAWHVSGGLSVDFLERPDELRG-QLTGESKAELE 587
5.  ------GWAVIRFIADNPGVWIMHCHLDAHISWGLAMAFLVE--NGNGVLQTIEQPPHDL 577
            : * :**** *::***:  *:. *::  *     :  .   :

1.              DLCPEYNAIEPEFQ---------------------- 520
2.              DLCPTYDARDPSDQ---------------------- 520
3.              DNCDAWRAYFPDNAPFPKDDSGLRSGVKAREVKMKW 619
4.              RVCREWKDWEAK-SPHGKIDSGLKQ--RRWDA---- 616
5.              PVC--------------------------------- 580
```

Figure 3.20 Sequence alignment of *Arabidopsis thaliana* (1), *Trametes versicolor* (2), *Neurospora crassa* (3), *Scytalidium thermophilum* (4), *Agaricus bisporus* (5) species copper-binding domains in laccase proteins. The four conserved metal-binding residues for copper types 1, 2 and 3, are shown in red color.

```
                        ↓
MaL   WPTN-PYPKIDSGL    KP-RRWVEES-EWLVR-  623
PaL   WPTN-PFPKIDSGL    KV-KKWVGEHPDWYIKN  621
NcL   FPDNAPFPKDDSGL    RSGVKAREVKMKW----- 619
ScL   EAK-SPHGKIDSGL    KQ--RRWDA----      616
```

Figure 3.21 Alignment of laccase sequence from *M. albomyces* (MaL), *P. anserina* (PaL), *N. crassa* (NcL) and *S. thermophilum* (ScL) possible C- terminus amino acid cleavage site.

3.5.2 N-terminal Processing of Fungal Propeptides

3.5.2.1 Dibasic Processing

The kexin family of proteases recognizes dibasic amino acid motifs and removes N-terminal propeptides. These kinds of proteases recognize Lys/Arg-Arg amino acid site. They are located both in trans golgi network or prevocuolar compartment. In filamentous fungi kexB recognize X-Arg ↓ where X can be Arg, Lys, Ala, Pro and Ser. (Table 3.3). Usually basic or aliphatic P_4 residue is needed for the effective activity of the enzyme.

In yeast kexin family *A. niger* endogalacturonase (*Pga*) family contain a dibasic cleavage site at the N-terminal propeptide except the *Pga*II which has a single arginine residue preceding the cleavage site. In *Aspergillus* species glucoamylase protein leader peptide sequence seems to be conserved. But this situation is not valid for all proteins. For example, unlike the lignin peroxidases, the manganese peroxidases of *P. chrysosporium* do not contain an apparent propeptide region despite both type of enzymes catalyse the H_2O_2-dependent oxidation of lignin (Gold & Alic, 1993). In the same way, nucleotide sequence-deduced putative amino acid sequences of the cellobiohydrolase I (CBH I) and CBH *Trichoderma reesi*, also reveal different potential target sites for posttranslational proteolytic modification, e.g., -R-A / -Q-CBHI; -E-R- / -Q-, CBH II (Figure 3.22). In spite of cellobiohydrolase II and I have the same function the processing sites are not related. Here, CBH I contains a processing target consisting of an only basic-hydrophobic amino acid sequence (-R-A- -Q).

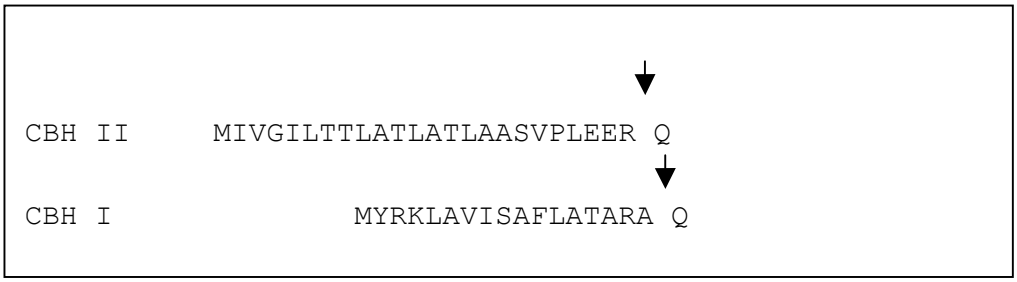

Figure 3.22 The processing preference of CBH I and CBH II. The arrows indicate the N-terminal amino acid of the secreted protein.

Fungal proteins that synthesized as prepropeptide, were found using the NCBI web site. In all, 134 fungal proteins were found. They were analyzed and categorized as the processing site of their propeptide. 20 of all show monobasic processing, and 54 of all show dibasic processing. The other 60 cannot be inserted in either two categories.

15 protein examples with dibasic processing were chosen and shown in the Table 3.4. Unlike the chitin deacetylase, other proteins are cleaved from K-R position. 5 of the examined protein possess Leu at the P4 position, where 3 of Val, 4 of Ile and 3 of Arg amino acids. This is appropriate for recognition by kexin family proteases. Among the data shows the proteins leader peptides that include dibasic motif appears to be most common among glucoamylases and lignin peroxidases (Table 3.4).

After the cleavage of preprosequence, the rest of N-termini mature part of fungal extracellular proteins have important role in export process. On the basis of protein analyzed, it is clear that P1' residue at the N-termini of the mature sequence is conserved. At the P1' position 6 different amino acids are determined. Ala appears to be the most abundant residue at the P1' position with ratio of almost 53.3 %. Apart from Ala amino acid, Val and Gly residue also appear in a ratio of 13.3 %. The other hydrophobic residues are not much encountered. In the total of the first 5 amino acid of mature proteins like the first position, Ala amino acid is the most abundant residue with a ratio of 20% (Table 3.8). The positively charge amino acids does not appear as in theP1' position is.

3.5.2.2 Monobasic Processing

An important number of fungal proteins include monobasic cleavage site. Dibasic processing is seen in specific proteins like ligninases and glucoamylases, on the contrary monobasic processing appears in a wide range of proteins (Table 3.5).

The length of the propeptide changes from 6 to 23 amino acids. The pro-hexapeptide of the polygalcturonase II of *A. niger* and *A. tubigensis* are completely conserved. *A. giganteus* propeptide region include extra two residues but the rest of the cleavage region shows high similarity. A comparison of the propeptide regions of proteins wih a monobasic cleavage site at their leader/mature protein junction suggests that the proposed pro-hexapeptide (Bussink et al., 1991b) is not completely conserved. While α-sarcin and restrictocin are 86% homologous (Lamy & Davies, 1991; Wnendt et al., 1993), the Glu at position P3 and Val at position P8 of α-sarcin is replaced byAsp and Ala respectively in the restrictocin.

The majority of the monobasic processing proteins show at the P2 position preference of Ala amino acid. This motif is similar to the recognition site of kexB. The others generally prefer amino acids that have hydrophobic side chains at the P2 position is much different from P2 amino acid preference of the dibasic processing from the point of view of that in the dibasic processing P2 amino acid should be R or K, both of which have hydrophilic side chains. But at the P4 position, the existance of amino acids having aliphatic side chains shows similary to dibasic processing.

Monobasic processing possessed proteins N- termini mature site is similar to that of the proteins possessing dibasic processing. At the P1' position and in the total of five amino acids Ala is shown the higher ratio (Table 3.9). Gly, D and Ser follow the Ala. Positively charged amino acids at P1' position and in the total of first five amino acids are not much encountered.

3.5.3 Propeptide Regions of Multicupper oxidases

In the multicupper oxidase family the propeptide cleavage site regions varies. The majority of these proteins do not show monobasic or dibasic processing. The length of these propeptides range from approximately 9-30 residues (Table 3.10).

These enzymes are not classified in short or long propeptides groups. Only laccase of *P. anserine* and glucose oxidase of *A. niger* possess monobasic and pyranose 2-oxidase of *T. versicolor* possess dibasic processing. At the same time only glucose oxidase of *A. niger* possess 6 amino acid short propeptide region.

When laccase proteins are compared with each other no important motif is seen in both leader peptide and mature N-terminus regions. *S. thermophilum* laccase shows similarity to *E. nidulans* (perfect form of *A. nidulans*) laccase in signal peptide processing region. But there isn't important similarity between propeptide processing site accept they are both cleaved from serine amino acid which has polar, uncharged R groups.

Since the amino acid terminal sequence of the mature *S. thermophilum* laccase protein was not determined, the exact length of the propeptide is not certain. The signal peptide and propeptide cleavage sites were only estimated by Novo Nordisk based on *N. crassa* laccase protein amino acid sequence. However, in spite of the high similarity of the mature amino acid sequence of the two protein, in the preprosequence they do not show important similarity.

S. thermophilum laccase protein possesses a high similarity with *E. nidulans* laccase protein preprosequence. The probable signal peptide cleavage site was also found by using Expasy web site. Both the sequence aligment, and bioinformatic results indicate same signal peptide cleavage site L-A / A-P. *S. thermophilum* laccase protein propeptide cleavage site is not clear. The sequence aligment point out the Ser amino acid. In spite the monobasic processing is not widespread among the muticopper oxidase family, *S. thermophilum* laccase protein can be also processed from Arg residue.

```
En.   MMKSFFSAAALLLGLVAPSAVLAAPSLPGVPREVTRDLLRPVEERQSSCHTAA 53
St.   -MKRFFINSLLLLAGLLNSGALAAPSTH--PRSNPDILLERDDHSLTSRQGSC 50
       ** **  : ***. :  *..*****  . **. .  **.  :.  :* : :.
```

Figure 3.23 Similarity of signal peptide processing site between laccases of *E. nidulans* and *S. thermophilum*. 1. *E. nidulans*, 2 *S. thermophilum*

Table 3.3 Kexin family

	Protease	Cleavage site specificity	Cellular location	Reference
mammalian kexins	Furin (PACE)	-Arg-X-Lys/Arg-Arg↓ I) P_1 arginine is essential, lysine is cleaved less efficient II) At least 2 residues of P_2, P_4, and P_6 have to be basic III) No P_1' amino acid with hydrophobic side chain	TGN/endosomal	Rockwell et al., 2002
	PC1 / PC3	-Lys/Arg-Arg↓	secretory granules	Seidah and Chrétien, 1997
	PC2	-Lys/Arg-Arg↓	secretory granules	
	PACE4	-Arg-X-Lys/Arg-Arg↓	TGN/endosomal	Rockwell et al., 2002
	PC4	-Lys-X-X-Arg↓	Testicular germ cells and ovarian macrophages	Basak et al., 2004
	PC5 / PC6	-Arg-X-X-Arg↓X-Lys-	A: secretory granules B: TGN/endosomal	Seidah and Chrétien, 1997
	PC7 (LPC)	-Lys/Arg-Arg↓	TGN/endosomal	Munzer et al., 1997
yeast kexins	Kex2	-Lys/Arg-Arg↓ I) Extremely stringent for P_1 arginine II) Proline is tolerated at P_2, but cleaved less efficient III) Basic or aliphatic P_4 residue needed	TGN/PVC	Rockwell et al., 2002
	Krp1	-Lys/Arg-Arg↓	N.D.	Davey et al., 1994
	Xpr6	-Lys/Arg-Arg↓	N.D.	Enderlin and Ogrydziak, 1994
k.f.f.	KexB	X-Arg↓ where X can be Arg, Lys, Ala, Pro and Ser	N.D.	Chapter 4

N.D = Not determined; TGN = Trans Golgi Network; PVC = prevacuolar compartment; k.f.f. = kexins of filamentous fungi

Table 3.4 Propeptide with a Dibasic Processing Site

Species	Protein	Leader peptide
C.neoformans	chitin deacetylase	MYGHLSLSALSLFAVVAA/APFRESWLQPRDSPVSQLFRR^TAPDP
F.neoformans	chitin deacetylase	MYGHLSLSTLSLLAVVAA/APFPESWLQPRDSDVSQLFRR^GAPDP
M. verrucaria	bilirubin oxidase	MFKHTLGAAALSLLFNSNA/VQASPVPETSPATGHLFKR^VAQIS
H. jecorina	acetylxylan esterase precursor	MPSVKETLTLLLSQAFLATG/SPVDGETVVKR^QCPAI
N. crassa	glucoamylase precurso	MHLVSSLLVVGAAFQAVLG/LPDPLHEKRHSDIIKR^SVDSY
A. niger	polygalacturonase	MVTSSSVIGLTLWAALVSA/SPVADPLVTPAPKLEDLEKR^ATSCT
A. album	chitinase	MLSFVKKSIALVAALQAVTALA/TPISSEAGVEKR^GSGFA
P. chrysosporium	Lignin peroxidase LG6).	MALKQLAAAVALALSİQAAQG/AAVKEKR^ATCSN
P. chrysosporium	LG2	MAFKQLFAAITVALSLTAANA/AVVKEKR^ATCAN

Table 3.4 Propeptide with a Dibasic Processing Site

Species	Protein	Leader peptide
P. chrysosporium	LIPB B	MAFKQLFAAISLALSLSAANA/AAVIEKR^ATCSN
	IPA A	MAFKQLVAAISLALSLTTAA/AVVKEKR^ATCSN
	H8 LPOA	MAFKQLFAAISLALLLSAANA/AAVIEKR^ATCSN
	LG5 GLG5	MAFKKLLAVLTAALSLRAAQG/AAVEKR^ATCSN
	GLG3	MAFKQLFAAISLALSLSAANA/AAVIEKR^ATCSN
	GLG4	MAFKQLLAALSVALTLQVTQA/APNLDKR^VACPD

Table 3.5 Propeptides with a Monobasic Processing Site

Species	Protein	Leader Peptide
N. crassa	catalas	MRVNALLPLSGLIGTALA/ACPFADPSALGRRAEGGEVDAR^QRLKE
A. flavu	polygalacturonase	MQLLQSSVIAATVGAALVAA/VPVELEAR^DSCTF
A. oryzae	polygalacturonase	MQLLQSSVIAATVGAALVAA/VPVELKAR^GSCTF
A. niger	endopolygalacturonase	MHSFASLLAYGLVAGATFASA/VPVELKAR^GSCTF
A. niger	endopolygalacturonase	MHSFASLLAYGLVAGATFASA/SPIEAR^DSCTF
A. tubigensis	polygalacturonase II	MHSFASLLAYGLAASATLASA/SPIEAR^GSCTF
A. giganteus	α-sarcin	MVAIKSLVLVALTAVTALA/VPSPLEAR^AVTWT
A..restrictus	restrictocin	MVAIKNLFLLAATAVSVLA/APSPLDAR^ATWTC
C. carbonum	polygalacturonase	MVAYALTSMLLSAGALVAAAP/SGLDAR^DGCTF

Table 3.5 Propeptides with a Monobasic Processing Site

Species	Protein	Leader Peptide
A. aculeatus	xylanase	MVQIKAAALAVLFASNVLA/NPIEPR^QASVSI
A. niger	xylanase II	MLTKNLLLCFAAAKAALA/VPHDSVAQRSDALHMLSER^STPSS
H. jecorina	hydrophobin	MKFFAIAALFAAAAVA/QPLEDR^SNGNG
T. punescens	pyranoseoxidase	MSTSSSDPFYNFAKTSFKSAAAQKASAT/SLPPLPGPDQK^VPGMD
A. niger	invertase	MKLQTASVLLGSAAA/ASPSMQTR^ASVVI
A. niger	glucose oxidase	MQTLLVSSLVVSLAAA/LPHYIR^SNGIE
Fusarium sp. NRRL2903	galactose oxidase	MKHLLTLALCFSSINAVA/VTVPHKAVGTGIPEGSLQFLSLR^ASAPI

Table 3.6 The First Five Residues at the N-terminus of Filamentous Fungal Extracellular Proteins Possessing Dibasic Processing

N-Terminal Residue	Overall Percentage
Ala	20
Ser	14.7
Thr	13.3
Cys	13.3
Asn	9.3
Pro	8
Asp	5.3
Val	4
Gly	4
Gln	2.7
Ile	2.7
Phe	1.3
Tyr	1.3

Table 3.7 The First Five Residues at the N-terminus of Filamentous Fungal Extracellular Proteins Possessing Monobasic Processing

N-Terminal Residue	Overall Percentage
Ser	17.5
Thr	13.75
Gly	10
Cys	8.75
Ala	7.5
Phe	7.5
Val	6.25
Asp	5
Ile	3.75
Pro	3.75
Asn	3.75
Trp	2.5
Gln	2.5
Glu	2
Leu	1.25
Met	1.25
Arg	1.25
Lys	1.25

Table 3.8 The First Residues at the N-terminus of Filamentous Fungal Extracellular Proteins Possessing Dibasic Processing

N-Terminal Residue	Overall Percentage
Ala	53.3
Gly	13.3
Val	13.3
Thr	6.6
Ser	6.6
Gln	6.6

Table 3.9 The First Five Residues at the N-terminus of Filamentous Fungal Extracellular Proteins Possessing Monobasic Processing

N-Terminal Residue	Overall Percentage
Ala	25
Gly	18.75
Asp	18.75
Ser	18.75
Gln	12.5
Val	6.25

Tablo 3.10 Propeptide cut sites of Multicupper oxidase

Species	Protein	Leader Peptide
N. crassa	laccase	MKFLGIAALVAGLLAPPLVLA/APAPGTEGVNLLTPVDKRQDSQAERYGG^GGGGG
P. anserine	laccase	MSTSSSDPFFNFTKSSFRSAAAQKASA/TSLPPLPGPDK^KVPGM
E. nidulans	laccase	MMKSFFSAAALLLGLVAPSAVLA/APSLPGVPREVTRDLLRPVEERQSS^CHTAA
T. versicolor	pyranose 2- oxidase	MFKHTLGAAALSLLFNSNA/VQASPVPETSPATGHLFKR^VAQIS
T. hirsuta	pyranose 2-oxidase	MSASSSDPFHSFAKTSFTSKAAKRATAH/SLPPLPGPG^DLPPG
C. glabrata	iron transport multicopper oxidase FET3	MMVPLLLSTYFITAVYG/
A. niger	glucose oxidase	MQTLLVSSLVVSLAAA/LPHYIR^SNGIE
S. thermophilum	laccase	MKRFFINSLLLLAGLLNSGALA/APSTHPRSNPDILLERDDHSLTS^RQGSC

CHAPTER 4

CONCLUSIONS

Cloning of *Scytalidium thermophilum* laccase gene will give an opportunity to overexpress the enzyme for future use in biotransformations and to be able to perform site-directed mutagenesis. In this study, PCR cloning studies were performed on the laccase gene of *Scytalidium thermophilum.*

Scytalidium thermophilum laccase gene (*lccS*) consists of an open reading frame of 2092 bp containing five introns. The gene encodes a protein of 616 amino acid that contains a signal sequence of 21 amino acids and a propeptide of 24 amino acids.

The lccS1 gene was ligated onto pAN52-4 vector without its signal and propeptide regions. This resulted in a construct where the *lccS* gene is fused to the signal-pro-peptide region of the *A. niger* glucoamylase gene. Furthermore the gene was put under the control of the constitutive gdpA promoter to allow high-level expression in the to allow high level expression in the presence of glucose & absence of an inducer. Accordingly, it was possible to express *S. thermophilum* laccase in *A. sojae* at a level of 70U/L, which is within the limits of laccase production in the literature.

In the future studies, heterologous expression in *A. sojae* will be optimized and the gene will be used in mutagenesis. This system will also allow the use of recombinant *A. sojae* culture supernatants in biocatalysis.

REFERENCES

Alexandre, G., Zhulin, I.B., 2000. Lactases are widespread in bacteria. Trends Biotechnol. 18, 41-42.

Alkorta, I., Garbisu, C., Llama, M. J., and Serra, J. L., 1998. Industrial applications of pectic enzymes: a review. *Process Biochem*. 33: 21-28

Aramayo, R., and W. E. Timberlake, 1990. Sequence and molecular structure of the *Aspergillus nidulans yA* (laccaseI) gene. Nucle,c Acids Res. 18:3415

Archer, D. B., 2000. Filamentous fungi as microbial cell factories for food use. *Curr. Opin. Biotechnol.* 11: 478-483.

Atlow, C. S., Bonadonna-Aparo, L., Klibanov, M. A., 1984, "Dephenolization of Industrial Wastewaters Catalysed by Polyphenol oxidase", *Biotechnology and Bioengineering*, Vol. XXVI, p. 599-603

Baker, D., Shiau, A. K., and Agard, D. A., 1993. The role of pro regions in protein folding. *Curr. Opin. Cell Biol.* 5: 966-970.

Bell, A.A., Wheeler, M.H., 1986. Biosynthesis and functions of fungal melanins. Annu. Rev. Phytopathol. 24, 411–451. Butler, M.J., Day, A.W., 1998. Fungal melanins: a review. Can. J. Microbiol. 44, 1115–1136.

Bollag J-M, Leonowicz, 1984;A. Comparative studies of extracellular fungal laccases. Appl Environ Microbiol 48:849-54

Butler, M.J., Lazarovits, G., Higgins, V.J., Lachance, M.-A., 1989. Identi.cation of a black yeast isolated from oak bark as belonging to genus Phaeococcomyces sp. Analysis of melanin produced by the yeast. Can. J. Microbiol. 35, 728–734.

Cardenas W. Dankert JR., 2000. Cresolase, catecholase and laccase activities in haemocytes of the red swamp crayfish. Fish Shellfish Immunol 10:33-46

Chang, P.-K., Ehrlich, K.C., Yu, J., Bhatnagar, D., Cleveland, T.E., 1995. Increased expression of *Aspergillus parasiticus aflR*, encoding a sequence-specific DNA binding protein, relieves nitrate inhibition of aflatoxin biosynthesis. Appl. Environ. Microbiol. 61, 2372–2377.

Chang, P.-K., Yu, J., Bhatnagar, D., Cleveland, T.E., 1999. The carboxy-terminal portion of the aflatoxin pathway regulatory protein AFLR of *Aspergillus parasiticus* activates *GAL1* :: *lacZ* gene expression in *Saccharomyces cerevisiae*. Appl. Environ. Microbiol. 65, 2508–2516.

Choi GH, Larson TG. Nuss DL., 1992. Molecular analysis of the laccase gene from the chestnut blight fungus and selective suppression of its expression in an isogenic hypovirulent strain. Mol. Plant Microb Interact 5:119-128

Claus H., 2003. Laccases and their occurrence in prokaryotes. Arch. Microbiol. 179, 145-150

Clutterbuck AJ., 1972. Absence of laccase from yellow-spored mutants of *Aspergillus nidulans*. J. Gen Microbiol 70:423-435

Clutterbuck AJ., 1990. The genetics of conidiosore pigmentation in *Aspergillus nidulans*. J. Gen Microbiol 136:1731-1738

Decker. H., Terwilliger, N., 2000. Cops and robbers: putative evolution of copper oxygen-binding proteins. J: Exp.Biol 203, 1777-1782

Duran, N., Rosa, A. M., D'Annibale, A., and Gianfreda, L., 2002, "Applications of laccases and tyrosinases (phenoloxidases) immobilized on different supports: a review", *Enzyme and Microbial Technology*, Vol. 31, p. 907-931

Dura´n, N., Esposito, E., 2000. Potential applications of oxidative enzymes and phenoloxidase-like compounds in wastewater and soil treatment: a review. Appl. Cat. B: Environ. 28, 83–99.

Enguita, F.J., Martins, L.O., Henrique, A.O., Carrondo, M.A., 1941. Crystal structure of a bacterial endospore coat component: a laccase with enhanced thermostability properties. J. Biol. Chem. 278,6-19425

Filip, Z., Claus, H., 1995. Effects of soil minerals on the microbial formation of enzymes and their possible use in remediation of chemically polluted sites. In: Huang, P.M., Berthelin, J., Bollag, J.M., McGill, W.B., Page, A.L. (Eds.), Environmental Impacts of soil component interactions, 30. CRC Press, Boca Raton, pp. 407–419,

Geiser, D.M., Pitt, J.I., Taylor, J.W.R., 1998. Cryptic speciation and recombination in the aflatoxin-producing fungus *Aspergillus flavus*. Proc. Natl. Acad. Sci. U.S.A. 95, 388–393.

Gouka, R. J., Punt, P. J., and van den Hondel, C. A. M. J. J., 1997. Efficient production of secreted proteins by *Aspergillus*: progress, limitations and prospects. *Appl. Microbiol. Biotechnol.* 47: 1-11

Henson, J.M., Butler, M.J., Day, A.W., 1999. The dark side of the mycelium: Melanins of phytopathogenic fungi. Annu. Rev. Phytopathol. 37, 447–471.
Hamilton, A.J., Gomez, B.L., 2002. Melanins in fungal pathogens. J. Med. Microbiol. 51, 189–191.

Jacobson, E.S., 2000. Pathogenic roles for fungal melanins. Clin. Microbiol. Rev. 13, 708–717.

Jacobson, E.S., Tinnell, S.B., 1993. Antioxidant function of fungal melanin. J. Bacteriol. 175, 7102–7104.

Klaus Piontek‡, Matteo Antorini§, and Thomas Choinowski, 2002. Crystal Structure of a Laccase from the Fungus *Trametes versicolor* at 1.90-Å Resolution Containing a Full Complement of Coppers. The Journal of Biological Chemistry
DOI 10.1074/jbc.M204571200

Kumar S. V. Suresh, Prashant S. Phale, S. Durani, Pramod P. Wangikar, 2003. Combined sequence and structure analysis of the fungal laccase family. Biotechnol. Bioeng. 83, 386-394.

Kurtzman, C.P., Smiley, M.J., Robert, C.J., Wicklow, D.T., 1986. DNA relatedness among wild and domesticated species in the *Aspergillus flavus* group. Mycologia 78, 955–959.

Kwon-Chung, K.J., Polacheck, I., Popkin, T.J., 1982. Melanin-lacking mutants of *Cryptococcus neoformans* and their virulence for mice. J. Bacteriol. 150, 1414–1421.

Langfelder, K., B. Jahn. H. Gehringer. A. Schmidt, G. Wanner, and A. A. Brakhage, 1998. Identification of a polyketide synthase gene (pksP) of *Aspergillus fumigatus* involved in conidial pigment biosynthesis and virulence Med. Microbiol. Immunol: (Berlin) 187:79-89

Leatham, G. F., and M. A. Stahmann, 1981. Studies on tha laccase of *Lentinus edodes*: specifity, localization and association with the development of fruiting bodies. J. Gen. Microbiol. 125:147-157

Lipkind, G. M., Zhou, A., and Steiner, D. F., 1998. A model for the structure of the P domains in the subtilisin-like prohormone convertases. *Proc. Natl. Acad. Sci. USA* 95: 7310-7315.

Lyons, G. A., McKay, G. J., and Sharma, H. S. S., 2000, "Molecular Comparison of *Scytalidium thermophilum* isolates using RAPD and ITS Nucleotide Sequence Analyses", *Mycological Research*, Vol. 104 (12), p. 1431-1438

Lyons, G. A., McKay, G. J., and Sharma, H. S. S., 2000, "Molecular Comparison of *Scytalidium thermophilum* isolates using RAPD and ITS Nucleotide Sequence Analyses", *Mycological Research*, Vol. 104 (12), p. 1431-1438

Matsushima, K., Yashiro, K., Hanya, Y., Abe, K., Yabe, K., Hamasaki, T., 2001. Absence of aflatoxin biosynthesis in koji mold (*Aspergillus sojae*). Appl. Microbiol. Biotechnol. 55, 771–776.

Maras, M., van Die, I. Contreras, R., and van den Hondel, C.A.M.J.J., 1999. Filamentous fungi as production organisms for glycoproteins of biomedical interest. *Glycoconj. J.* 16: 99-107.

Molloy, S. S., Bresnahan, P. A., Leppla, S. H., Klimpel, K. R., and Thomas, G. 1992. Human furin is a calcium-dependent serine endoprotease that recognizes the sequence Arg-X-X-Arg and efficiently cleaves anthrax toxin protective antigen. *J. Biol. Chem.* 267: 16396-16402.

Nakayama, K., 1997. Furin: a mammalian subtilisin/Kex2p-like endoprotease involved in processing of a wide variety of precursor proteins. *Biochem. J.* 327: 625-635.

Olsen, V., Cawley, N. X., Brandt, J., Egel-Mitani, M., and Loh, Y. P., 1999. Identification and characterization of Saccharomyces cerevisiae yapsin 3, a new member of the yapsin family of aspartic proteases encoded by the YPS3 gene. *Biochem. J.* 339: 407-411.

Payne, G.A., Brown, M.P., 1998. Genetics and physiology of aflatoxin biosynthesis. Annu. Rev. Phytopathol. 36, 329–362.

Perng-Kuang Chang, 2003; Lack of interaction between AFLR and AFLJ contributes to nonaflatoxigenicity of *Aspergillus sojae*. Journal of Biotechnology 107 (2004) 245–253

Piontek K., Antorini M., Choinowski T., 2002. Crystal structure of a laccase from the fungus *Trametes versicolor* at 1.90-A resolution containing a full complement of coppers. The Journal of Biological Chemistry. DOI 10.1074

Pomerantz, H., Warner, H.V., 1967. 3,4-DOPA as the tyrosinase cofactor. Occurrence in melanoma and binding constant. J. Biol. Chem. 242, 5308–5314.

Eric Record, Peter J. Punt, Mohamed Chamkha, Marc Labat, Cees A. M. J. J. ven den Hondel and Marcel Asther; 2202. Expression of the *Pycnoporus cinnabarinus* laccase gene in *Aspergillus niger* and characterization of the recombinant enzyme. Eur. J. Biochem 269, 602-609.

Sakemi, S., Inagaki, T., Kaneda, K., Hirai, H., Iwata, E., Sakakibara, T., Yamauchi, Y., Norcia, M., Wondrack, L.M., Sutcli.e, J.A., et al., 1995. CJ-12,371
and CJ-12,372, two novel DNAgyrase inhibitors. Fermentation, isolation, structural elucidation andm biological activities. J. Antibiot. (Tokyo) 48, 134–142.

Samson, R.A., 1992. Current taxonomic schemes of the genus *Aspergillus* and its teleomorphs. In: Bennett, J.W., Klich, M.A. (Eds.), *Aspergillus* – Biology and Industrial Applications. Butterworth – Heinemann, Stoneham, Mass, pp. 355–390.

Scherer, M. And Fischer, R., 1998 Purification and characterization of laccase II of *Aspergillus nidulans*. Arch. Microbiol. 170,78-84.

Schechter, I., and Berger, A., 1967. On the size of the active site in proteases. I. Papain. *Biochem. Biophys Res. Commun.* 27: 157-162.

Seidah, N. G., Hamelin, J., Mamarbachi, M., Dong, W., Tadros, H., Mbikay, M., Chretien, M., and Day, R., 1996. cDNA structure, tissue distribution, and chromosomal localization of rat PC7, a novel mammalian proprotein convertase closest to yeast kexin-like proteinases. *Proc. Natl. Acad. Sci. USA* 93: 3388-3393.

Shinde, U., and Inouye, M., 2000. Intramolecular chaperones: polypeptide extensions that modulate protein folding. *Semin. Cell Dev. Biol.* 11: 35-44.

Siezen, R. J., and Leunissen, J. A. M., 1997. Subtilases: The superfamily of subtilisin-like serine proteases. *Protein Sci.* 6: 501-523.

Takahashi, T., Chang, P.-K., Matsushima, K., Yu, J., Abe, K., Bhatnagar, D.Cleveland, T.E., Koyama, Y., 2002. Nonfunctionality of *Aspergillus sojae aflR* in a strain of *Aspergillus parasiticus* with a disrupted *aflR* gene. Appl. Environ. Microbiol. 68, 3737–3743.

Thurston CF, The structure and function of fungal laccases, 1994. Microbiology; 140; 19-26

Tran-Dinh, N., Pitt, J.I., Carter, D.A., 1999. Molecular genotype analysis of natural toxigenic and nontoxigenic isolates of *Aspergillus flavus* and *A. parasiticus*. Mycol. Res. 103, 1485–1490.

Watson, A.J., Fuller, L.J., Jeenes, D.J., Archer, D.B., 1999. Homologs of aflatoxin biosynthesis genes and sequence of *aflR* in *Aspergillus oryzae* and *Aspergillus sojae*. Appl. Environ. Microbiol. 65, 307–310.

Wheeler, M.H., Bell, A.A., 1988. Melanins and their importance in pathogenic fungi. In: McGinnis, M.R. (Ed.), Current Topics in Medical Mycology. Springer Verlag, New York, NY, pp. 338– 387.

Wicklow, D.T., 1984. Adaptation in wild and domesticated yellow-green Aspergilli. In: Kurata, H., Ueno, Y. (Eds.), Toxigenic Fungi: their Toxins and Health Hazards. Elsevier, Amsterdam, pp. 78–86

Wiegant, W. M., 1992, " Growth Characteristics of the Thermophilic Fungus *Scytalidium thermophilum* in Relation to Production of Mushroom Compost", *Applied and Environmental Microbiology*, Vol. 58, p. 1301-1307

Wiegant, W. M., 1992, " Growth Characteristics of the Thermophilic Fungus *Scytalidium thermophilum* in Relation to Production of Mushroom Compost", *Applied and Environmental Microbiology*, Vol. 58, p. 1301-1307

Wilcox C. A., Redding, K., Wright, R., and Fuller, R. S., 1992. Mutation of a tyrosine localization signal in the cytosolic tail of yeast Kex2 protease disrupts Golgi retention and results in default transport to the vacuole. *Mol. Biol. Cell* 3: 1353-1371.

APPENDIX A

CHEMICALS, ENZYMES AND THEIR SUPPLIERS

Chemical or Enzyme	**Supplier**
λ.DNA/*Eco*RI+*Hin*dIII MBI	Fermentas
β-mercaptoethanol	Merck
ABTS	Sigma
Agar	Acamedia
Agarose	Sigma
Ampicilin	Mustafa Nevzat İlaç San.
ATP	MBI Fermentas
BamHI	MBIFermentas
Biotin	Sigma
Calcium Chloride	Merck
Casein Hydrolysate	Fluka
Chloroform	Merck
$CuSO_4$	Sigma
dNTP mix	MBIFermentas
*Eco*RI	MBI Fermentas
EDTA	Merck
Ethanol	Gurup Deltalar

Chemical or Enzyme	Supplier
Ethidium Bromide	Sigma
$FeSO_4$	Sigma
Glacial Acetic Acid	Merck
Glycerol	Merck
Glucose Monohydrate	Merck
Hind III	MBIFermentas
Hydrochloric Acid	Merck
IPTG	MBI Fermentas
Isopropanol	Merck
KCl	Merck
K_2HPO_4	Merck
Klenow Enzyme	Boehringer Mannheim
Mineral Oil	Sigma
$MgSO_4.7H_2O$	Merck
NaCl	Merck
NaOH	Merck
Nicotinamide	Sigma
Pantothenic acid	Sigma
Phenol:Chloroform:Isoamylalcohol	AppliChem
Pfx DNA Polymerase	Invitrogen
Pyridamine	Aldrich
RNase A	Roche
Riboflavin	Fluka
SDS	Merck

Chemical or Enzyme	Supplier
Sodium Acetate	Merck
Sodium Citrate	Merck
Sorbitol	Sigma
Spermidine	Sigma
Sucrose	Merck
T4 DNA Ligase	Roche
Taq DNA Polymerase	MBI Fermentas
Tetracycline	Mustafa Nevzat İlaç San.
Tyrptone	Merck
Tris	Merck
Triton X-100	Sigma
Tryptone	Difco
Tween 20	Sigma
Tween 80	Sigma
Uracil	Sigma
Uridine	Sigma
X-gal	MBI Fermentas
Yeast Extract	Merck

APPENDIX B

PREPARATIONS OF GROWTH MEDIA, BUFFERS AND SOLUTIONS

1. Agarose Gel 0. 8 % (w/v)

0.8 g agarose and 2 ml 50 X TAE are added and dissolved in 100 ml distilled water by microwave.

2. Acetate Buffer

Stock Solutions:
A: 0.2 M solution of sodium of acetic acid
B: 0.2 M solution of sodium acetate
14.8 ml of A and 35.2 ml of B diluted a total of 100 ml to adjust the pH5

3. Binding Solution

125 of 6M sodium iodide solution

4. Biotin (0.2 mg/mL)

5 mg biotin is dissolved in 25 ml distilled water. Sterilized by autoclave. Stored at 0^{o}C.

5. Buffer P1 (Resuspension Buffer)

50 mM Tris-Cl, pH = 8.0
10 mM EDTA
100 µg/ml RNase A

6. Buffer P2 (Lysis Buffer)

200 mM NAOH
1 % SDS

7. Buffer P3 (Neutralization Buffer)

3.0 M potassium acetate, pH = 5.5

8. Buffer QBT (Equilibration Buffer)

750 mM NaCl
50 mM MOPS, pH = 7.0
15 % isopropanol
0.15 % Triton X-100

9. Buffer QC (Wash Buffer)

1.0 M NaCl
50 mM MOPS, pH = 7.0
15 % isopropanol

10. Buffer QF (Elution Buffer)

1.25 M NaCl
50 mM Tris·Cl, pH = 8.5;
15 % isopropanol

11. CaCl2 (1 M, 50 ml)

5.55 g of CaCl2 is dissolved in 40 ml H2O. The volume is adjusted to 50 ml and sterilized by filtration.

12. Concentrated Washing Buffer:

15 ml of concentrated solution of Tris, NaCl and EDTA for making wash buffer

13. DNase free RNase

RNase A is dissolved in 0.01 M Sodium acetate (pH 5.2) to give a final concentration of 10 mg/ml. The solution is heated to 100ºC for 15 min in a boiling water bath for the inactivation of DNase. It is cooled slowly to room temperature. 0.1 volume of 1 M Tris-HCl (pH 7.4) is added until the pH of the solution is 7.0. The solution is dispensed into aliquots and stored at -20ºC.

14. EDTA (0.5M, pH 8.0)

186.1 g of ethylenedinitrilotetraacetic acid disodium salt dihydrate added to 800 ml of distilled water. It is stirred vigorously on a magnetic stirrer while the pH is adjusted to 8.0 with NaOH pellets. The solution is dispensed into aliquots and sterilized by autoclaving.

15. Ethanol (70%, 100 ml)

70 ml absolute ethanol mixed with 30 ml distilled sterile water.

16. Glucose (25% w/v, 10 ml)

2.5 g of glucose is dissolved in 8 ml H2O. The volume is adjusted to 10 ml and sterilized by filtration.

17. IPTG (100 mM)

0.24 g IPTG is dissolved in 10 ml H_2O, filter sterilized, dispensed into aliquots and stored at 4^oC.

18. $MgSO_4$ (10 mM, per Liter)

2.46 g $MgSO_4.7H_2O$ is dissolved in 1 liter distilled water. The solution is sterilized by autoclaving.

19. LB Broth (per Liter)

10 g tryptone
5 g yeast extract
10 g NaCl

Final volume is adjusted to 1 liter with distilled water and PH is adjusted to 7.0 with NaOH and autoclaved. 150 µl / 100 ml ampicilin is added whenever it is used. The medium is stored at 4 ^oC.

20. LB Agar (per Liter 75 % ampicilin)

10 g tryptone
5 g yeast extract
10 g NaCl
20 g agar
Final volume is adjusted to 1 liter with distilled water and PH is adjusted to 7.0 with NaOH and autoclaved. When it cools to 55 ^{o}C 150 µl / 100 ml ampicilin is added and poured to petri dishes. The plates stored at 4 ^{o}C

21. LB Tetracycline Agar (per Liter)

10 g NaCl
10 g tryptone
5 g yeast extract
20 g agar

22. Lysis Buffer

40 mM Tris-HCl
10 mM EDTA
0.2 M NaCl
1.5 % SDS

23. NaCl (5M)

292.2 g NaCl is dissolved in 800 ml distilled water. The volume is adjusted to 1 liter and sterilized by autoclaving.

24. NaAC (3 M, per Liter)

408.3 g of NaAC.3H2O is dissolved in 800 ml of H2O. The pH is adjusted to the desired value with glacial acetic acid. The volume of the solution is then adjusted to 1 liter with distilled water, dispensed into aliquots and sterilized by autoclaving.

25. NaOH (10 N, 100 ml)

40g of NaOH pellets is added slowly to 80 ml of H2O. When the pellets have dissolved completely, the volume is adjusted to 1 liter with H2O. The solution is stored at room temperature. Sterilization is not necessary.

26. PEG Buffer (25 % PEG-6000)

2.5 g PEG-6000 is weighted, 7.5 ml TC is added and dissolved by heating to approximately 60 ^{o}C in a microwave oven (when prepared fresh), or filter sterilized (0.2 μm membrane)

27. Pantothenic Acid (1 mg/mL)

5 mg pantothenic acid is dissolved in 5 mL distilled water. Sterilized by autoclave and stored in –20 ^{o}C.

28. SDS (10% w/v, per Liter)

100 g of SDS is dissolved in 900 ml of H2O. The solution is heated to 68°C and stirred with a magnetic stirrer to assist dissolution. If necessary, pH is adjusted to 7.2 by adding a few drops of concentrated HCl. The volume of the solution is adjusted to 1 liter with H2O. It is stored at room temperature. Sterilization is not necessary. Do not autoclave.

29. Saline Tween (ST)

0.8 % NaCl
0.005 % Tween-80 (1:100 dilution from 0.5% (v/v) Tween-80 stock.

30. Silica Powder Suspension:

1.5 ml of specially prepared glass beads suspension in water.

31. STC Buffer

1.33 M sorbitol in TC

32. Stabilized Minimal Medium Agar

70 mM $NaNO_3$
7 mM KCl
11 mM $KH2PO_4$
2 mM Mg_2SO_4
1 % (w/v) glucose
1000 X Trace elements
1000 X Vitamins
325.2 g sucrose (0.95 M)
The pH is adjusted to 6.5. 1.2 % agar is added, an case of top agar 0.6 % is used.

33. TAE Buffer (50X, per Liter)

242 g of Tris base is dissolved in 600 ml distilled waster. The pH is adjusted to 8.0 with approximately 57 ml glacial acetic acid. Then 100 ml 0.5 M EDTA (pH 8.0) is added and the volume is adjusted to 1 liter.

34. TC Buffer

50 mM $CaCl_2$
10 mM Tris/HCl pH 7.5

35. TE Buffer

10 mM Tris- HCl (pH 8.0)
mM EDTA (pH 8.0)

36. Tris HCl Buffer

121.1 g Tris base is dissolved in 800 ml of distilled water. The pH is adjusted to the desired value with concentrated hydrochloric acid. The solution is cooled to room temperature before making final adjustment to pH. The volume of the solution is then adjusted to 1 liter with distilled water and sterilized by autoclaving.
15.X- gal (2%)

37. Transformation (complete) medium

70 mM $NaNO_3$
7 mM KCl
11 mM $KH2PO_4$
2 mM Mg_2SO_4
1 % (w/v) glucose
0.5 % (w/v) yeast extract
0.2 % (w/v) casamino acids
1000 X Trace elements
1000 X Vitamins
10 mM uridine
10 mM uracil

Final volume is adjusted to 250 ml with distilled water. pH is adjusted to6.5 with KOH. Sterilized by autoclaving. MgSO4 and glucose are added after autoclave.

38. Trace Elements

76 mM $ZnSO_4$
178 mM $H3BO_3$
25 mM $MnCl_2$
18 mM $FeSO_4$
7.1 mM $CoCl_2$
6.4 mM $CuSO_4$
6.2 mM Na_2MoO_4
174 mM EDTA

Trace elements solution was dissolved in 80 mL and the volume brought to 100 mL. Sterilized by autoclave. Stored at 4-8 ^oC

39. TTE Buffer

10 mM Tris –HCl (PH=8.5)
10 mM EDTA (PH =8)
4 mM spermidine
10 mM β-mercaptoethanol
0.5 M sucrose
36 mM KCl
0.25 % Triton X-100

40. Vitamins

100 mg/liter thiamin
100 mg/liter riboflavin
100 mg/liter nicotinamide

50 mg/liter pyridoxine

10 mg/liter pantothenic acid

0.2 mg/liter biotin

41. YpSs Broth Media

Yeast extract 4.0 g/L

K_2HPO_4 1.0 g/L

$MgSO_4.7H_2O$. 5.0 g/L

$CuSO_4.5H_2O$ 0.1 g/L

Glucose monohydrate 44.0 g/L

APPENDIX C

DNA SIZE MARKER

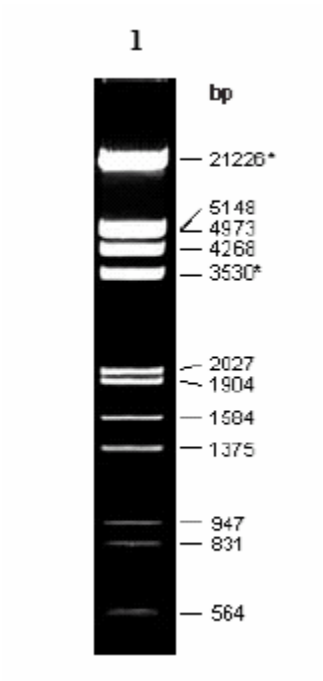

Figure C.1 Lambda DNA/*Eco*RI+*Hin*dIII Marker

APPENDIX D

NUCLEOTIDE AND AMINO ACID SEQUENCE OF THE *SCYTALIDIUM THERMOPHILUM* LACCASE GENE

```
ctgaatttaaatacaggaagatcgcattcaatccagcctagactgcacaatg

gttctgcacgaccgtcgcacacctgccaatagtgttaataacggcctgaattc

ATG AAG CGC TTC TTC ATT AAT AGC CTT CTG CTT CTC GCA        39
 M   K   F   F   I   N   S   L   L   L   L   A   G        13

GGG CTC CTC AAC TCA GGG GCC CTC GCG GCT CCG TCT ACA        78
 L   N   S   G   A   L   A   A   P   S   T   H   P        26

CAT CCC AGA TCA AAC CCC GAC ATA CTG CTT GAA AGA GAT        117
 R   S   N   P   D   I   L   L   E   R   D   D   H

GAC CAC TCC CTT ACG TCT CGG CAA GGT AGC TGT CAT TCT        156
 S   L   T   S   R   Q   G   S   C   H   S   P   S

CCA AGC AAC CGC GCC TGT TGG TGC TCT GGC TTC GAT ATC        195
 N   R   A   C   W   C   S   G   F   D   I   N   T

AAC ACG GAT TAT GAG ACC AAG ACT CCA AAC ACC GGA GTG        234
 D   Y   E   T   K   T   P   N   T   G   V   V   R

GTG cggcgggttagtatcccaagttacgtttgaccaagaaatggacgtga       284
       R

agtgtgctgactctcccgctag  TAC ACC TTT GAT ATC ACC GAA       327
                         Y   T   F   D   I   T   E

GTC GAC AAC CGC CCC GGT CCC GAT GGG GTC ATC AAG GAG        366
 V   D   N   R   P   G   P   D   G   V   I   K   E

AAG CTC ATG CTT ATC AAC GAC AAA CTC CTG gtagggtcctc       407
 K   L   M   L   I   N   D   K   L   L

Tcgaacgcctgcgtctgccacacagcgtaaaactaacgaaccgctag           454

GGC CCG ACA GTC TTC GCA AAC TGG GGC GAC ACC ATC GAG        493
 G  P   T   V   F   A   N   W   G   D   T   I   E

GTG ACC GTC AAC AAC CAC CTG AGA ACC AAC GGA  gtaag    531
 V   T   V   N   N   H   L   R   T   N   G
```

```
cgttcggacacaaagcccagcaacctagacacactcaactgaccaagtag          569


ACC TCC ATC CAC TGG CAC GGC TTG CAC CAA AAA GGA ACC          608
 T   S   I   H   W   H   G   L   H   Q   K   G   T

AAC TAC CAC GAC GGC GCC AAC GGC GTG ACC GAG TGT CCC          647
 N   Y   H   D   G   A   N   G   V   T   E   C   P

ATC CCG CCC GGT GGC TCC CGA GTC TAC AGC TTC CGA GCG          686
 I   P   P   G   G   S   R   V   Y   S   F   R   A

CGC CAA TAT GGA ACG TCA TGG TAC CAC TCC CAC TTC TCC          725
 R   Q   Y   G   T   S   W   Y   H   S   H   F   S

GCC CAG TAT GGC AAC GGC GTG AGC GGC GCC ATC CAG ATC          764
 A   Q   Y   G   N   G   V   S   G   A   I   Q   I

AAC GGA CCC GCC TCC CTG CCC TAC GAC ATC GAC CTC GGC          803
 N   G   P   A   S   L   P   Y   D  I   D   L   G

GTC CTC CCG CTG CAG GAC TGG TAC TAC AAG TCC GCC GAC          842
 V   L   P   L   Q   D   W   Y   Y   K   S   A   D

CAG CTC GTC ATC GAG ACC CTG GCC AAG GGC AAC GCT CCG          881
 Q   L   V   I   E   T   L   A   K   G   N   A   P

TTC AGC GAC AAC GTC CTC ATC AAC NGC ACC GCA AAG CAC          920
 F   S    D   N   V   L   I   N   G   T   A   K   H

CCC ACC ACT GGC GAA GGG GAG TAC GCC ATC GTG AAG CTC          959
 P   T   T   G   E   G   E   Y   A   I   V   K   L

ACC CCG GGC AAA CGC CAT CGC CTG CGG CTC ATC AAC ATG          998
 T   P   D   K   R   H   R   L   R   L   I   N   M

TCG GTG GAG AAC CAC TTC CAG GTC TCG CTG GCG AAG CAC         1037
 S   V   E   N   H   F   Q   V   S   L   A   K   H

ACC ATG ACG GTC ATC GCG GCG GAC ATG GTC CCC GTC AAC         1076
 T   M   T   V   I   A   A   D   M   V   P   V   N

GCC ATG ACC GTC GAC AGC CTG TTT ATG GCC GTC GGG CAG         1115
 A   M   T   V   D   S   L   F   M   A   V   G   Q

CGG TAT GAT GTT ACC ATC GAC GCG AGC CAG GCG GTG GGG         1193
 R   Y   D   V   T   I   D   A   S   Q   A   V   G

AAT TAC TGG TTC AAC ATC ACC TTT GGA GGG CAG CAG AAG         1232
 N   Y   W   F   N   I   T   F   G   G   Q   Q   K

TGC GGC TTC TCG CAC AAT CCG GCG CCG GCA GCC ATC TTT         1271
 C   G   F   S   H   N   P   A   P   A   A   I   F

CGC TAC GAG GGC GCT CCT GAC GCT CTG CCG ACG GAT CCT         1310
 R   Y   E   G   A   P   D   A   L   P   T   D   P

GGC GCT GCG CCA AAG GAT CAT CAG TGC CTG GAC ACT TTG         1343
 G   A   A   P   K   D   H   Q   C   L   D   T   L
```

```
GAT CTT TCA CCG GTG GTG CAA AAG AAC GTG CCG GTT GAC        1382
 D   L   S   P   V   V   Q   K   N   V   P   V   D

GGG TTC GTC AAA GAG CCT GGC AAT ACG CTG CCG GTG ACG        1421
 G   F   V   K   E   P   G   N   T   L   P   V   T

CTC CAT GTT GAC CAG GCC GCG GCT CCA CAC GTG TTT ACG        1460
 L   H   V   D   Q   A   A   A   P   H   V   F   T

TGG AAG ATC AAC GGG AGC GCT GCG GAC GTG GAC TGG GAC        1499
 W   K   I   N   G   S   A   A   D   V   D   W   D

AGG CCG GTG CTG GAG TAT GTC ATG AAC AAT GAC CTG TCT        1538
 R   P   V   L   E   Y   V   M   N   N   D   L   S

AGC ATT CCG GTC AAG AAC AAC ATT GTG AGG GTG GAC GGA        1577
 S   I   P   V   K   N   N   I   V   R   V   D   G

GTC AAC GAG TGG ACG TAC TGG CTC GTC GAA AAC GAC CCG        1616
 V   N   E   W   T   Y   W   L   V   E   N   D   P

GAG GGC CGC CTC AGT TTG CCG CAT CCG ATG CAT CTA CAC        1655
 E   G   R   L   S   L   P   H   P   M   H   L   H

Gtaagtcacatcccccactaccattcggaatgaccaccaggtactgacacc       1706


ctcctcctcaatag  GGA CAC GAT TTC TTT GTC CTA GGC CGC        1747
                 G   H   D   F   F   V   L   G   R

TCC CCC GAC GTC TCG CCC GAT TCA GAA ACC CGC TTC GTC        1786
 S   P   D   V   S   P   D   S   E   T   R   F   V

TTT GAC CCG GCC GTC GAC CTC CCC CGT CTG CGC GGA CAC        1825
 F   D   P   A   V   D   L   P   R   L   R   G   H

AAC CCC GTC CGG CGC GAC GTC ACC ATG CTT CCC GCG CGC        1864
 N   P   V   R   R   D   V   T   M   L   P   A   R

GGC TGG CTG CTG CTG GCC TTC CGC ACG GAC AAC CCG GGC        1903
 G   W   L   L   L   A   F   R   T   D   N   P   G

GCG TGG TTG TTC CAC TGC CAC ATC GCG TGR CAC GTG TCG        1942
 A   W   L   F   H   C   H   I   A   W   H   V   S

GGC GGG TTA AGC GTC GAC TTT CTG GAG CGG CCG GAC GAG        1981
 G   G   L   S   V   D   F   L   E   R   P   D   E

CTG CGC GGG CAG CTG ACG GGA GAG AGC AAG GCG GAG TTG        2020
 L   R   G   Q   L   T   G   E   S   K   A   E   L

CAG CGT GTT TGT CGC GAG TGG AAG GAT TGG GAG GCG AAG        2059
 E   R   V   C   R   E   W   K   D   W   E   A   K

AGC CCG CAT GGG AAG ATC GAT TCG GGG TTG AAG CAG CGG        2098
 S   P   H   G   K   I   D   S   G   L   K   Q   R

CGA TGG GAT GCG tgaggtagttgggcggattgtttaacacgtagtggg      2146
 R   W   D   A
```

```
taaggttggggcgggtttgtttggcgttttcaggggttggggtgcggatgct       2198

ggtcatccgggaaacggctctacaactggtgtcaatagactaatatagagtg       2250

atcaaagaactgaggttctgaaagaggcgtggaagtcgcgttgtgactccct       2302

ttgccatgttgggaagtgtggctcaacattgtgttcaggtttgctcagggtg       2354

atntcgaactgacgtnttgatgagggttattgcntaga                    2392
```

Figure D.1 Nucleotide and amino acid sequence of the *Scytalidium thermophilum* laccase gene

SEQUENCE ALIGNMENTS OF *SCYTALIDIUM THERMOPHILUM* LACCASE GENE CLONED ONTO pUC19 VECTOR AMPLIFIED WITH PRIMER lacS2

```
lc  CTGAATTTAAATACAGGAAGATCGCATTCAATCCAGCCTAGACTGCACAATGGTTCTGCA 60
sf  ------------------------------------------------------------


lc CGACCGTCGCACACCTGCCAATAGTGTTAATAACGGCCTGAATTCATGAAGCGCTTCTTC 120
sf ------------------------------------------------------------


lc ATTAATAGCCTTCTGCTTCTCGCAGGGCTCCTCAACTCAGGGGCCCTCGCGGCTCCGTCT 180
sf ---------------------------------GTTANGCGGCA---GTGATTNCTCGT 23
                                        *  * ***    * *  * *   *

lc ACACATCCCAGATCAAACCCCGACATACTGCTTGAAAGAGATGACCACTCCCTTACGTCT 240
sf CTCCATCCCAGCTCAA-CCCCGACATACTGCTTGAAAGAGATGACCACTCCCTTACGTCT 82
       ******** **** ****************************************

lc CGGCAAGGTAGCTGTCATTCTCCAAGCAACCGCGCCTGTTGGTGCTCTGGCTTCGATATC 300
sf CGGCAAGGTAGCTGTCATTCTCCAAGCAACCGCGCCTGTTGGTGCTCTGGCTTCGATATC 142
    ************************************************************

lc AACACGGATTATGAGACCAAGACTCCAAACACCGGAGTGGTGCGGCGGGTTAGTATCCCA 360
sf AACACGGATTATGAGACCAAGACTCCAAACACCGGAGTGGTGCGGCGGGTTAGTATCCCA 202
    ************************************************************

lc AGTTACGTTTGACCAAGAAATGGACGTGAAGTGTGCTGACTCTCCCGCTAGTACACCTTT 420
sf AGTTACGTTTGACCAAGAAATGGACGTGAAGTGTGCTGACTCTCCCGCTAGTACACCTTC 262
    ************************************************************

lc GATATCACCGAAGTCGACAACCGCCCCGGTCCCGATGGGGTCATCAAGGAGAAGCTCATG 480
sf GATATCACCGAAGTCGACAACCGCCCCGGTCCTGATGGGGTCATCAAGGAGAAGCTCATG 322
    ******************************** ***************************

lc CTTATCAACGACAAACTCCTGG-TAGGGTCCTCTCGAACGCCTGCGTCTGCCACACAGCG 539
sf CTTATCAACGACAAACTCCTGGGTAGGGTCCTCTCGAACGCCAGCGTCTGCCACACAGCG 382
    ********************** ****************** ****************

lc TAAAACTAACGAACCGCTAGGGCCCGACAGTCTTCGCAAACTGGGGCGACACCATCGAGG 599
sf TAAAACTAACGAACCGCTAGGGCC-GACAATCTTCGCAAACTGGGGCGACACCATCGAGG 441
    ************************ **** ******************************


lc TGACCGTCAACAACCACCTGAGAACCAACGGAGTAAGCGTTCGGACACAAAGCCCAGCAA 659
sf TGACCGTCAACAACCACCTGAGGAC-AACGG-GTAA-CGTTCGGACACAAAGCCCAGCA- 497
    ********************** ** ***** **** **********************
```

```
lc CCTAGACACACTCAACTGACCAAGTAGACCTCCATCCACTGGCACGGCTTGCACCAAAAA 719
sf CCTAGACACACTCA-CTGAC-AAGTANACCTCCTCCC--TGGCACGGCTGGCNCCAAAGG 553
    ************** ***** ***** ******  **  ********** ** *****

lc GGAACCAACTACCACGACGGCGCCAACGGCGTGACCGAGTGTCCCATCCCGCCCGGTGGC 779
sf ACCACTNCCCGNCGC---------------------------------------------- 568
       **   *   * *

lc TCCCGAGTCTACAGCTTCCGAGCGCGCCAATATGGAACGTCATGGTACCACTCCCACTTC 839
sf ------------------------------------------------------------
```

Figure D. 2 DNA sequence alignments of *Scytalidium thermophilum* laccase gene cloned onto pUC19 vector amplified with primer lacS2

SEQUENCE ALIGNMENTS *SCYTALIDIUM THERMOPHILUM* LACCASE GENE CLONED ONTO pUC19 VECTOR AMPLIFIED WITH PRIMER lacS3

```
lc CTAGCATTCCGGTCAAGAACAACATTGTGAGGGTGGACGGAGTCAACGAGTGGACGTACT 1680
Sr ----------------------------------------------------------GNN 3


lc GGCTCGTCGAAAACGACCCGGAGGGCCGCCTCAGTTTGCCGCATCCGATGCATCTACACG 1740
sr TNNTGTTTTAAAANGACC-GGAGGTC--NCTCAGTT--GCTCATCCGA-GCATTTACACG 57
      *  *  **** **** ***** *   *******   * ******* **** ******

lc TAAGTCACATCCCCCACTACCATTCGGAATGACCACCAGGTACTGACACCCTCCTCCTCA 1800
sr TA-GTCAC--TCCCCACTNCC--TTNGAAT-ACCNCCAG-TACTGACCCCTCTCTC---- 106
   ** *****   ******* **  *  **** *** **** ******* **   ***

lc ATAGGGACACGATTTCTTTGTCCTAGGCCGCTCCCCCGACGTCTCGCCCGATTCAGAAAC 1860
sr ATGNGGACACGATTT-TTTGTCTTAGGCCGNTCCCN-GAGGTNTCCCCGGATTCAGAAAC 164
   **  ********** ****** ******* ****  ** ** ** ** **********

lc CCGCTTCGTCTTTGACCCGGCCGTCGACCTCCCCCGTCTGCGCGGACACAACCCCGTCCG 1920
srCCGTTTTTTTTCGACCCGGCCGTCGACNTCCCCCGTNTGCGCGGGCACAACCCCGTCCG 223
   *** **  * ** ************** ******** ******* **************

lc GCGCGACGTCACCATGCTTCCCGCGCGCGGCTGGCTGCTGCTGGCCTTCCGCACGGACAA 1980
sr GCGCGACGTCGCCATGCTTCCCGCTCGGGGTTGGCTGCTGCTGGCCTTCCGCACGGACAA 283
   ********** ************* ** ** ****************************
```

```
lc CCCGGGCGCGTGGTTGTTCCACTGCCACATCGCGTGRCACGTGTCGGGCGGGTTAAGCGT 2040
sr CCCGGGCGCGTGGTTGTTCCACTGCCACATCGCCTGGCACGTGTCGGGCGGGTTCAGCGT 343
   ********************************** ** ********************* *****

lc CGACTTTCTGGAGCGGCCGGACGAGCTGCGCGGGCAGCTGACGGGAGAGAGCAAGGCGGA 2100
sr CGACTTTCTGGAGCGGCCGGACGAGCTGCGCGGGCAGCTGACGGGAGAGAGCAAGGCGGA 403
   ************************************************************

lc GTTGGAGCGTGTTTGTCGCGAGTGGAAGGATTGGGAGGCGAAGAGCCCGCATGGGAAGAT 2160
sr GTTGGAGCGCGTTTGTCGCGAGTGGAAGGATTGGGAGGCGAAGAGCCCGCATGGGAAGAT 463
   ********* ************************************************

lc CGATTCGGGGTTGAAGCAGCGGCGATGGGATGCGTGAGGTAGTTGGGCGGATTGTTTAAC 2220
sr TGATTCGGGGTTGAAGCAGCGGCGATGGGAT-CGTGAGAATTCGAGCTCGGTACCCGGGG 522
    ***************************** ******       *   * *

lc ACGTAGTGGGTAAGGTTGGGGCGGGTTTGTTTGGCGTTTTCAGGGGTTGGGGTGCGGATG 2280
sr ATCCTCTAGAGTCGACCTGCAGGCATCAAGCTGGCGAATCATGCAA-------------- 568
   *     * *    *    *   *  *     *****  *   *

lc CTGGTCATCCGGGAAACGGCTCTACAACTGGTGTCAATAGACTAATATAGAGTGATCAAA 2340
sr ------------------------------------------------------------
```

Figure D. 3 DNA sequence alignments *Scytalidium thermophilum* laccase gene cloned onto pUC19 vector amplified with primer lacS3

SEQUENCE ALIGNMENTS *SCYTALIDIUM THERMOPHILUM* LACCASE GENE CLONED ONTO pAN52-4 VECTOR AMPLIFIED WITH PRIMER pAN-pro

```
lc CTGAATTTAAATACAGGAAGATCGCATTCAATCCAGCCTAGACTGCACAATGGTTCTGCA 60
pr ------NCCNNNNNNNNNNNAGCTTTCCCACTTCATCGCAGCTTGACTAACAG--CTACC 52
                      * *     ** * ** *  **  **   **  *  ** *

lc CGACCGTCGCACACCTGCCAATAGTGTTAATAACGGCCTGAATTCATGAAGCGCTTCTTC 120
pr CCGCTTGAGCAGACATCACCATGGGCTTCCTA----TCTCTACTCGCCCTGAGCGGCCTC 108
   *  *    *** ** *  * ** *  **  **     **  * **     * **  * **

lc ATTAATAGCCTTCTGCTTCTCGCAGGGCTCCTCAACTCAGGGGCCCTCGCGGCTCCGTCT 180
pr GTCTGCAACGGGTTGG--CAAATGTGATTTCCAAGCGCGCTGGATCCGAAGCTTTCACCG 166
    *    * *    **   *      *  * *  * * *   **  *    *  * *  *
```

```
lc A-CACATCCCAGATCAAACCCCGACATACTGCTTGAAAGAGATGACCACTCCCTTACGTC 239
pr ATCCCATCGCTAAACAATCCGCC-CAGAATTCTC---ACGCATC-CCATCGCTAAACAAT 221
   * * **** *  * *** ** *  ** * * **    *   **  ***   *   **

lc TCGGC--AAGGTAGCTGTCATTCTCCAAGCAACCGCGCCTGTTGGTGCTCTGGCTTCGAT 297
pr CCGCCCAAAGCTTCCTGTCATTCTCCAAGCAACCGCGCCTGTTGGTGCTCTGGCTTCGAT 281
    ** *  *** *  **********************************************

lc ATCAACACGGATTATGAGACCAAGACTCCAAACACCGGAGTGGTGCGGCGGGTTAGTATC 357
pr ATCAACACGGATTATGAGACCAAGACTCCAAACACCGGAGTGGTGCGGCGGGTTAGTATC 341
   ************************************************************

lc CCAAGTTACGTTTGACCAAGAAATGGACGTGAAGTGTGCTGACTCTCCCGCTAGTACACC 417
pr CCAAGTTACGTTTGACCAAGAAATGGACGTGAAGTGTGCTGACTCTCCCGCTAGTACACC 401
   ************************************************************

lc TTTGATATCACCGAAGTCGACAACCGCCCCGGTCCCGATGGGGTCATCAAGGAGAAGCTC 477
pr TTCGATATCACCGAAGTCGACAACCGCCCCGGTCCTGATGGGGTCATCAAGGAGAAGCTC 461
   ** ******************************** ************************

lc ATGCTTATCAACGACAAACTCCTGG-TAGGGTCCTCTCGAACGCCTGCGTCTGCCACACA 536
pr ATGCTTATCAACGACAAACTCCTGGGTAGGGTCCTCTCGAACGCCTGCGTCTGCCACACA 521
   ************************ ***********************************

lc GCGTAAAACTAACGAACCGCTAGGGCCCGACAGTCTTCGCAAACTGGGGCGACACCATCG 596
pr GCGTAAAACTAACGAACTGCTAGGGCC-GACAATCTTCGCAAACTGGGGCGACACCATCG 580
   ***************** ********* **** ****************************

lc AGGTGACCGTCAACAACCACCTGAGAACCAACGGAGTAAGCGTTCGGACACAAAGCCCAG 656
pr AGGTGACTGTCAACAACCACCTGAGGACCAACGG-GTAAGCGTTCGGACACAAAGCCCAG 639
   ******* ***************** ******** ************************

lc CAACCTAGACACACTCAACTGACCAAGTAGA-CCTCCATCCACTGG-CACGGCTTGCACC 714
pr CAACCTAGACACACTCAACTGACCAAGTAGAACCTCCATCCACTGGGCACGGCTTGCACC 699
   ******************************** ************** *************

lc AAAAAGGAACCAACTACCACGACGGCGCCAACGGCGTGACCGAGTGTCCCATCCCGCCCG 774
pr AAAAAGGAAACAACTACCACGACGGCGCTA-CGGCGTGACCGAGTGTCCCATCCCGCCCG 758
   ********* ****************** * ****************************

lc GTGGCTCCCGAGTCTACAGCTT-CCGAGCGCGCCAATATGG-AACGTCATGG-TACCACT 831
pr GCGGCTCCCGAGTCTACAGCTTTCCGAGCGCGCCAATATGGGAACGTCATGGGTACCACT 818
   * ******************* ***************** ********** *******


lc CCCACTTCTCCGCCCAGTATGGCAA-CGGCGTGAGCGGCGCCATCCAGATCAACGGACCC 890
pr CTCACTTCTCTGCCCAGTATGGCAAACGGCGT--------------------------- 850
   * ******** ************** ******
```

```
lc GCCTCCCTGCCCTACGACATCGACCTCGGCGTCCTCCCGCTGCAGGACTGGTACTACAAG 950
pr ------------------------------------------------------------
```

Figure D. 4 DNA sequence alignments *Scytalidium thermophilum* laccase gene cloned onto pAN52-4 vector amplified with primer pAN-pro

SEQUENCE ALIGNMENTS *SCYTALIDIUM THERMOPHILUM* LACCASE GENE CLONED ONTO pAN52-4 VECTOR AMPLIFIED WITH PRIMER 52478R1

```
lc CGCACAATCCGGCGCCGGCAGCCATCTTTCGCTACGAGGGCGCTCCTGACGCTCTGCCGA 1380
74 ------------------------------------------------------------


lc CGGATCCTGGCGCTGCGCCAAAGGATCATCAGTGCCTGGACACTTTGGATCTTTC-ACCG 1439
74 --------------------AGGATCATCAGTGCCTGGACACTCTGGATCTTTCCACCG 39
                        ********************** ********** ****

lc GTGGTGCAAAAGAACGTGCCGGTTGACGGGTTCGTCAAAGAGCCTGGCAATACGCTGCCG 1499
74 GTGGTGCAAAAGAACGTGCCGGTTGACGGGTTCGTCAAAGAGCCTGGCAATACGCTGCCG 99
   ************************************************************

lc GTGACGCTCCATGTTGACCAGGCCGCGGCTCCACACGTGTTTACGTGGAAGATCAACGGG 1559
74 ATGACGCTCCATGTTGAGCAGGCCGTGGCTCCACACGTGTTTACGTGGAAGATCAACGGG 159
    *************** ******* **********************************

lc AGCGCTGCGGACGTGGACTGGGACAGGCCGGTGCTGGAGTATGTCATGAACAATGACCTG 1619
74 AGCGCTGCGGACGTGGACTGGGACAGGCCGGTGCTGGAGTATGTCATGAACAATGACCTG 219
   ************************************************************

lc TCTAGCATTCCGGTCAAGAACAACATTGTGAGGGTGGACGGAGTCAACGAGTGGACGTAC 1679
74 TCTAGCATTCCGGCCAAGAACAACATCGTGAGGGTGGACGGAGTCAACGAGTGGACGTAC 279
   ************* ************ *********************************

lc TGGCTCGTCGAAAACGACCCGGAGGGCCGCCTCAGTTTGCCGCATCCGATGCATCTACAC 1739
74 TGGCTCGTCGAAAACGACCCGGAGGGTCGCCTCAGTTTGCCTCATCCGATGCATCTACAC 339
   ************************** ************** ******************
```

```
lc GTAAGTCACATCCCCCACTACCATTCGGAATGACCACCAGGTACTGACACCCTCCTCCTC 1799
74 GTAAGTCACATCCCCCACTACCATTCGGAATAACCACCAGGTACTGACACCCTCCTCCTC 399
   ******************************** ****************************

lc AATAGGGACACGATTTCTTTGTCCTAGGCCGCTCCCCCGACGTCTCGCCCGATTCAGAAA 1859
74 AATAGGGACACGATTTCTTCGTCCTAGGCCGCTCCCCCGACGTCTCGCCCGATTCAGAAA 459
   ****************** ****************************************

lc CCCGCTTCGTCTTTGACCCGGCCGTCGACCTCCCCCGTCTGCGCGGACACAACCCCGTCC 1919
74 CCCGCTTCGTCTTCGACCCGGCCGTCGACCTCCCCCGCCTGCGCGGGCACAACCCCGTCC 519
   ************ ********************** ******** *************

lc GGCGCGACGTCACCATGCTTCCCGCGCGCGGCTGGCTGCTGCTGGCCTTCCGCACGGACA 1979
74 GGCGCGACGTCGCCATGCTTCCCGCTCGGGGTTGGCTGCTGCTGGCCTTCCGCACGGACA 579
   *********** ************* ** ** ****************************


lc ACCCGGGCGCGTGGTTGTTCCACTGCCACATCGCGTGRCACGTGTCGGGCGGGTTAAGCG 2039
74 ACCCGGGCGCGTGGTTGTTCCACTGCCACATCGCCTGGCACGTGTCGGGCGGGTTCAGCG 639
   ********************************** ** ***************** ****

lc TCGACTTTCTGGAGCGGCCGGACGAGCTGCGCGGGCAGCTGACGGGAGAGAGCAAGGCGG 2099
74 TCGACTTTCTGGAGCGGCCGGACGAGCTGCGCGGGCAGCTGACGGGAGAGAGCAAGGCGG 699
   ************************************************************

lc AGTTGGAGCGTGTTTGTCGCGAGTGGAAGGATTGGGAGGCGAAGAGCCCGCATGGGAAGA 2159
74 AGTTGGAGCGCGTTTGTCGCGAGTGGAAGGATTGGGAGGCGAAGAGCCCGCATGGGAAGA 759
   ********** ************************************************

lc TCGATTCGGGGTTGAAGCAGCGGCGATGGGATGCGTGAGGTAGTTGGGCGGATTGTTTAA 2219
74 TCGATTCGGGGTTGAAGCAGCGGCGATGGGATCGGTGAAAGCTTGAGATCCACTTAACGT 819
   ********************************  ****     *  *    * *

lc CACGTAGTGGGTAAGGTTGGGGCGGGTTTGTTTGGCGTTTTCAGGGGTTGGGGTGCGGAT 2279
74 TACTGAAATCATCAAACAGCTGANGATNNNN---------------------------- 850
    **  *     * *    *  *  * *

lc GCTGGTCATCCGGGAAACGGCTCTACAACTGGTGTCAATAGACTAATATAGAGTGATCAA 2339
74 ------------------------------------------------------------
```

Figure D. 5 DNA sequence alignments *Scytalidium thermophilum* laccase gene cloned onto pAN52-4 vector amplified with primer 52478R1

APPENDIX E

pAN52-1 VECTOR MAP

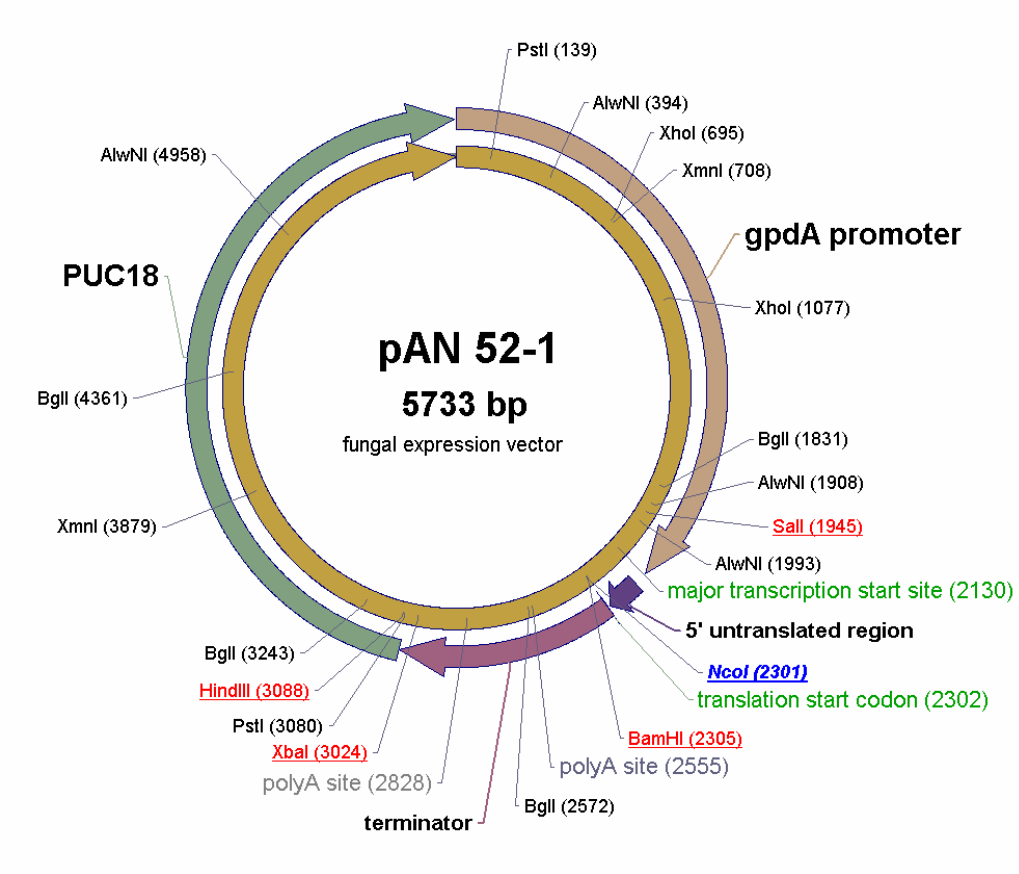

Figure E.1 pAN52-1 restriction map

Enzymes with unique restriction sites are shown in red color

pAN52-4 VECTOR MAP

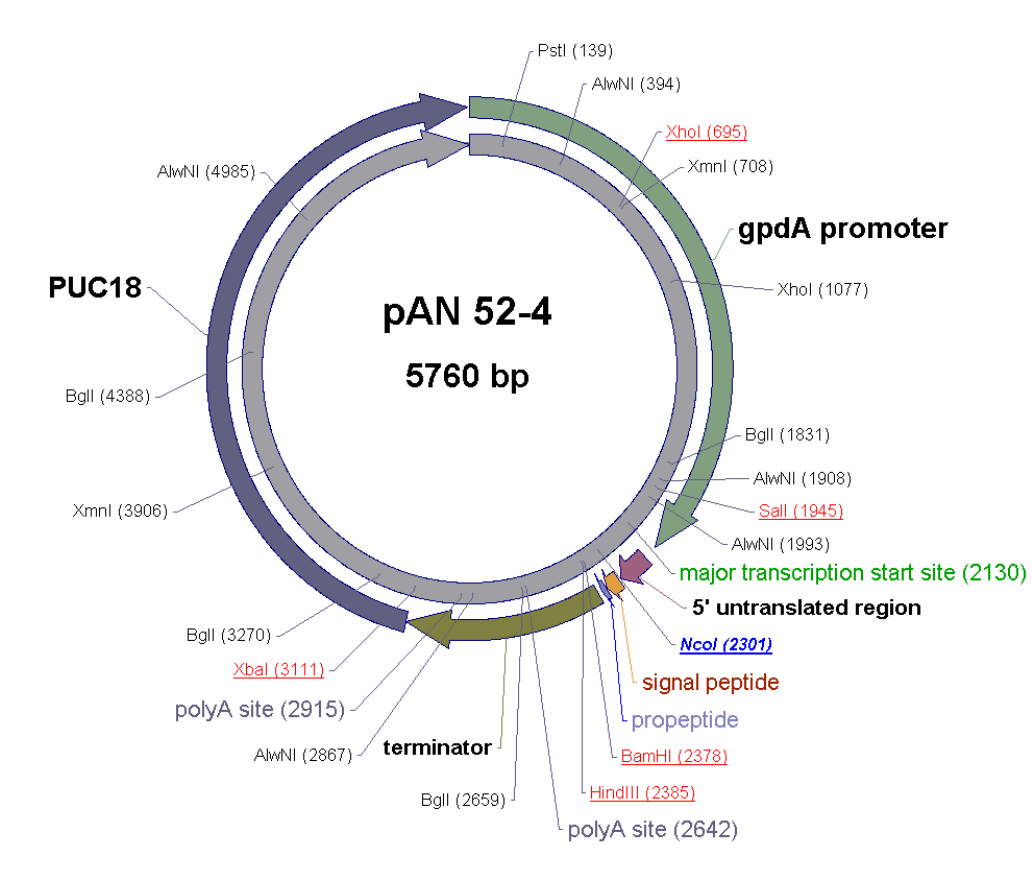

Figure E.2 pAN52-4 restriction map

Enzymes with unique restriction sites are shown in red color

pUC 19 VECTOR MAP

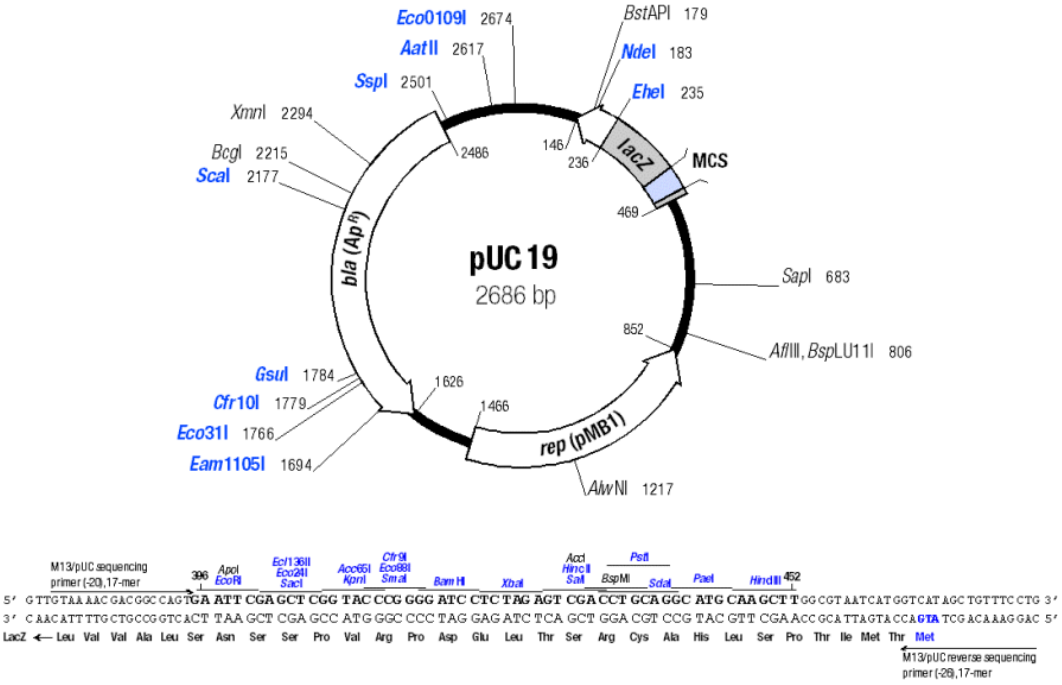

Figure E.3 pUC19 restriction map

APPENDIX F

SEQUENCES OF THE PRIMERS

Name of the primer	Sequence of the primer (5’ to 3’)
315	AGGGTTTTCCCAGTCACGAC
pAN-pro	TCCCATCCCTTATTCCTTTG
52478R1	GACATCGACACCAACGATCT

Figure F.1 Sequence of primers