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Trametes versicolor laccase: random mutagenesis and heterologous expression in *Pichia pastoris*

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Sammanfattning

Lackas är ett blått multikopparoxidas, som har bred bioteknisk potential, vilket leder till ökat intresse att studera enzymets egenskaper. Den lackas-kodande genen lcc2 från vitrötesvampen *Trametes versicolor* muterades med två olika metoder för slumpmässig mutagenes: dels med s.k. error-prone PCR mutagenes och dels med en metod där man använder en *E. coli* stam (ES1301 mutS) som ger upphov till mutationer. Som templat för error-prone PCR användes vektorn pPICZB med lcc2-genen som insättning. För *E. coli*-metoden användes vektorn pBluescript SKII med lcc2-genen insatt för transformation av *E. coli*-stammen ES1301 mutS. Mutagenes-produkterna klonades in i *Pichia pastoris*-vektorn pPICZB och *P. pastoris*-stammen SMD1168 transformerades. Transformanterna spreds på agarplattor innehållande zeocin. Lackas-utsöndrande transformanter selekterades genom lackas förmåga att oxidera substraten ABTS [2,2´-azinobis-(3-etylbenstiazolin-6-sulfonsyra)] och syringaldazin [N,N´-bis(3,5-dimetoxi-4-hydroxibensyliden)hydrazin], som ger upphov till produkter med grön respektive lila färg. Ett tjugotal transformanter från de två mutagenes-metoderna plockades över till plattor innehållande 1 mM ABTS respektive 1 mM syringaldazin. Ingen transformant gav upphov till någon färg. Kontrolltransformanter (pPICZB med icke-muterad lcc2) spreds också på båda substraten. De växte bra och utvecklade tydlig färg med båda substraten. ABTS gav upphov till grön färg efter ett dygn, medan det tog tre dygn för syringaldazin att utveckla lila färg. Försök med flera olika substrat visade att ABTS och syringaldazin var bäst lämpade. Remazol Brilliant Blue och Phenol Red är två substrat som efter optimering skulle kunna vara alternativ för selektion av lackas-utsöndrande transformanter.

Abstract

Laccase is a blue multi-copper oxidase. It has a broad biotechnical potential which increases the interest to study the enzyme further. A laccase-encoding gene from the white-rot fungus *Trametes versicolor* (lcc2) was mutated using two different methods for random mutagenesis: error-prone PCR and a method based on an *E. coli* strain (ES1301 mutS) that introduces random mutations. For the error-prone PCR reaction, the vector pPICZB with the lcc2 gene inserted was used as template. The *E. coli* strain ES1301 mutS was transformed with the vector pBluescript SKII with the lcc2 gene as insert. The mutagenesis products were cloned into the *Pichia pastoris* expression vector pPICZB for transformation of *P. pastoris* SMD1168. The transformants were spread on agar plates containing zeocin. Laccase-secreting transformants were selected by their ability to oxidize the substrates ABTS [2,2´-azinobis-(3-ethylbenzthiazoline-6-sulfonic acid)] and syringaldazine [N,N´-bis(3,5-dimethoxy-4-hydroxybenzylidene)hydrazine], the products of which give green and purple colour, respectively. Around 20 transformants from each of the mutagenesis methods were transferred to plates containing 1 mM ABTS or 1 mM syringaldazine. None of the transformants produced any colour. Control transformants (pPICZB with unmutated lcc2) were also spread on plates with either ABTS or syringaldazine. The transformants gave rise to green colour after 24 hours on the ABTS plates and to purple colour after 72 hours on the syringaldazine plates. Experiments with different chromogenic substrates indicated that ABTS and syringaldazine were best suited for screening of mutants. Remazol Brilliant Blue and Phenol Red are two substrates that after optimisation can serve as alternatives for the selection of laccase-secreting transformants.

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List of abbreviations

ABTS	2,2-Azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid)
BMMH	Buffered minimal methanol medium
DMSO	Dimethyl sulfoxide
EP-PCR	Error-prone polymerase chain reaction
HBT	1-Hydroxybenzotriazole
PhR	Phenol red
RBB	Remazol brilliant blue
Syringaldazine	N,N´-bis(3,5-dimethoxy-4-hydroxybenzylidene)hydrazine
YPD	Yeast extract peptone dextrose
YPDS	Yeast extract peptone dextrose sorbitol

1. Introduction

1.1 White-rot fungi

1.1.1 *Trametes versicolor*

There are three different kind of wood-rotting fungi; white-rot, brown-rot and soft-rot fungi. *Trametes (Coriolus, Polyporus) versicolor* belongs to the white-rot fungi and is a lignin-degrading basidiomycete that can be found in temperate and sub-tropical forests all over the world [1]. This is one of the best studied white-rot fungi [1,2]. When *T. versicolor* decays wood, it first attacks the cell wall and removes lignin from the secondary wall. This is followed by rapid degradation of cellulose. White-rot fungi are often good producers of laccase [2] and several isoforms of the enzyme are secreted by *T. versicolor* [3].

Figure 1 The fungus *Trametes versicolor*. Photo by courtesy of Dr Leif J. Jönsson.

1.1.2 Laccase

Laccase belongs to a group of enzymes called blue multi-copper oxidases. Since laccase is often found in wood-destroying fungi that can degrade lignin, it is believed to play an important role in lignin degradation. In 1883, Yoshida discovered laccase in the latex of lacquer trees [3]. Apart from laccase, the group blue multi-copper oxidases contains two other

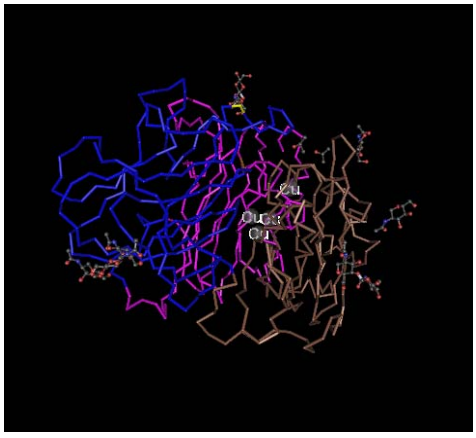

Figure 2 Laccase molecule made in Cn3D 4.1.

enzymes; the plasma protein ceruloplasmin and the plant protein ascorbate oxidase [4]. Laccase is the simplest and contains four copper ions per protein molecule [5] and has a chain length of about 500 amino-acid residues [6]. It is one of the best understood oxidases, at least in terms of its spectroscopic properties.The four copper ions are of three different types, and there is one type 1 Cu, one type 2 Cu and two type 3 Cu. The copper ions are essential for the catalytic activity of these enzymes [3]. All proteins that contain copper are involved in electron-transfer processes [7]. Laccase is involved in the coupling of the one-electron oxidation of a reducing substrate with the four-electron reduction of O_2 to H_2O [7], without releasing reactive oxygen species, as for example H_2O_2 or hydroxyl radicals [8].

Laccase are found in many fungi and plants. Laccase-like enzymes have also been reported in bacteria and insects [9]. The physiological role of laccase is not clear and may depend on the type of organism. As discussed before, *T. versicolor* secretes different isoforms of laccase, and it is possible that these isoforms have different functional roles [10].

The list of what laccase can be used for could be made long and some of the possible applications for the enzyme are: (1) removal of lignin in paper manufacturing, (2) detoxification of lignocellulose hydrolysates in ethanol production, (3) drug analysis, and (4) detoxification of industrial waste water [9]. Laccase from *T. versicolor* is divided in two different chromatographic fractions, A and B [3]. This work deals with laccase A and the gene that encodes for that protein, namely lcc2 [11].

1.2 Expression

In earlier studies, heterologous expression of laccase genes from *T. versicolor* has been performed using a number of host organisms including *A. niger*, *P. pastoris* and *S. cerevisiae* [12]. In this study, the lcc2 gene of *T. versicolor* was expressed in the methylotrophic yeast *Pichia pastoris*.

There are many different reasons why it is of interest to have a rapid and efficient system for heterologous expression of proteins in yeast. This would be of interest in the search for different forms of mutants, in the production of larger quantities of an enzyme and for the construction of yeast strains with novel properties, such as improved resistance against phenolic inhibitors [2].

1.2.1 *Pichia pastoris*

P. pastoris is a homothallic ascomycetous yeast. During the past 15 years, *P. pastoris* has been developed into a highly successful system for the production of a variety of heterologous proteins. The system gives high-level expression of proteins. *P. pastoris* can grow on cheap carbon sources. It can use methanol as its sole carbon source and is therefore a methylotrophic yeast. Three basic steps are necessary in the expression of a foreign gene in *P. pastoris*: 1) cloning of the gene into an expression vector; 2) transformation of the expression vector into the genome of *P. pastoris*; and 3) examination of potential expression strains for the foreign gene product [13]. Foreign proteins expressed in *P. pastoris* can be produced either intracellularly or extracellularly [13].

The strain used in this work was SMD1168, which has been shown to be effective in reducing degradation of some foreign proteins. SMD1168 is a protease-deficient pep4 mutant. It can be used in the selection of zeocin-resistant expression vectors to generate strains without protease A activity [14]. The disadvantage of this strain is that it has a slower growth rate and is more difficult to transform than wild type strains [13].

1.2.2 Vector pPICZB

For intracellular expression, pPICZ is a vector of choice, while pPICZα is a vector designed for extracellular expression [15]. pPICZB was the vector that was used in this project. It consists of 3328 bp and contains the AOX1 promoter for methanol-induced expression. It has a multiple-cloning site [16]. There is a zeocin-resistance gene for selection of *P. pastoris* transformants.

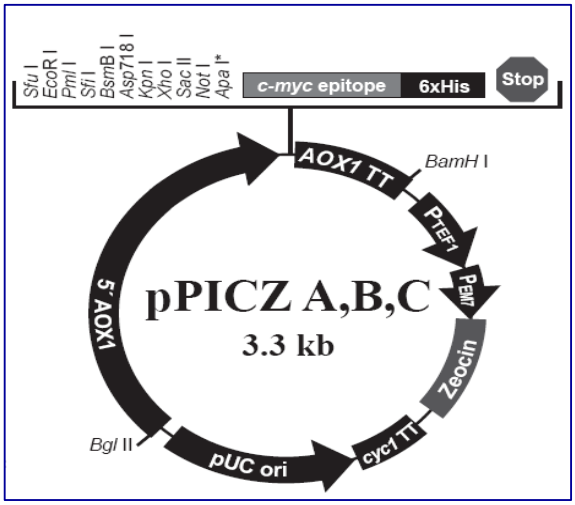

Figure 3 Vector pPICZA. In pPICZB, the Apa I site is exchanged for an Xba I site [16].

Zeocin is an antibiotic that shows strong toxicity against bacteria, fungi (including yeast), plants and mammalian cells. Zeocin is isolated from *Streptomyces* and belongs to a family of structurally related bleomycin/phleomycin-type antibiotics. Antibiotics in this family have a broad spectrum and

can be used both as antibacterial and anti-tumour drugs. Zeocin is a copper-chelated glycopeptide that is basic and water-soluble and has the formula $C_{55}H_{83}N_{19}O_{21}S_2Cu$ [14].

The mechanism by which zeocin works is not known, but it is thought to be the same as other antibiotics in the family. When the antibiotic enters the cell, the copper cation is reduced from Cu^{2+} to Cu^{+} and is removed by sulfhydryl compounds in the cell. Upon removal of the copper, zeocin is activated to bind DNA and cleavage of DNA causes cell death [15].

1.2.3 Screening

Maybe the most universal type of assay used in enzyme discovery is direct expression screening using substrates with bonds attacked by the enzyme of interest. The method of detection can for example be colour change, fluorescence intensity or wavelength change, polarization, resonance energy transfer or any other phenomenon easily detectable in a screening format [17]. The main purpose of small-scale expression is to identify a recombinant *P. pastoris* clone that is expressing the correct protein [15]. Laccase has a low specificity with regard to the reducing substrate, and a large number of different substances can be oxidized [3]. Some of these substrates develop colour in the reaction, which can be used to screen for transformants that express active laccase. Substrates that were used in the screening experiments in this study included Phenol Red (PhR), Remazol Brilliant Blue (RBB), syringaldazine (N,N´-bis(3,5-dimethoxy-4-hydroxybenzylidene)hydrazine), ABTS (2,2-azino-bis-(3,5-ethylbenzthiazoline-6-sulfonic acid)) and Bromophenol Blue. Syringaldazine is a substrate for easy and quick detection of laccase. It will go from yellow to dark purple during treatment with laccase and air. ABTS is one of the most frequently used substrates for screening of laccase. In an agar plate with ABTS, a green zone will appear around a colony that produces laccase. RBB and Bromophenol Blue are blue compounds and the reaction with laccase causes a discolouring. PhR will develop a red/orange colour. The intensity in colour and/or how quick the colour develops indicates the amount of laccase present. In this case, it also shows if the substrate can be used in the screening of laccase.

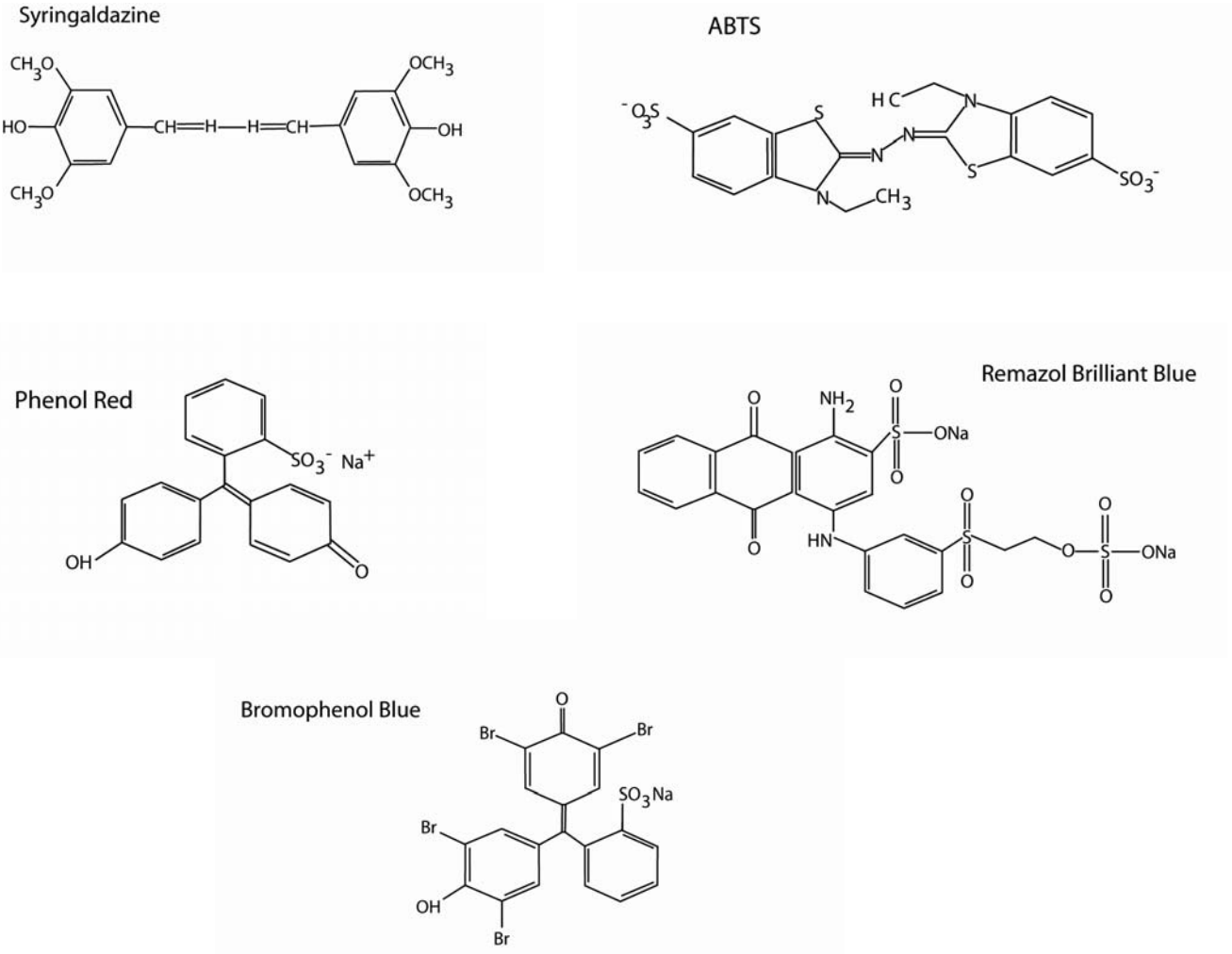

Figure 4 Different substrates used for the screening of laccase activity.

1.3 Mutations

The fact that new mutants appear in a population is an essential part in the evolution of a species. Many, if not all, spontaneous mutations arise as errors in DNA replication, recombination or repair [18]. During the DNA synthesis, a nucleotide can be placed in the wrong position, which will lead to a base pair that does not match. If this incorrect pair is not repaired, there will be a mutation. This occurs spontaneously at a frequency of 1 per 10^9-10^{10} base pairs per cell division. An incorrect insertion does not always result in a mutation because the proofreading exonuclease activity of DNA polymerase edits the mistake. If there should still be an error after the replication, it will probably be corrected by mismatch repair [19]. Mutations can also be used to learn more about a specific protein. Mutations can be introduced by using site-directed mutagenesis or by random-mutagenesis methods.

1.3.1 Random mutagenesis

Cloning techniques can be used not only to overproduce proteins, but to produce protein products with different properties compared to the native form. The alteration of an enzyme can alter the catalytic rate, thermostability, binding affinity and specificity. In understanding the relationship between protein structure and function, random mutagenesis is a good tool. The functional and structural roles of amino-acid residues in a protein of interest can be studied by comparison with a mutant form of the protein carrying changes in the amino-acid sequence. When site-directed mutagenesis is used, mutations are normally introduced in positions known to have functional importance. With random mutagenesis, many mutations may be silent, but you can also find new unexpected mutations of importance for the function of the protein. To use random mutagenesis can also be a first step to define positions in a protein that are of interest to change by site-directed mutagenesis. In this work, two different strategies to generate random mutations were employed. These two methods are discussed in the following sections.

1.3.2 *E. coli* MutS ES1301

In the mismatch-repair process, some proteins are essential in the detection of mismatch and direction of the repair. Those are MutS, MutL and MutH. The protein that recognises the mismatched base on the new strand is MutS and it binds to the error. MutH binds a hemimethylated GATC sequence on the daughter strand but it will stay latent until it is activated by contact with the MutL protein. MutL binds to MutS and works as a mediator between MutH and MutS. MutH nicks the daughter strand close to the mismatch and DNA helicase II separates the two strands. Different types of exonucleases will digest the strand and the single-stranded gap created can be repaired by DNA polymerase III, which uses the other strand as template. The final step is to seal the strand with DNA ligase and methylate the daughter strand, a reaction performed by Dam methylase [20].

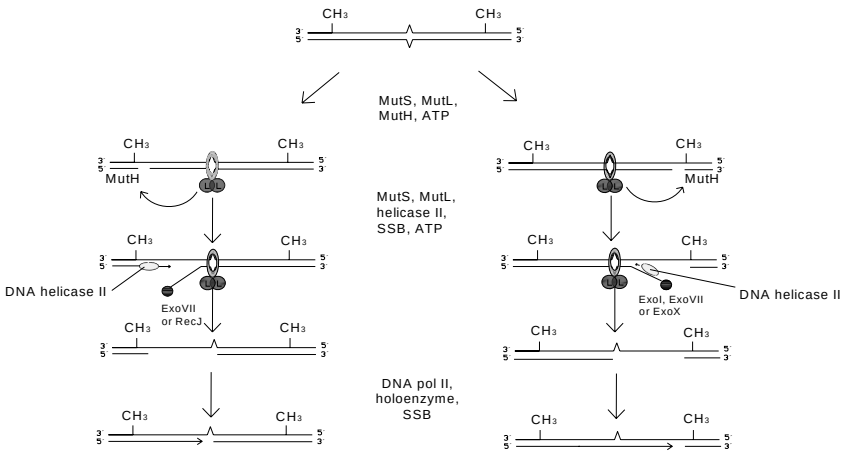

Figure 5 The mechanism of mismatch-repair.

MutS ES1301 is a mismatch repair-minus strain of *E. coli* with defective DNA repair systems, and suitable for use in *in vitro* mutagenesis systems. The use of a MutS strain prevents repair of the newly synthesized unmethylated strand, leading to high mutation efficiencies [21]. As discussed before, MutS is one of the proteins responsible for mismatch repair and when this is defected it will be easier for mutations to appear in the DNA strain. This has been shown to increase significantly spontaneous *in vivo* mutagenesis. A plasmid containing the gene of interest can be introduced into such a strain to produce random mutations. Although the mutation rate for this kind of *E. coli* strain is low compared to that of PCR-based mutagenesis methods, the simplicity of this approach is very attractive [22].

1.3.3 Error-prone PCR

The introduction of PCR has made both DNA synthesis and DNA mutagenesis *in vitro* to very efficient processes. PCR undergoes multiple heating and/or cooling cycles, each having three phases: denaturation at about 95˚C, annealing around 55˚C and extension at about 72˚C. At the denaturation phase, the two template strands are separated to allow the primers to anneal to them. Under the annealing phase, the two primers bind to one strand each. A good primer for DNA synthesis should be free of strong secondary structures, such as

hairpins, stemloops or direct repeats. Under the extension phase, the annealed primers are extended according to the template strands. Many cycles are then repeated in the same way. Thus, both the original templates and their products, which after several cycles predominate over the original templates, become templates in subsequent rounds of DNA synthesis. In this way, templates are amplified exponentially by around 2^{20}-fold to yield linear double-stranded PCR products. In comparison to single-stranded DNAs, double-stranded DNAs are easier to prepare, and gene inserts are in general more stable with double-stranded DNAs. It can sometimes be difficult to digest PCR products, leading to low efficiency of ligation or even failure. It is especially hard when restriction sites are built too close to the end of a PCR product. Those sites will digest with difficulty, even when the ends of the products are complete [22].

PCR-based mutagenesis is very important in molecular biology and protein engineering studies, since it is both efficient and cost-effective. Error-prone PCR is a random-mutagenesis technique for generating amino-acid substitutions in proteins by introducing mutations into a gene during PCR. The error-prone PCR reaction was performed with GeneMorph® II Random Mutagenesis Kit (Stratagene). There will be mutations because of the use of error-prone DNA polymerases and/or the reaction conditions. Mutazyme® II DNA polymerase is an error-prone-PCR enzyme blend, which is made to give useful mutation rates. This blend gives less biased mutations, since the mutation rates of A/T and G/C base pairs are equivalent. Therefore, this method would generate greater mutant representation compared to libraries prepared using other enzymes. The blend consisted of two error-prone DNA polymerases, Mutazyme I DNA polymerase and a novel *Taq* DNA polymerase mutant that exhibits increased misinsertion and misextension frequencies compared to wild-type *Taq* DNA polymerase. A mutation rate of 1-16 mutations per kb can be achieved. The mutation rate can be controlled by changing the initial amount of target DNA or by changing the number of amplification cycles. Low mutation frequencies can be achieved by using higher DNA template concentrations and/or lower numbers of PCR cycles. For the same PCR yield, amplification of low amounts of target DNA results in more duplications than amplification of high amounts of target DNA. The more times a target is replicated, the more errors accumulate [23]. The mutated PCR products are then cloned into an expression vector and the resulting mutant library can be screened for changes in the protein activity.

1.4 The aim of this study

The goal of this study was to introduce random mutations into the lcc2 gene of *T. versicolor*. The two different mutagenesis methods described in the previous sections were used and compared to each other, for example with regard to the mutation efficiency and how easy they are to work with. The gene was then expressed in *P. pastoris* SMD1168, which was followed by screening for transformants that expressed laccase mutants with other properties than the native form of the enzyme. Five different substrates were also examined with respect to use in the screening of laccase-producing colonies on agar plates. The most commonly used substrate is ABTS, but it would be of interest to find new ways to screen for activity.

2 Materials and Methods

2.1 Examination of different substrates for laccase screening

Different substrates were incorporated into BMMH (buffered minimal methanol medium) plates in different concentration. ABTS or syringaldazine were added to the plates to a final concentration of 0.2, 1 or 3 mM. ABTS was dissolved in methanol. Syringaldazine was dissolved in DMSO (dimethyl sulfoxide). Bromophenol Blue, Phenol Red, and Remazol Brilliant Blue were added to the plates to a final concentration of 0.05, 0.2 or 0.8 mM. Phenol Red and Remazol Brilliant Blue were dissolved in water and Bromophenol Blue was dissolved in methanol. To plates with Phenol Red, Remazol Brilliant Blue or Bromophenol Blue, 0.5 mM HBT (1-hydroxybenzotriazole) was added as a mediator. Plates with 0.2 mM Remazol Brilliant Blue were examinated further with different HBT concentrations; 0.1 mM, 0.5 mM, 1 mM and 5 mM. Phenol Red (0.8 mM and 1.2 mM) was also tried in agar plates in combination with HBT in different concentrations (same as for Remazol Brilliant Blue). Solutions of laccase (laccase T from *Trametes* spec. 100 kU, 938.1 U/g, Jülich Fine Chemicals, Jülich, Germany) with the approximate activity of 15.0 mM/min (1), 1.50 mM/min (2), 150.0 µM/min (3) or 15.0 µM/min (4) were added as droplets on those plates.

Determination of laccase activity was performed by using a spectrophotometer (UV-2101, Shimadzu, Kyoto, Japan). The reaction mixture contained 200 µl 2 mM ABTS, 200 µl 50 mM sodium acetate buffer (pH 5.2) and 50 µl laccase solution with an appropriate dilution.

Water was added to a total volume of 1 ml. The change in absorbance at 414 nm was recorded for 1-5 min at room temperature (see Appendix 3 for calculations [24]).

2.2 Preparation of DNA

For preparation of DNA, the plasmid pPICZB (Invitrogen, Carlsbad, CA, USA) with and without the lcc2 gene, was transformed into *E. coli* XL-1 Blue (Stratagene, La Jolla, CA, USA). Approximately 2 µg of pPICZB were mixed with 200 µl of a suspension of competent *E. coli* XL-1 Blue cells and put on ice for 20 min. The cells were heat shocked at 42˚C for 90 sec, and were then put on ice for 1 min. Then, 800 µl of low-salt LB medium were added. The mix was incubated with shaking at 37˚C for 30 min (model G25, New Brunswick Scientific, Edison, New Jersey, USA).

Approximately 0.4 µg of pPICZB/lcc2 were mixed with 200 µl of a suspension of competent *E. coli* XL-1 Blue cells. The procedure followed was the same as above except that SOC medium was added instead of low-salt LB medium. There were problems to get the transformants to grow on the agar plates and SOC medium contains more nutrients, which facilitates cell recovery. The cell suspension was centrifuged at 10000 rpm for 3 min (Eppendorf Mini Spin Plus, Eppendorf, Hamburg, Germany). The pellet was dissolved in 450 µl of the supernatant. The transformants were spread on low-salt LB plates containing 25 µg/ml zeocin (Invitrogen). The plates were put upside down at 37°C over night. One colony was picked and inoculated in 3 ml LB medium with 25 µg/ml zeocin and incubated for 8 hours at 37°C with shaking. Fifty ml LB medium without zeocin was inoculated with 100µl from the preculture. The E flask with the culture was then incubated at 37°C with shaking over night. The plasmid was then purified with HiSpeed Plasmid Midi Kit (Qiagen, Hilden, Germany) and concentrated by adding sodium acetate (pH 5.2) to a final concentration of 0.3 M and 2 volumes of ice-cold ethanol. This mixture was stored at -20°C over night. Determination of the DNA concentration was performed by a spectrophotometric measurement at 260 nm. At this wavelength one absorbance unit corresponds approximately to 50 µg DNA per ml [25]. To check the DNA preparation, a restriction enzyme digestion was performed with EcoR I and Xba I (both from Roche). The plasmid without the inserted lcc2 gene was digested with EcoR I. The digests were analyzed using 1% agarose gels.

2.3 Error-prone mutagenesis

Approximately 850 ng of the vector pPICZB with the gene lcc2 inserted were used as template. The mutation-PCR reaction was performed with GeneMorph II Random Mutagenesis Kit (Stratagene) using the primers Error L 5´-AAACGAGGAATTC-ACCATG-3´ and Error R 5´-TGAGATGAGTTTTTGTTCTAGATTA-3´. The PCR (Techne, Duxford, Cambridge, UK) was carried out according to the following scheme: 95°C 2 min (once) + 95°C 30 sec, 50°C 30 sec, 72°C 2 min (30 cycles) + 72°C 10 min (once). A new PCR was used to amplify the error-prone PCR product. The same primers were used but now with normal *Taq* DNA polymerase (Roche) according to the following scheme: 95°C 2 min (once) + 95°C 30 sec, 50°C 30 sec, 72°C 2 min (25 cycles) + 72°C 10 min (once). The PCR product was then purified with the PCR-purification kit of Qiagen. To check that it was the right fragment, the PCR product was analyzed on an 1% agarose gel.

2.4 *E. coli* ES1301 mutS mutagenesis reaction

E. coli XL-1 Blue cells carrying the pBluescript/lcc2 plasmid were grown in LB medium containing 100 µg/ml ampicillin. The plasmid was purified from the *E. coli* cells by HiSpeed Plasmid Midi Kit (Qiagen). The vector was then used for transformation of *E. coli* mutS strain ES1301. One hundred µl of a suspension of *E. coli* mutS cells were mixed with 20 µl of a solution of pBluescript/lcc2 and put on ice for 20 min. The cells were then heat shocked at 37˚C for 10 min. Then, 200 µl SOC medium and 0.5 µl 2 M $MgCl_2$ were added. The cells were spread on LB plates containing 100 µg/ml ampicillin and grown over night at 37˚C. Some of the colonies were picked and grown in 3 ml LB/ampicillin medium over night. The plasmids were purified with a HiSpeed Plasmid Midi Kit (Qiagen). Agarose-gel electrophoresis was used to check that the right fragment (4.6 kb) had been obtained.

2.5 Cloning into pPICZB

A spot test [26] was performed to determine the DNA concentration and to calculate the volumes for the digestion reaction. Around 10 µg of DNA (pPICZB, EP-PCR product or

product from MutS 1301) was digested with 20 U EcoR I and 20 U Xba I. The reactions were incubated at 37˚C for 4 h and thereafter purified with a PCR Purification Kit (Qiagen). Precipitation with ethanol was used to concentrate the DNA samples (see section 2.2). The digestions were analyzed on 1% agarose gels. After another spot test (see above) to determine the concentration, the DNA fragments were ligated. Approximately 3 µg of EP-PCR product and 8 µg of pPICZB were mixed together with 2 units of T4 DNA ligase, 1.2 µl ligase buffer and 0.55 µl water in a total volume of 12 µl. Approximately 5.5 µg mutS product and 8 µg pPICZB were mixed together with 2 units of T4 DNA ligase, 1.2 µl ligase buffer and 0.55 µl water to an total volume of 12 µl. Both those mixtures were incubated at 16˚C over night. After the ligation, the DNA was purified with the PCR Purification Kit (Qiagen) and the products were dissolved in 30 µl water.

2.6 Linearization of the construct

After the ligation, there would be a circular construction of the size 4.9 kbp. The plasmid with the gene incorporated was digested with Sac I to make it linear for the transformation of *P. pastoris*. The DNA preparations that were digested were the mutS construct, the EP-PCR construct and control pPICZB/lcc2 (unmutated). In all these reactions, the amount of DNA was approximately 10 µg and 20 units of Sac I were used. The mixtures were incubated at 37˚C for 4 h. Ethanol precipitation (see section 2.2) was used to concentrate the DNA and the products were analyzed on 1% agarose gels.

2.7 Preparation of *P. pastoris* SMD1168 for electroporation

The yeast cells were grown on YPD (yeast extract peptone dextrose) plates and incubated at 30°C. One colony was picked to inoculate 5 ml YPD medium, and the culture was incubated at 30°C with shaking over night. 0.5 ml of the over-night culture was used to inoculate 500 ml YPD medium. The culture was grown to an OD_{600} of 1.3-1.5. The cells were harvested by centrifugation at 4500 rpm in 5 min at 4°C and redissolved in 500 ml ice-cold water. Then, the cells were centrifuged again (as above) and the pellet was resuspended in 250 ml ice-cold water. The cells were centrifuged again (as above) and the pellet was resuspended

in 20 ml 1 M sorbitol. Finally, the cells were centrifuged again (as above) and resuspended in 1 ml 1 M sorbitol. The cells were then stored on ice until the electroporation (according to ref. 15).

2.8 Transformation of *P. pastoris* SMD1168

Transformation of *P. pastoris* SMD 1168 was performed with electroporation. Eighty µl of the cells from the step before (section 2.7) were mixed with 15 µl of a solution of linear pPICZB/lcc2 DNA and transferred to an electroporation cuvette (Gene Pulser® Cuvette, 0.2 cm electrode, Bio-Rad Laboratories, Hercules, CA, USA), which was put on ice for 5 min. The cells were then pulsed using 1.5 kV, 25 µF and 400 Ω in a Gene Pulser (Bio-Rad). After the electrical pulse, 1 ml 1 M sorbitol was immediately added and the cell suspension was transferred to 15 ml tubes and the cells were allowed to recover for 2 h. The cells were spread on YPDS (yeast extract peptone dextrose sorbitol) plates containing 100 µg/ml zeocin. The plates were incubated upside down at 30˚C until colonies could be seen (according to ref. 13).

2.9 Expression in *P. pastoris* and detection of laccase activity

The colonies that could grow on zeocin plates were picked and plated on a BMMH-plate with 1 mM ABTS and another plate with 1 mM syringaldazine. The plates were stored upside down in room temperature. To analyze the transformants, a colony PCR was performed [26]. The reaction mixture contained 10x*Taq* polymerase buffer, 5 units *Taq* DNA polymerase, 10 mM dNTPs, 10 pmol of each primer and water up to 50 µl. One colony was picked and added to each PCR tube. Error L 5´-AAACGAGGAATTC-ACCATG-3´ and Error R 5´-TGAGATGAGTTTTTGTTCTAGATTA-3´ and ANN6 L 5´-ACAGCTACCCCGCTTG-3´ and ANN6 R 5´-TCAAGCTGTTTGATGATTTC-3´ were used as primers. Error primers anneal both to the plasmid and to the gene, but ANN6 only anneals to the plasmid. PCR was carried out according to the following scheme: 95°C 4 min (once) + 95°C 1 min, 50°C 1 min, 72°C 2 min (35 cycles) + 72°C 10 min (once). The PCR products were controlled on a 1% agarose gel to check if it was the right fragment.

3. Results and discussion

3.1 Screening with different substrates

Different substrates were tested for screening of laccase activity on agar plates. ABTS is the most common substrate in this kind of work, but we wanted to search for other good alternatives to choose between. The experiment was performed by putting droplets of a laccase solution on BMMH plates.

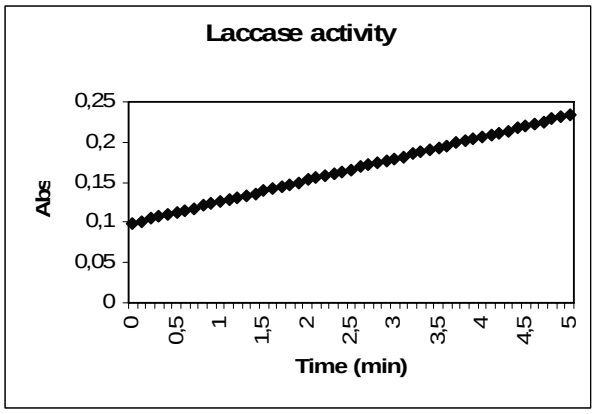

Figure 6 Absorbance measurement of the most diluted laccase solution. The activity was then calculated from this measurement (see appendix 3).

The plates contained different substrates in various concentrations. The compounds examined were ABTS, syringaldazine, Phenol Red, Remazol Brilliant Blue and Bromophenol Blue. Each substrate was first evaluated separately, with respect to the concentration of substrate, the laccase activity and the time required for colour development. Then, the different substrates were compared.

3.1.1 ABTS

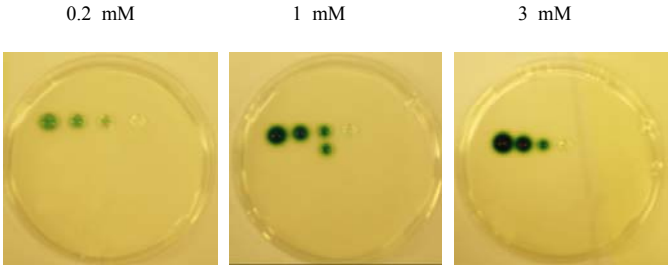

Figure 7 4 hours after laccase was added to the ABTS plates.

The experiments with ABTS showed, as expected, that good results may be obtained with this substrate. The colour development started directly when laccase was added to the plates. All the three different concentrations of ABTS resulted in a clear colour development (Fig. 7). However, the colour on the plates containing 1 mM and 3 mM ABTS was stronger and appeared faster than on the plate with 0.2 mM. Since there was not any big difference between 1 mM and 3 mM, there was no reason to choose 3 mM, because that would just lead to consumption of more substrate. For further work with the transformants, 1 mM ABTS was used in the plates. The ABTS experiments are summarized in Table 1.

Table 1 Comparision of different concentrations of ABTS. The results are indicated on a scale 0-5, in which 5 represents the clearest colour development. The colour development was controlled after 4 hours. 1-4 indicate the four different laccase activities, of which 1 is the highest activity.

ABTS conc (mM)	1	2	3	4
0.2	3	2	1	0
1	4.5	4	3.5	0.5
3	5	4.5	4	0.5

3.1.2 Syringaldazine

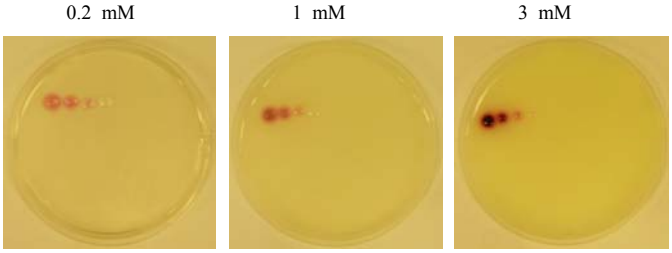

Figure 8 4 hours after laccase was added to syringaldazine plates.

As can be seen in Fig. 8 syringaldazine gave good results. As was also the case for ABTS, the colour appeared directly when laccase was added to the plates. Syringaldazine could not be dissolved in water or methanol as the other substrates, but DMSO worked. The question whether cells can grow and express protein on plates containing DMSO is addressed in section 3.4. With increasing concentration of syringaldazine, there was an increasing concentration of DMSO in the plates. To avoid too high DMSO concentrations, 1 mM was used for further experiments. There is still much work that can be done to optimize syringaldazine as a substrate. One interesting thing is to see how the cells will react when they grow on plates with even higher DMSO concentrations than 1 mM and if syringaldazine can be dissolved in another solvent that is totally harmless to the cells. As Fig. 8 shows, the colour is stronger with 3 mM syringaldazine and even higher concentrations may therefore be of interest. The syringaldazine experiments are summarized in Table 2.

Table 2 Comparision of different concentrations of syringaldazine. The results are indicated on a scale 0-5, in which 5 represents the clearest colour development. The colour development was controlled after 4 hours. 1-4 indicate the four different laccase activities, of which 1 is the highest activity.

syringaldazine conc (mM)	1	2	3	4
0.2	3	2	1.5	0.5
1	4	3	2.5	0.5
3	5	4.5	3	1

As can be seen in Table 2, there was a slight colour development at laccase activity 4 on plates with 0.2 mM syringaldazine. But with the lowest concentration of ABTS, there was no colour development. This suggests that syringaldazine gives higher sensitivity. However, further work is needed to confirm that.

3.1.3 Phenol Red

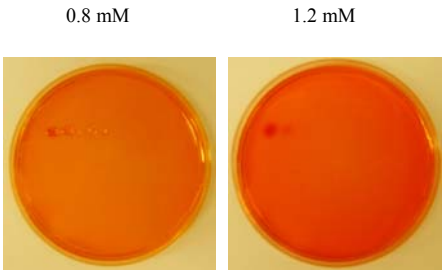

Figure 9 4 hours after laccase was added to PhR plates.

In experiments with plates with phenol red (PhR) in concentrations from 0.05 mM to 0.8 mM, only the plate with 0.8 mM PhR developed colour. Because of that, a higher concentration (1.2 mM) of PhR was also examined. The plate with 1.2 mM substrate showed colour after ½ hour. After 4 hours, (Fig. 9) a dark red colour appeared on plates with 0.8 and 1.2 mM PhR.

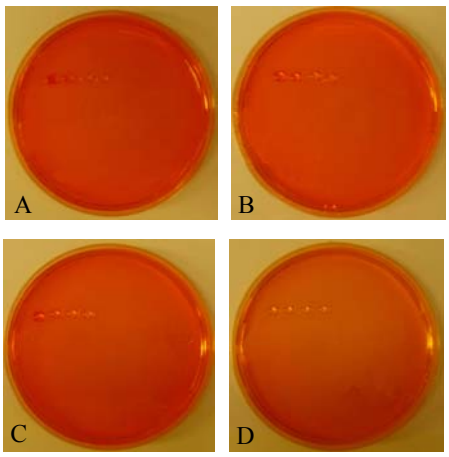

Figure 10 0.8 mM PhR with different HBT concentrations (after 4 hours). A 0.1 mM HBT; B 0.5 mM HBT; C 1 mM HBT; D 5 mM HBT.

The colour was stronger on the plate with 1.2 mM PhR. Both these concentrations were used in the experiment with different HBT concentrations. As can be seen in Fig. 10 and 11, the best results were obtained with low HBT concentration (0.1 mM). The best result was obtained with the plate with 1.2 mM PhR and 0.1 mM HBT. An experiment without HBT was performed and after 45 minutes colour appeared. This result suggests that mediator may not be necessary. The PhR experiments are summarized in Table 3.

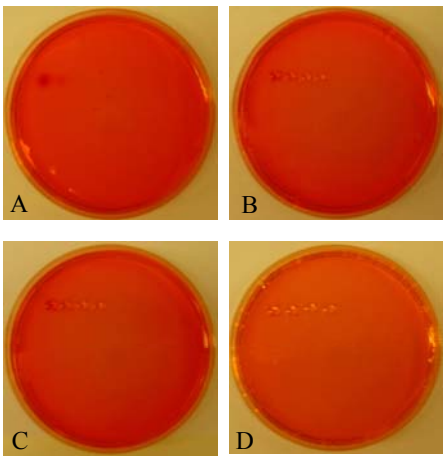

Figure 11 1.2 mM PhR with different HBT concentrations (after 4 hours). A 0.1 mM HBT; B 0.5 mM HBT; C 1 mM HBT; D 5 mM HBT.

Table 3 Comparision of different concentrations of PhR. The results are indicated on a scale 0-5, in which 5 represents the clearest colour development. The colour development was controlled after 4 hours. 1-4 indicate the four different laccase activities, of which 1 is the highest activity.

HBT 0.5 mM	PhR conc. (mM)	1	2	3	4
	0.05	0	0	0	0
	0.2	0	0	0	0
	0.8	5	3	0	0

Different HBT conc. (mM)	PhR conc. (mM)	1	2	3	4
0.1	**0.8**	4	1	0	0
0.5	**0.8**	1	0.5	0	0
1	**0.8**	1	0.5	0	0
5	**0.8**	0	0	0	0
0.1	**1.2**	5	2	0	0
0.5	**1.2**	2	1	0	0
1	**1.2**	1	1	0	0
5	**1.2**	0	0	0	0

More optimization is needed for plates containing PhR. As Table 3 shows, there was no colour development when the laccase activity was low. That indicates that the detection of low expression levels can be difficult. It is therefore of interest to try even higher concentrations of PhR to obtain faster and stronger colour development. The question whether HBT is necessary also needs to be further examined. PhR was not used further in the experiments with the transformants.

3.1.4 Remazol Brilliant Blue

Some discolouring could be seen after ½ hour and it was most clear on the plate with 0.2 mM Remazol Brilliant

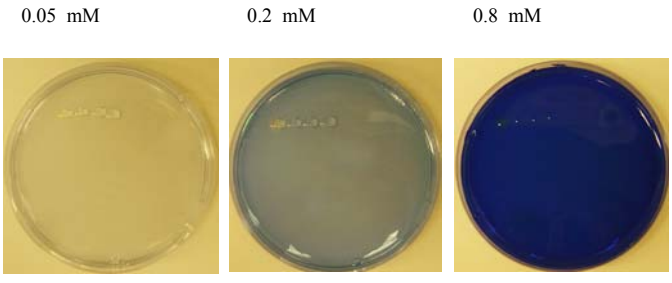

Figure 12 Four hours after addition of laccase to plates with RBB.

Blue (RBB). Fig. 12 shows the reaction after 4 hours.

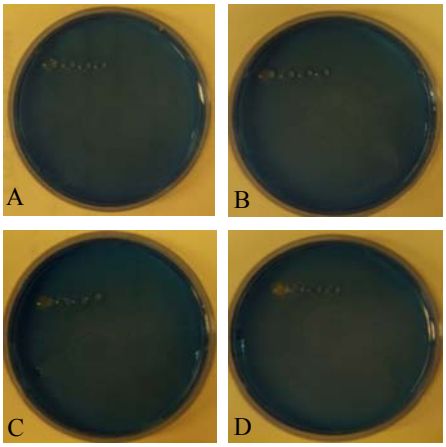

Figure 13 0.2 mM RBB with different HBT concentrations (after 4 hours). A 0.1 mM HBT; B 0.5 mM HBT; C 1 mM HBT; D 5mM HBT.

This concentration was used in the experiment with different HBT concentrations. For RBB, the best results were obtained with high HBT concentrations. The clearest discolouring was observed on the plate with 5 mM HBT. RBB was also examined in plates without the mediator HBT and after 45 minutes the reaction was visible. This indicates that the mediator is needed. The RBB experiments are summarized in Table 4.

Table 4 Comparision of different concentrations of RBB. The results are indicated on a scale 0-5, in which 5 represents the clearest colour development. The colour development was controlled after 4 hours. 1-4 indicate the four different laccase activities, of which 1 is the highest activity.

HBT 0.5 mM	RBB conc. (mM)	1	2	3	4
	0.05	4	3.5	0	0
	0.2	5	3.5	0	0
	0.8	4	0	0	0

Different HBT conc. (mM)	RBB conc. (mM)	1	2	3	4
0.1	**0.2**	2	0	0	0
0.5	**0.2**	3	0	0	0
1	**0.2**	4	0	0	0
5	**0.2**	5	1	0	0

RBB was not used in further experiments, but it could be an interesting substrate after optimization. It is probably no need to investigate higher or lower concentrations, because both 0.05 and 0.8 mM were not as good as 0.2 mM. But it could be of interest to examine if

HBT can make the result clearer and if higher HBT concentrations can make the substrate more sensitive to lower enzyme concentrations.

Experiments were also performed with Bromophenol Blue as substrate but without any good results (not shown). After 24 hours, a very slight discolouring was visible around the drop with the highest laccase activity. It would be very time-consuming to use Bromophenol Blue as a screening substrate and no further work with Bromophenol Blue was done. ABTS and syringaldazine were selected as the best substrates, because they showed colour directly after addition of laccase to the plates. Both ABTS and syringaldazine were used in the screening of laccase-secreting transformants. RBB and Phenol Red could serve as alternatives after further optimization.

3.2 Preparation of DNA

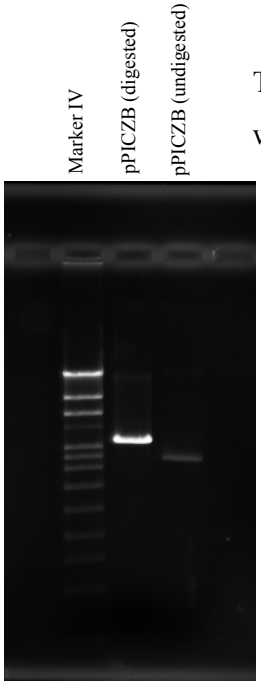

Figure 14 pPICZB digested with EcoR I.

The vector pPICZB and a plasmid consiting of pPICZB with the lcc2 gene inserted were transformed into *E. coli* XL-1 blue. Each type of transformants was spread on LB plates containing zeocin. One colony was picked and inoculated in LB medium and plasmid DNA was prepared. A digestion with restriction enzymes was performed to assure that it was the correct DNA. Fig. 14 shows digestion of the preparation of the pPICZB vector. The band is approximately at 3.3 kbp and that is the expected size of the plasmid. Fig. 15 shows digestion of pPICZB/lcc2 with EcoR I and Xba I. The band at ~ 1.6 kbp correlates with the expected size of the lcc2 gene. The band at ~ 3.3 kbp should be the

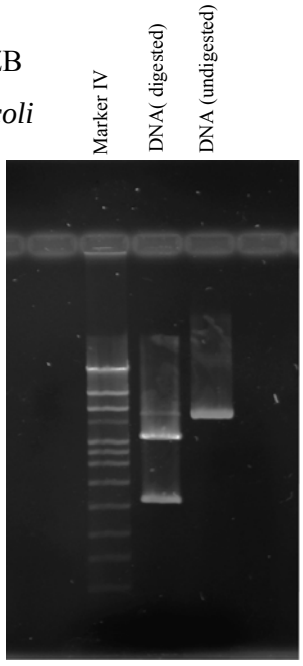

Figure 15 pPICZB/lcc2 digested with EcoR I and Xba I.

vector without insert. The weak band visible at ~ 4.9 kb represents undigested pPICZB/lcc2. The analysis suggests that the DNA preparation was correct. To determinate the DNA concentration, a spectrophotometric measurement was performed (Table 5).

Table 5 Spectrophotometric determination of DNA concentrations.

DNA	Absorbance	Concentration (µg/ml)
pPICZB/lccc2	3.2	160
pPICZB	2.0	100

The concentrations were used to calculate the DNA addition to the error-prone PCR in order to get the right mutation frequency. 500-1000 ng of DNA were added to the PCR reaction to get a mutation frequency of 0-4.5 mutations per kbp. This frequency was chosen to get many transformants with a functional lcc2 gene and hopefully some with different properties. A higher mutation frequency could give more diverse transformants but there would be a risk that they could not express a functional enzyme. After the PCR reaction, the DNA was analyzed on an agarose gel and it showed that the amount of PCR product was almost the same as the amount of template. This indicates that it was too much template from the beginning. Maybe it would have been more accurate to instead of a absorbance measurement use a spot-test, that gives more precise results. The amount of DNA template in the beginning should not affect the final amount of PCR product, but the mutation frequency could be lower than wanted.

3.3 Cloning of the mutagenesis product into pPICZB

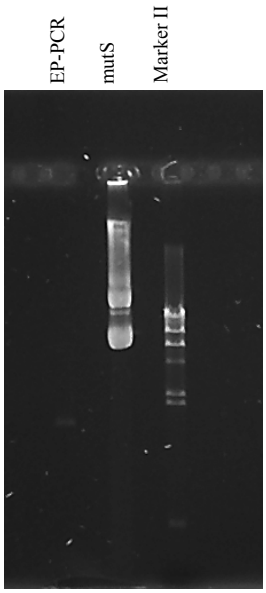

Figure 16 Error-prone PCR product and *E. coli* mutS product.

Since only small amounts of product were obtained in the PCR-mutagenesis reaction, the product was amplified by a new PCR. The amplified product was purified and analyzed using a 1% agarose gel. A band corresponding to the gene (~ 1.6 kbp) was observed (Fig. 16).

Plasmids were purified from the *E. coli* ES 1301 mutS cells with the pBluescript/lcc2 plasmid and were then analyzed on a 1% agarose gel

(Fig. 16).

The product from the *E. coli* mutS strain should be the vector (pBluescript) (3.0 kbp) plus the inserted gene (1.6 kbp) and the size of this fragment should therefore be 4.6 kbp. The gel (Fig. 16) shows a band of this size. The concentration was determined by a spot test and a restriction digest was performed.

Both mutagenesis products were digested with EcoR I and Xba I. The error-prone PCR product needed to be digested to take away a few nucleotides from the ends. These nucleotides needed to be removed because the primers overlapped the junctions between the gene and the vector and the products were longer than the gene. It could be difficult to digest PCR products when the recognition site of the restriction enzyme is very close to the end of the fragment. To make the digestion more thorough, the reaction time was prolonged and more enzyme than normally considered necessary was used. The mutS product was digested to cut out the insert from the vector. The pPICZB vector was digested as a preparation for the insertion of the mutated laccase genes. The digestion was performed with EcoR I and Xba I to create sticky ends to facilitate ligation (Fig. 17).

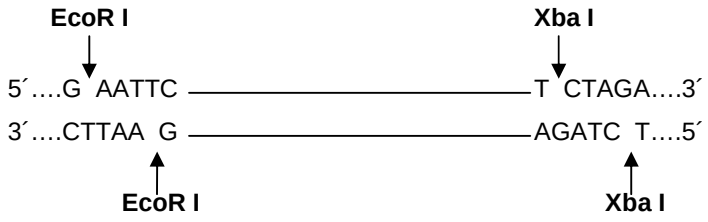

Figure 17 Digestion with EcoR I and Xba I.

The digestion of the PCR product, the products from the mutS strain and the vector pPICZB were analyzed with agarose-gel electrophoresis (Fig. 18). To check if the digestion was succesful for the error-prone PCR product could be difficult, because just a few nucleotides should be removed by the restriction enzymes, so the size of the digested and the undigested DNA should be very similar. This should not be a problem in the digestion of the product from the mutS strain and the vector pPICZB. The PCR product can be seen as a band around 1.6 kbp (Fig. 18), but as discussed above, it is impossible to say if it is correctly digested. The lane with digested mutS product showed a band at 4.6 kbp, which is probably undigested plasmid, and a fragment with the size of the plasmid (3.0 kbp). There is also a week band at 1.6 kbp. The plasmid pPICZB seems to have been digested correctly. After the digestion, another spot test was performed to determine the DNA concentration prior to the ligation reaction.

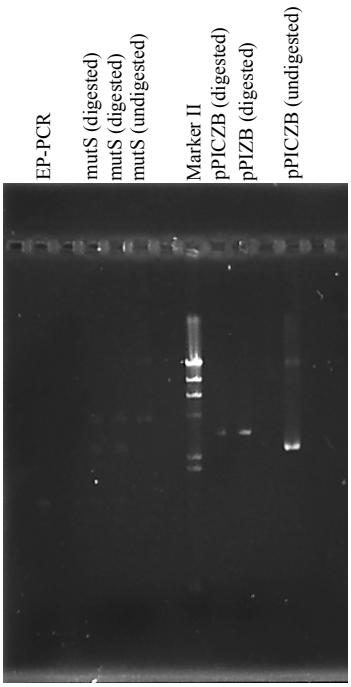

Figure 18 Control of the digestion (1% agarose gel)

The ligation could be critical, since it is necessary to have enough DNA and a balance between the vector and the insert. It would be best to have a surplus of the mutagenesis product to make sure that there is enough to clone into the plasmid. The ligation could also fail if the digestion was unsuccesful, so that the ends of the fragments would not be compatible.

3.4 Linearization and transformation of *P. pastoris*

After ligation, the DNA was linearized with Sac I. The DNA was concentrated by sodium acetate precipitation before it was checked by agarose gel electrophoresis. As can be seen in Fig. 19, there are many bands in the lane with the product from PCR mutagenesis. There is one band at around 1.6 kbp, which implicates that not all of the PCR product has been ligated. There are two bands under 3.3 kb, the size of the vector. One of these bands is probably open plasmid that has been digested with Sac I. Two of the PCR products could

have been ligated, which would give a fragmant of around 3.2 kbp. The band around 3.3 kbp could be unligated plasmid. One band is approximately 4.9 kbp and could be the ligation construct. The remaining bands could be circular ligation products, where the Sac I digestion has not been succesful. The lane with the *E. coli* mutS product also shows many bands (Fig. 19) and the reason could be the same as for the PCR product ligation mixture. Fig. 19 shows that the control seems to have been correctly digested. The DNA was then used for transformation of *P. pastoris* SMD1168 and the cells were spread on YPDS-zeocin plates. After three days, there were approximately 50 colonies from each ligation reaction visible. They were incubated for a couple of days to obtain larger colonies. Then, around 20 colonies were picked and transferred to BMMH plates with either ABTS or syringaldazine.

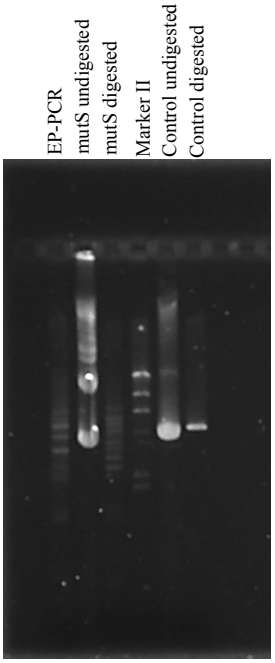

Figure 19 Linearization of EP-PCR product, *E. coli* mutS product and control.

3.5 Analysis of transformants

After one day (Fig. 20A), a green colour was visible around the control colonies on plates containing 1 mM ABTS. After three days (Fig. 20C), the first purple colour could be seen on the plates with 1 mM syringaldazine. Fig. 20B and 20D show the plates after one week. As discussed before, it was not clear how the yeast cells would perform on agar plates containing DMSO, which was present in plates with syringaldazine. As can been seen in Fig. 20C and D, the cells could grow and express laccase on those plates. When the ABTS concentration was higher than the normal concentration 0.2 mM, the screening was more effective. The colour appeared already after one day, while it took longer time with lower concentrations. Syringaldazine seems to work as good as ABTS as a substrate for screening of laccase-expressing transformants. With 1 mM syringaldazine in the plates, colour development took longer time than with 1 mM ABTS. But as showed in the initial test, syringaldazine plates were better with higher concentrations and that should be

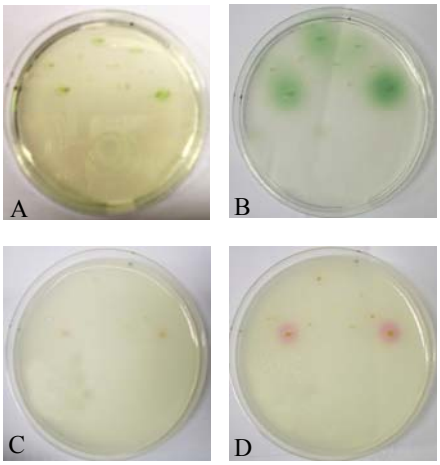

Figure 20 Colour development on control plates. A: One day after growth on plates with 1 mM ABTS, B: One week after growth on plates with 1 mM ABTS, C: Three days after growth on plates with 1 mM syringaldazine, D: One week after growth on plates with 1 mM syringaldazine.

interesting to try in screening experiments. Maybe they also have higher sensitivity to lower laccase activity.

On the plates with transformants with mutagenesis products no colour development was observed even after 3 weeks. To analyze the transformants, a colony PCR was performed. The PCR products were analyzed on 1% agarose gel without any results. This indicates that only the plasmid without gene insert was transformed into *P. pastoris*. What happend to the gene is a question that can have many answers. First, with regard to the mutagenesis PCR, something went wrong when the product amount was that low. If the start amount of DNA was too large, the mutation frequency would have been lower than wanted. The result of this would be larger amount of funtional genes. In the ligation reaction the balance between gene and plasmid was not as wanted. In the end this caused too many transformants with just the plasmid inserted. Maybe there were transformats with the plasmid plus the gene but they were not picked and analyzed. As mentioned in section 2.2.1, the *P. pastoris* strain used has a slower growth rate and is more difficult to transform than wild-type strains. But it does not seem like that was the problem here, because there was no problem with the control transformants.

Since no transformants were expressing laccase, it was not possible to compare the mutation efficiency of both mutagenesis methods. One problem with the error-prone PCR product is that the recognition site for the digestion is just a few nucleotides from the end. This is not a problem for the *E. coli* mutS strain. In conclusion, both of the methods are easy to perform, but it seems to be difficult to continue with the products.

4. Acknowledgements

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Appendix content

1) Recipes
2) Markers
3) Results from absorbance measurement and calculations of laccase activity.

Appendix 1

Recipes

YPD medium
1% Yeast extract
2% Peptone
2% Dextrose
For plates add ± 2% agar

YPDS medium
1% Yeast extract
2% Peptone
2% Dextrose
1 M Sorbitol
For plates add ± 2% agar

LB medium (1l)
10 g Tryptone
10 g NaCl
5 g Yeast extract
Adjust pH to 7.5 with NaOH
For plates add ± 2% agar
For low-salt LB add 5 g NaCl instead of 10 g

BMMH plates
100 mM Potassium phosphate pH 6.0
1.34% YNB
$4x10^{-5}$% Biotin
0.5% Methanol
1 M Sorbitol
0.1 mM $CuSO_4$
For plates add ± 2% agar

10xTBE Buffer (1l)
1 g NaOH
108 g TrisBase
55 g Boric acid
7.5 g EDTA

1% agarose gel
1% agarose in 1xTBE buffer

SOC medium (1l)
20 g Tryptone
5 g Yeast extract
0,5 g NaCl
10 ml 250 mM KCl solution
Adjust pH to 7.0 with NaOH.
5 ml 2 M $MgCl_2$
20 mM glucose

Sodium acetate precipitation
50 µl DNA-solution
1/10 vol. 3M NaAc pH 5,2
2 volumes of EtOH

Agarose-gel marker
8 µl λ DNA (2 µg)
1 µl Hind III
2 µl Buffer
9 µl water
Incubate in 37˚C for two hours
Use 3 µl of this on the gel → 300 ng

Appendix 2

Marker IV (Roche) (bp)	Marker II (Roche) (bp)
19329	23130
7743	9416
5526	6557
4254	4361
3140	2322
2690	2027
2322	564
1882	
1489	
1150	
925	
697	
421	

Appendix 3

Four laccase solutions were used in the examination of different substrates for laccase screening (section 2.1). Solution number 4 had the lowest activity, solution 3 was 10 times more concentrated, solution 2 was 100 times stronger and solution 1 was 1000 times more concentrated. To calculate the approximate laccase activity, an absorbance measurement was performed. These are the results from the absorbance measurements on solution number 1.

Time	Abs
0	0.0987
0.1	0.102
0.2	0.1044
0.3	0.1072
0.4	0.1096
0.5	0.1122
0.6	0.1148
0.7	0.1175
0.8	0.1205
0.9	0.1236
1	0.1261
1.1	0.128
1.2	0.1306
1.3	0.1332
1.4	0.1364
1.5	0.1391
1.6	0.1416
1.7	0.1442
1.8	0.1468
1.9	0.1497
2	0.1527
2.1	0.1555
2.2	0.1582
2.3	0.1609
2.4	0.1637
2.5	0.1662

Time	Abs
2.6	0.1688
2.7	0.172
2.8	0.1744
2.9	0.1765
3	0.1794
3.1	0.1818
3.2	0.1852
3.3	0.1882
3.4	0.1911
3.5	0.1934
3.6	0.196
3.7	0.1987
3.8	0.2013
3.9	0.204
4	0.2067
4.1	0.2093
4.2	0.212
4.3	0.2143
4.4	0.2168
4.5	0.2195
4.6	0.2225
4.7	0.2253
4.8	0.2284
4.9	0.2311
5	0.2335

The activity can then be calculated from the following formula:

A_1-A_0/ 1(l)*3.6*10^4(ε)*time (min) = Activity (M/min, (U/L))

Lambert beers law

$A = C*l* \varepsilon = C = A/(l* \varepsilon)$

ε from ref. 24

Activity calculation of solution four

$(0.2335-0.0987)/(1*3.6*10^4*5)=7.5*10^{-7}$ M/min

The laccase solution was diluted 20 times in the cuvette, so the activity in solution number 4 was:

$7.5*10^{-7}*20= 1.5*10^{-5}$ M/min