SCIENCE AND TECHNOLOGY FACULTY. LEIOA

DEGREE ENDING PROJECT

CLONING AND ANALYSIS OF LACCASES FROM *ACINETOBACTER BAUMANNII* ISOLATES

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Date: 24th June 2014

Director:

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Academic year:

2013/2014

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1. INTRODUCTION AND OBJECTIVES

Laccases (benzenediol: oxygen oxidoreductases; EC 1.10.3.2) are widespread in nature. They are usually found in higher plants and fungi (Thurston 1994; Mayer and Staples 2002), but recently some bacterial laccases have also been found. The first laccase studied was from *Rhus vernicifera* in 1883, a Japanese lacquer tree, from which the name laccase was derived (Yoshida, 1883).

These enzymes belong to the group of blue multi-copper oxidases (MCOs). They usually contain four copper atoms located in three distinct sites. Each site reacts differently to light. The Type 1 (T1) site copper atom absorbs intensely at 600 nm and emits the blue light, the Type 2 (T2) site copper atom is not visible in the absorption spectrum and last, the Type 3 (T3) site has two copper atoms and absorbs at 330 nm (Santhanam *et al*., 2011; Quintanar *et al*., 2007). The protein structure acts as a complex ligand for the catalytic coppers, providing them the right structure where changes between the reduction states are thermodynamically possible (Dubé, 2008). These enzymes oxidize a surprisingly wide variety of organic and inorganic compounds like, diphenols, polyphenols, substituted phenols, diamines and aromatic amines, with concomitant reduction of molecular oxygen to water (Thurston, 1994).

Laccases as oxidative biocatalysts are very appealing because unlike many other oxidoreductases, do not require cofactors such as NAD(P)H, and unlike peroxidases, laccases do not produce toxic peroxide intermediates (Santhanam *et al*., 2011). Due to their capacity to oxidize a wide range of toxic industrial compounds, laccases show a huge potential for using in many processes such as biopulping, biobleaching, bioremediation, food processing a waste water treatment and therefore, their use could serve as a more eco-friendly alternative to the currently used chemical processes (Abadulla *et al.*, 2000; Breen and Singlston, 1999; Collins *et al*., 1996; Reid, 1998; Dwivedi *et al*., 2011; Xu, 1999).

The laccase mediated catalysis has been well studied, but the electron transfer mechanism within the enzyme is not completely understood. It is thought that the electron transfer cycle starts at the T1 site where four substrate molecules are oxidized, producing four electrons. These electrons are shuttled to the trinuclear cluster where the cycle is ends up with the reduction of O_2 to two molecules of water. (Santhanam *et al*, 2011)

However, the use of biological processes based on laccases biotechnology is still very limited because of the relatively high production costs of hese enzymes, the low yield, the low stability under industrial conditions and the inefficacy to process some dangerous effluents. Even though some laccases posses a very high specificity towards their substrates the complete oxidation is not always reached due to the low enzymatic efficiency when some phenolic compounds are used as substrates (Viswanath et al., 2006; Xu, 1996). This is one of the reasons why it is still necessary to search for enzymes with high specificity towards some substrates. Apart from that, for the industrial application of laccases, a big quantity of enzyme is required at a low cost.

1.1 FUNGAL LACCASES

Most of the known laccases that have been characterized up to now originate from fungi. Typical fungal laccases are extracellular proteins of approximately 60–70 kDa. They generally operate in the mesophilic temperatures ranging from 30 ℃ to 55 ℃ and acidic conditions (pH 4). They are generally glycosylated, with a rate of glycosylation between 10 and 25% and only in a few cases the degree of glycosilation is higher than 30% (Mayer and Staples, 2002; Morozova *et al*., 2007). The problem of the fungal laccases is that the quantity of enzyme produced by fungal strains is not high enough to afford their commercial use. Moreover, the hetereologus expression of fungal laccases, especially of those that have a high redox potential, is difficult due to eukaryotic post- translational barriers. (Rodgers *et al*., 2010)

1.2 BACTERIAL LACCASES

The first bacterial laccase was discovered in 1993 in non-motile strains of *Azospirillum lipoferum* isolated from the rhizosphere of rice while measuring the phenoloxidase activity. After this, different laccases have been found in diverse bacteria, particularly in different genres of streptomycetes, *Bacillus* spp. and *Pseudomonas* spp (Santhanam. *et al.* 2011). During the past 10 years, *in silico* biology has opened a new way of discovery for laccases in bacteria. Starting from non-redundant protein sequence databases and incomplete microbial genomes and using fungal laccase amino acid sequences as queries, bioinformatic approaches have revealed several putative prokaryotic laccases. However, only a few bacterial laccase isoforms have been discovered. The best studied bacterial laccase is CotA laccase from *Bacillus subtilis.* (Alexandre, Zhulin, 2000)
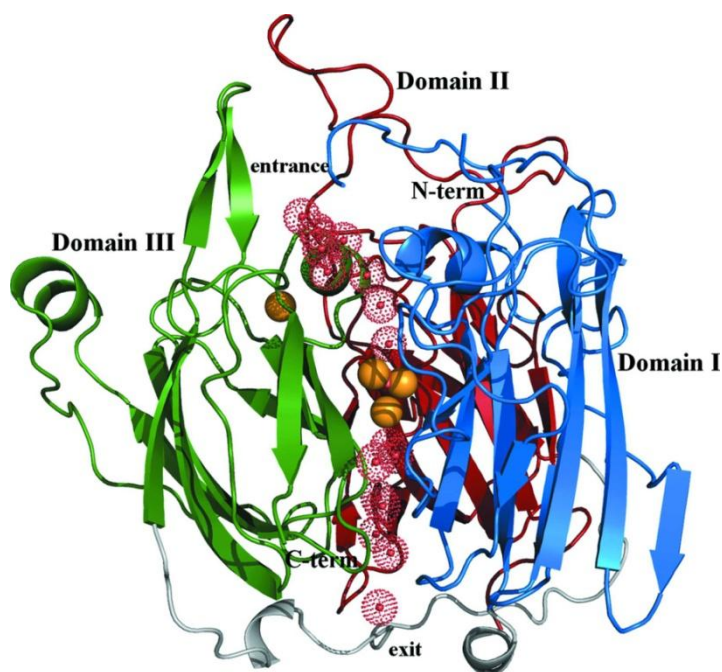
Figure 1: Representation of the three-dimensional structure of the *Bacillus subtilis* laccase CotA.

1.2.1 Molecular characteristics and cellular localization

Prokaryotic laccases can be very variable in size (28–180 kDa) and can function as monomers, trimers or tetramers. However, whether they undergo post-translational modifications such as glycosilation is still unknown (Kumar *et al.,* 2003). Bacterial laccases can be both, extracellular or intra-cellular and the cellular localization of laccases varies considerably in bacteria. High thermostability, alkaline pH optimum and halotolerance are properties that distinguish prokaryotic laccases from eukaryotic laccases, which adds to their potential as robust industrial biocatalysts. (Santhanam. *et al.* 2011)

1.2.2 Structure of bacterial laccases

When it comes to the sequence homology between fungal and bacterial laccases it is pretty low. Nevertheless, the geometry of their active sites is very similar. The structure consists of highly conserved features and the active site containing four copper atoms is specifically structured. In general, laccases are monomers with three interconnected domains twisted together to form a globular structure. (Skálová *et al.,* 2009; Durao *et al.,* 2006)

1.2.3 Reduction potential

T1 Cu of laccases accepts electrons from reducing substrates. Those substrates that can be directly oxidized by laccases must have either lower or slightly higher ionization potentials than the standard reduction potential of the T1 Cu. Bacterial laccases have low reduction potentials (usually below 0.5V) compared with most fungal laccases (in the range of 0.5-0.8 V). This characteristic constrains their industrial application. (Xu *et al.,* 1998; Alcalde, 2007)

There are many hypotheses why the reduction potential of bacterial laccases is lower than fungal laccases. One of them points out that the position of one of the ligands of

the T1 Cu plays an important role in the redox potential of the enzyme. Once the crystal structure of some bacterial laccases was obtained, it was seen that the T1 Cu is attached by two histidines, one cysteine and one methionine along with a vacant position for the binding of the reducing substrate, while fungal laccases have a leucine or a phenylalanine in the place of the methionine. Site-directed mutagenesis of CotA laccase from *Bacillus subtilis* showed that replacement of the methionine residue with leucine and phenylalanine resulted in an increased reduction potential reaching 60 to 90 mV, respectively (Santhanam. e*t al.,* 2011).

1.2.4 Mediators

The substrate specificity of laccases can be broadened by mediators, which are small molecular-mass compounds that are oxidized into radicals by laccases. These mediators can either oxidize compounds of high molecular weight or high ionization potential. Lignin is an example of the high molecular weight substrates that can be oxidized. There are many different mediators. The use of synthetic mediators like ABTS has been well studied by using fungal laccases. However, many mediators are very expensive for being used in industrial processes and create toxic compounds in the redox reactions. Natural mediators have been tested as cheaper and more environment-friendly alternatives. Fungal metabolites like 3-hydroxyanthranilic and natural substituted phenols related to lignin have been studied so far. (Santhanam. e*t al.,* 2011; Xu *et al.,* 2000; Canas, Camarero, 2010).
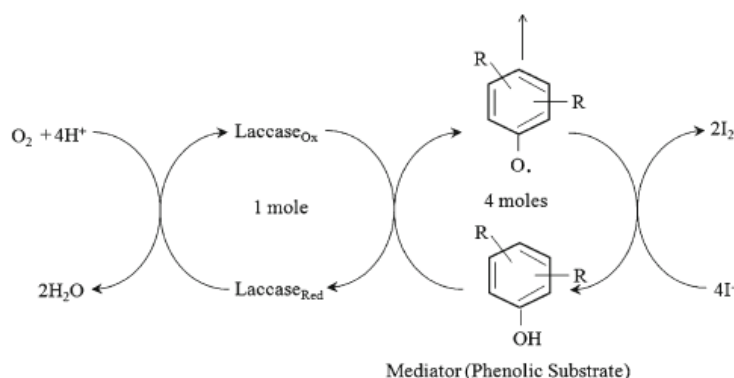

Figure 2: The electron transfer mechanism mediated by laccases. The acceptor of the electrons is the molecular oxygen and the donor of electrons is the mediator that reduces the enzyme.

1.2.5 Applications of laccases in the cellulosic biofuel industry

The effect of mediators in the treatment of pretreated softwood to remove lignin with fungal laccases and its effect on the enzymatic hydrolysis was investigated (Palonen *et al*. 2004). Without a mediator, solubilized aromatic compounds derived from lignin were polymerized and the glucose release rate by enzymatic hydrolysis of cellulose was increased by 11–13%, no matter the laccase treatment was applied prior to or simultaneously with cellulose hydrolysis. In the presence of a mediator, *N*-hydroxy-*N*-phenylacetamide (NHA), it was found that both simultaneous and sequential treatments with laccases and cellulases enhance the extent of hydrolysis. Thus laccases alone or in combination with non-toxic mediators, are very likely to enhance the efficiency of cellulosic production processes such as pretreatment, fermentation and by-product recovery (Santhanam *et al*. 2011). This implies that laccase-mediated treatment could provide a viable and environmentally friendly approach for the removal of lignin often removed by other methods such as chemical treatment.

1.2.6 Engineering of laccases

One of the advantages of bacterial laccases is that their hetereologus expression in *E. coli* is simpler, which makes it easier to obtain a high level of enzyme production at a reasonable cost, thereby avoiding the high cost expected while obtaining these enzymes from fungi (Santhanam *et al*., 2011). Moreover, the laccases of fungal origin require post-translational modifications, such as glicosilations, that only eukaryotic microorganism can perform. On the other hand, such modifications are not essential for the enzymatic activity of the laccases of bacterial origin. This is the major reason why the hetereologus expression of bacterial laccases is easier.

Another reason why the bacterial laccases have attracted such interest is that prokaryotic laccases are more amenable to improvements in activity, selectivity and expression levels through protein engineering (Santhanam *et al*. 2011).

Taking all this into account, the main objective from now on will be the identification and characterization of new laccases of bacterial origin that will have a potential application in biotechnology and industry, such as bioremediation of waste waters and contaminated soils, industrial effluents, etc. because of their potential to oxidize a great variety of compounds like phenols, polyaromatic hydrocarbons, estrogens and biopolimers.

1.2.7 *Acinetobacter baumannii*

This work is focused on laccases likely expressed in clinical strains of *Acinetobacter baumannii.*

Acinetobacter baumannii is gram negative coccobacilli, catalase positive, oxidase-negative, non- fermenter, strictly aerobic and with a GC content of 38- 47 %. Different species of the genus *Acinetobacter* have been isolated from various environments, such as hospitals, soil, water, natural environment, food and infected tissues.

Outside the hospital *A.baumannii* has been isolated from various environmental locations. *A. baumannii* has been isolated from soil, vegetables, and public areas like tables in parks. It has been recovered from soil contaminated with petroleum hydrocarbons, and several *Acinetobacter* species, including *A. baumannii*, are able to degrade diesel fuel (Eveillard *et al.,* 2013). According to *The laccase and multicopper oxidase engineering database* there is evidence of laccase activity in bacteria like *Acinetobacter baumannii.* The identification of laccases in these bacteria models would be very useful for the characterization of the activity of these enzymes that could be used for industrial applications, for example in the bioremediation of waste water and effluents (degradation of petrochemical compounds), of soils and effluents contaminated with heavy metals (textile industry), production of biopolimers and biosurfactants (paper industry, among others) (Doughari *et al.,* 2011).

The main environmental source of *Acinetobacter baumannii* is the hospital setting. *Acinetobacter baumannii* is a nosocomial pathogen responsible for severe infections such as pneumonia, with high mortality rates (up to 35%), chronic bronchitis, bacteriemia, meningitis, urinate tract infections, endocarditis… that affect especially immunocompromised patients. Other risk factors to develop these infections are advanced age, patients with pre-existing diseases, under broad-spectrum antibiotic treatment, with mechanical ventilation, hospitalized for extended periods, patients with burn wounds (Perez *et al.,* 2007). Community-acquired infections include mainly pneumonia and wound infections in unusual situations, such as in victims of an earthquake or war-related wounds in Iraq and Afghanistan (Eveillard *et al.,* 2013).

Regarding to its pathogenicity and virulence factors, *Acinetobacter* colonizes the skin and the respiratory tract due to its capacity of adherence to the host cells and to its resistance to the immune responses of skin and mucosal surfaces; it grows forming a biofilm, regulated by "quorum sensing". It has different virulence factors to damage the host tissues and to evade the immune response: a great capacity of adhesion, the production of the polysaccharide (capsule), the production of enzymes that damage soft tissues, endotoxins, lipopolysaccharide of the cell envelope and lipid A, and the production of slime, that inhibit neutrophils migration and increase the virulence in mixed infections. The factors that promote colonization and damage the host are the induction of the inflammatory response, citotoxicity (*omp*A produces damages in epithelial cells), iron uptake and resistance to complement activity. (Towner, 2009).

Infections are treated with different group or antibiotics, but in many cases they are very difficult to treat as multidrug-resistant phenotypes have been described worldwide, leaving limited therapeutic options that sometimes can lead to the death of the patient. (Neonakis *et al.,* 2011). In fact, it is one of the six most important multidrug-resistant microorganisms in hospitals worldwide (Antunes *et al.,* 2013).

The problem of *Acinetobacter* at hospitals is that it is widely spread due to its ability to contaminate floors, sinks, tabletops, doors, patient's charts, telephone handless, mattresses, pillows, bed linen and curtains with a great persistence of the strains along time, as they are very difficult to eradicate (Gerischer, 2008). Inanimate surfaces have often been described as the source for outbreaks of nosocomial

infections. An estimated 20% to 40% of hospital acquired infections have been attributed to cross infection via the hands of health care personnel, who have become contaminated from direct contact with the patient or indirectly by touching contaminated environmental surfaces (Weber *et al.,* 2010).

Its survival in the clinical environment is due to its great resistance to dry conditions, disinfectants and antibiotics, which is related to the biofilm formation, and its capacity of using different metabolic sources (Gerischer, 2008). *A. baumannii* can survive on inanimate surfaces for months, and can thereby be a continous source of transmission due to its capacity to persist despite attempts to disinfect or remove them (Kramer *et al.,* 2006; Otter *et al*., 2011). Different species of *Acinetobacter* can even survive to the exposure to disinfectants usually used in surface cleaning like clorhexidine, gluconate and phenols. This could be, in part, related to the production of laccases able to oxidize these compounds; so the identification of laccases in *Acinetobacter* could contribute to the development of new targets to eliminate these microorganisms from the hospital environment.

One remarkable fact is that the majority of *A. baumannii* infections are caused by three main population clones with worldwide distribution. Comparative typing of *A. baumannii* strains from different European hospitals demonstrated the occurrence of three clones, originally named "European clones I-III". These three European clones have subsequently been identified worldwide and accordingly re-named as "international clones I-III" being ICII by far the most widely spread, already found in at least 34 countries (Zarrilli *et al.,* 2013). In addition to these major clones, a wide geographic distribution of some other clones has been reported (Kempf *et al.,* 2012).

In the Basque Country, studies carried out by our research group have analyzed the presence and genetic characteristics of *A. baumannii* isolates collected from a hospital in Bilbao since 1999. These three international clones were also present in our hospital environment, together with sporadic ones, at the beginning of the study. Along the years isolates belonging to the international clone I have become predominant (together with an acquisition of antibiotic resistance genes encoded in integrons and plasmids), followed by clone II. The sporadic clones have disappeared. (Canduela *et al.* 2006; Rosales *et al.* 2012)

The persistence along the years of these clones in our hospital environment can be related to the capacity of *A. baumannii* to survive resisting the action of disinfectants. Again it could be due to the capacity to degrade certain toxic compounds by laccases. For these reasons, to go deeper in the study of the laccases produced by the two main clones of *A. baumannii* present in our hospital environment could offer new tools for its control.

1.3 **OBJECTIVES**

Laccases are multicopper oxidases that have the capacity to oxidize a wide variety of organic and inorganic compounds and can be used in many biotechnology applications. Most of the known laccases that have been characterized up to now are of fungal origin but these enzymes are not robust enough to operate at industrial conditions, and the costs of the production are very high.

The laccases obtained from prokaryotes offer advantages because they are easier to produce and to improve using genetic engineering.

Acinetobacter baumannii is a microorganism that has the capacity to survive in environments contaminated with different toxic compounds, and in the hospital setting where it can be detected on inanimate surfaces for months, resisting the action of disinfectants like clorhexidine, gluconate and phenols. These characteristics make this organism very attractive for the study of laccases.

For all these reasons, the objectives of this project are:

- Characterization of putative laccase encoding genes in *Acinetobacter baumannii*
- Cloning of putative laccase encoding genes of *Acinetobacter baumannii*
- Expression of laccase encoding genes from *Acinetobacter baumannii* in *E.coli*
- Measurement of the activity of the recombinant laccase polypeptides

2. DEVELOPMENT

2.1 MATERIALS AND METHODS

2.1.1 Organisms and vectors

Acinetobacter baumannii was the chosen microorganism for this research project. The clone ATCC 19606 was chosen as the control clone. Two representative isolates belonging to international clones I and II were chosen for laccase characterization. The strain 5A/11 belongs to clone I and strain SM28 belongs to clone II. Diverse *E. coli* strains (DH5α, Rosetta and BL21) were used as a host for the recombinant plasmid cloning and for the overexpression of the recombinant protein. Plasmid pBAD/Mic-His C was used as a vector for gene expression.

The *E. coli* Rosetta strain is designed to facilitate the expression of proteins that contain codons that are not frequently used in *E .coli.* It is a derivative of BL21. This strain supplies tRNAs for AGG, AGA, AUA, CUA, CCC, GGA codons on a compatible chloramphenicol-resistant plasmid. This helps to express heterologous proteins in *E. coli* at high level.

The BL21 was also tested because it was mentioned in an article that this strain had been used successfully for the expression of a protein originating from *Acinetobacter baumannii* (Sikarwar, 2013).
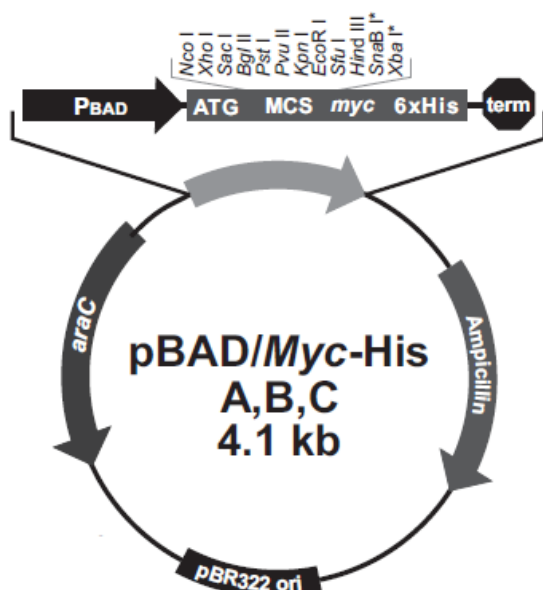

Figure 3: pBAD/Myc-His C is the expression vector selected for the heterologous expression of the recombinant protein. The vector has an inducible promoter araBAD, a multiple cloning site, a C-terminal myc epitope tag, a histidine tag and an ampicillin resistance gene.

2.1.2 Detection of laccase coding genes

2.1.2.1 Polymerase chain reaction

The laccase encoding genes were amplified by PCR using primers based on the genomic sequences obtained from The Laccase Engineering Database LccED, (http://www.lcced.uni-stuttgart.de) and verified in GeneBank, the NIH genetic database (http://www.ncbi.nlm.nih.gov/genbank/). The genes of *A. baumannii* that encode laccases were amplified by PCR using specific primer pairs that were complementary to the sequences flanking each gene.

First, genomic DNA was extracted from the selected strains. According to the protocol, 100 µl of sterile miliQ water were aliquoted in a sterile eppendorf tube and microorganisms taken from the agar plates were resuspended in the water. The cell suspension was heated at 100 ºC for 15 min. After the heating another 900 µl of miliQ water were added to the suspension and it was centrifuged (12000 rpm) for 5 min. At the end of the spinning, the supernatant containing DNA was collected. The structural gene was amplified from total DNA extract of the strains 5A/11 and SM28 of *Acinetobacter baumannii* by polymerase chain reaction (PCR) using the following primers:

5´- ATGGAATTTGTTCAAGGTTTACC-3´ (F) and

 5´-CTACATAAAAACAAAAGTTGCCA-3´(R)

The PCR conditions were these: The samples were heated for 2 min at 94 ºC and then the cycles started. The cycle was formed with a 1 min denaturalization time at 94 ºC, a 1 min hybridization time at 55 ºC and an elongation time of 1 min at 72ºC. The PCR was designed to do 25 cycles. At the end of the cycles, there was a 10 min period at 72 ºC and to end the PCR the samples were cooled down to 4 ºC.

To check if the PCR product obtained with these primers had the expected size, an aliquot of PCR reaction mix was analyzed by gel electrophoresis.

2.1.3 Sequencing

Plasmids containing *A. baumannii* laccase genes were extracted by using the GeneJET Plasmid Miniprep Kit and aliquots of plasmid DNA were sent for sequencing to the sequencing and genotyping service of UPV/EHU (SGIKer). The sequencing was made by Sanger methodology. The sequencing kit used is the BigDye v3.1 (Applied Biosystems)

2.1.4 Cloning of the laccase genes

The structural genes of 5A/11 and SM28 were again amplified from total DNA extract, but this time using different primers.

5´- AATTAACCATGGAATTTGTTCAAGGTTTACCTCAAGGCG -3´ (F)

5´- TTGTTCTACGTACATAAAAACAAAAGTTGCCATACGGCC -3´ (R)

Due to the presence of extra nucleotides at the 5' end of the forward primer (F) introduced a *Nco*I site (underlined) on the beginning part, and the reverse primer (R) introduced a *Sna*BI (underlined) at the end of it. To check if the PCR product obtained with these primers had the predicted size, a DNA gel electrophoresis was performed.

2.1.4.2 Double digestion of PCR product

The amplification products were digested with *Nco*I-*Sna*BI as described below. Namely, after mixing 10 μl of PCR product, 16 μl of miliQ water, 2 μl of FastDigest buffer. 1 μl of each enzyme was added and the mix was incubated for 1 h at 37 ºC. Once the digestion was finished the enzymes were inactivated by incubating at 65 ºC for 15 min.

2.1.4.2 Double digestion of Plasmids

The pBAD/Myc-His C plasmid was digested with *Nco*I-*Sna*BI as described below. Namely, after mixing 10 μl of PCR product, 24 μl of miliQ water, 4 μl of FastDigest buffer. 1 μl of each enzyme was added and the mix was incubated for 1 h at 37 ºC.

Once the digestion was finished the enzymes were inactivated by incubating them at 65 ℃ for 15 min. When the plasmid was digested it was treated with alkaline phosphatase.

2.1.4.3 Purification of digested PCR product and digested plasmid

The *Nco*I-*Sna*BI treated PCR products and plasmids were purified from agarose gels. The digested PCR products were run in a 1.2% agarose gel (1.2 g of agarose in 100 ml of TBE 1x and 10 µl of Gel Red as the staining agent) and the digested plasmids were run in a 0.8% agarose gel (0.8 g of agarose in 100 ml of TBE 1x and 10 µl of diluted Gel Red as a staining agent as the staining agent). Once the DNA bands of interest were identified, they were cut from the gels. After extraction from gel, DNA was purified using the GeneJET^{TM} Gel Extraction Kit, according to the manufacturer's instructions.

2.1.4.4 Ligation of digested PCR product and digested plasmid

The ligation mixture was prepared using the following formula:

100 ng vector + 3x (molar ratio) insert+ 4 µl Rapid ligation buffer
+ 1µl T4 DNA ligase + nuclease free water (up to 20 µl)

$$3 \times 100 \; ng \; vector \times \frac{0.7 \; kb}{4.1 \; kb} = x \; ng \; insert$$

The ligation mixture was incubated for 30 min at a temperature of 22 ℃. The products of the ligation were purified using the GeneJET^{TM} Gel Extraction Kit, as described above.

2.1.5 Transformation

To simplify the expression of the recombinant laccases, the plasmid was transferred to an *E. coli* strain.

The transformation of *E. coli* was made using the TransformAid^{TM} Bacterial Transformation Kit. To prepare competent cells, 2 ml of C-medium were inoculated with cells from a single colony and incubated at 37 ℃ overnight in a shaker. Next

day the overnight culture was transferred to a fresh C-medium and it further incubated for at least 20 min in at 37 ℃ as well. During incubation two Mueller Hinton agar plates with ampicillin were put in the incubator to pre-warm.

When the culture had incubated for more than 20 minutes in the incubator, the transformation procedure was started. The bacterial cells of the C medium were pelleted by 1 min of centrifugation (12000 g), and the supernatant was discarded. Then the cells were resuspended in ice-cold 300 μl of T solution prepared by mixing equal volumes of solution A and B and incubated for 5 min on ice. After the incubation, the cells were again pelleted by 1 min of centrifugation, resuspended in 120 μl of T solution prepared as described above and incubated for 5 min on ice.

During incubation, 5 μl of each ligation mixture (5A/11 and SM28) were put in two eppendorf tubes and incubated on ice for 2 min. When the 5 min of the cells incubation were finished 50 μl of the cell solution were mixed with each ligated DNA sample and they were incubated on ice for another 5 min. After that, the transformed cells were immediately plated onto two pre-warmed plates and incubated overnight at 37 ℃.

2.1.6 Culture conditions

The transformed strains were selected in Mueller Hinton agar containing 50 μg/ml of ampicillin. When the strains were grown in liquid medium, they were grown in Mueller Hinton broth containing 40 μg/ml cocentation of ampicillin.

2.1.7 Clone testing

2.1.7.1 PCR analysis

First of all, single colonies of the transformed clones were selected on Mueller-Hinton agar and from each isolated colony DNA extraction was made by boiling the cell culture. Aliquotes of DNA were anamyzed by PCR to determine whether the

cloned sequence was present in the plasmid or not. To see the results of the PCR analysis the samples were run in an agarose gel.

2.1.7.2 Sequencing

The PCR product obtained from each clone was sent for sequencing to the sequencing and genotyping service of UPV/EHU, SGIKer.

2.1.7.3 Protein overexpression

For the overexpression of the recombinant protein, the transformed clones were cultured with ampicillin overnight, and 500 µl of the overnight cultured media were used to inoculate 5 ml of fresh medium with ampicillin 50 µg/ml, which was further incubated until OD (600 nm) = 0.5. When the required culture density was reached the first sample of 1 ml was taken from each culture and used as a control (t_0=0 h). Then, the promoter araBAD of the vector was induced by adding arabinose (0.2%) and the cultures were incubated further. The culture samples were taken after t_1= 2 h and t_2= 3 h. The samples were centrifuged at 12000 rpm for 1 min and the supernatant was discarded. Then, the pellet was resuspended in 40 µl of TE buffer and after that, 60 µl of protein loading buffer were added. To finish the treatment the samples were heated at 95 ºC for 5 min and then stored at -20 ºC. To see if there was any overexpression or not the protein samples were run in a 15% polyacrilamide SDS gel (200 V and 45 mA) and compared to a control. The same procedure was repeated with the cells overexpressing enolase. These additional controls were prepared to make sure that the induction protocol works.

2.1.8 Protein determination

2.1.8.1 Western Blot

Cells were grown until OD_{600} 0.4-0.6 was reached and protein overexpression was induced by 0.2% arabinose. After 180 min of overexpression protein samples were prepared as described above and loaded onto a SDS-polyacrylamide gel. Proteins were resolved at 125 V for 2 h and transferred to a PVDF membrane. Recombinant

laccase polypeptides were probed with anti-6His antibodies and visualized by an ECL detection kit.

2.1.9 Zymography

A normal SDS-PAGE was performed using a 15% polyacrilamide gel. Zymograms of the proteins were obtained by renaturing the gels in buffer containing 10 mM Tris-HCl (pH 7) for 2 h. The bands containing laccase polypeptides were revealed by ncubating in laccase reaction buffer (2.5 mM ABTS in 0.1 M acetate buffer (pH 5)) (Zapata, 2012).

2.2 RESULTS AND DISCUSSION

2.2.1 Laccase encoding gene amplification

By searching in several databases, we found some putative genes encoding *Acinetobacter baumannii* laccases. One of them is the *yfiH*, encoding a certain multi-copper oxidase with high homology to laccases.

Using polymerase chain reaction (PCR) the laccase encoding genes of both strains 5A/11 (clone I) and SM28 (clone II) were amplified. Results shown in Figure 4:
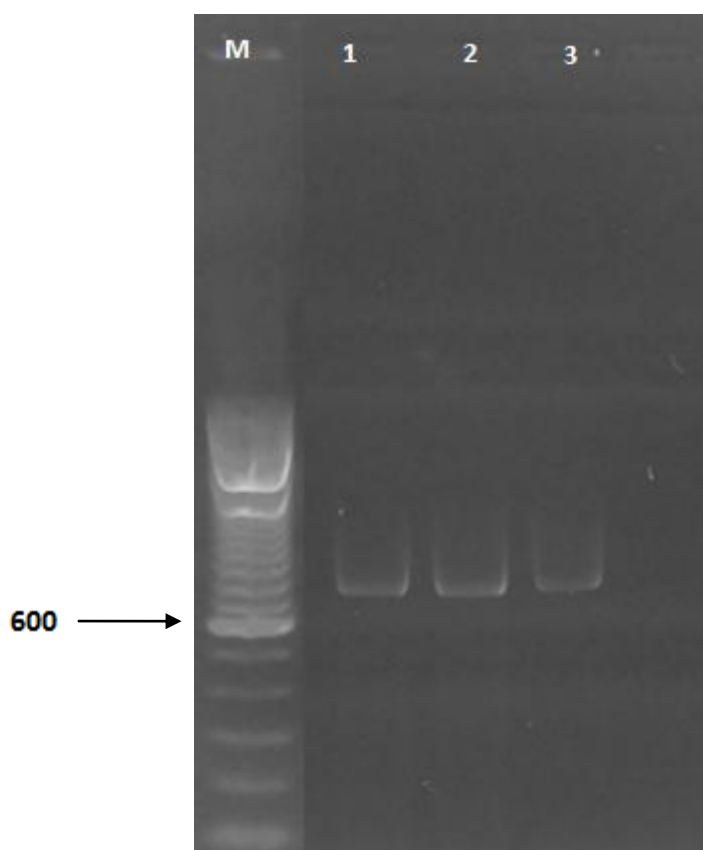
Figure 4: M: 100 bp DNA ladder, Invitrogen. Lane 1: amplified ATCC19606 *yfiH* gene. Lane 2: amplified SM28 *yfiH* gene. Lane 3: amplified 5A/11 *yfiH* gene

As it can be seen in the picture the amplification of the gene was successful. The obtained result was a 738 bp amplicon.

2.2.2 Sequencing of the laccase coding genes

When the *yfiH* gene of both SM28 and 5A/11 were amplified, the first thing to do was to check them for sequencing. Identity and orientation of the laccase encoding genes were confirmed by sequencing of the PCR product.

The obtained results for 5A/11 are shown in Figure 5:
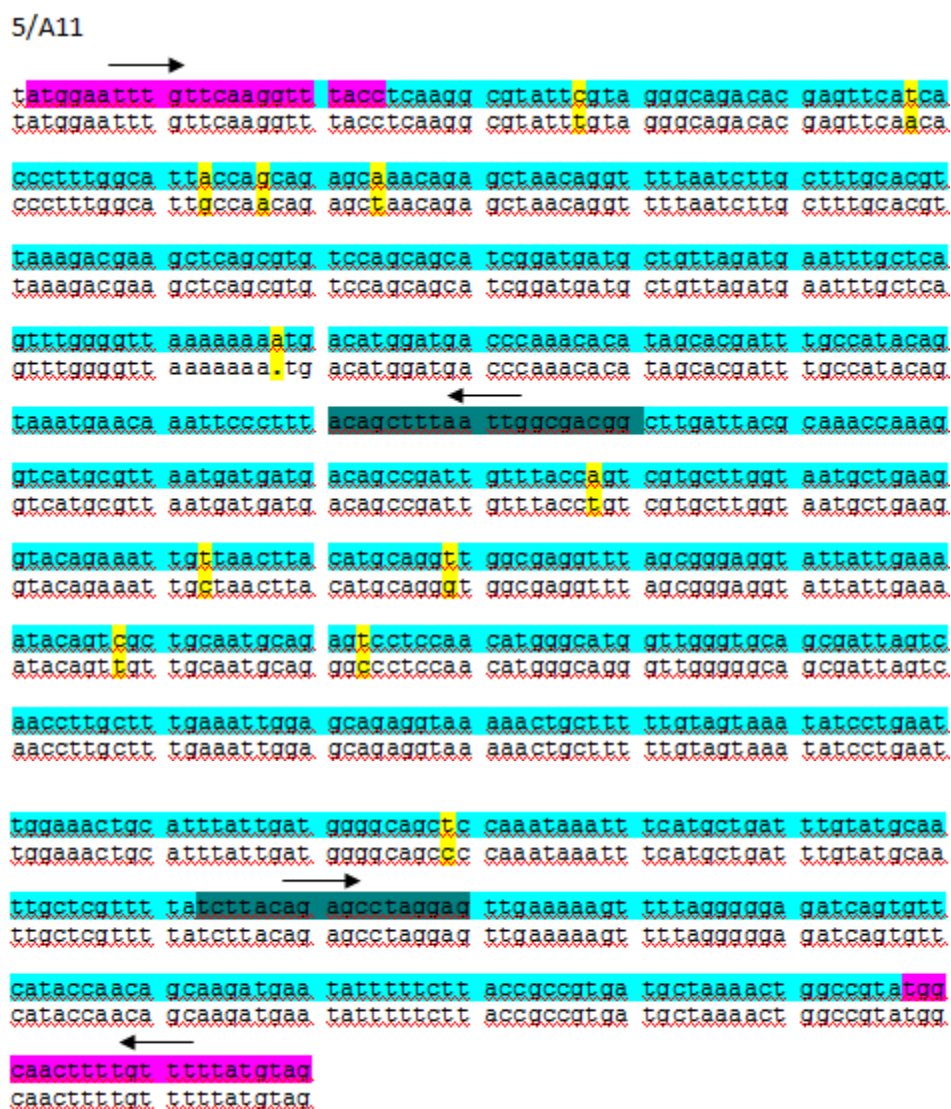
Figure 5: Sequence in blue: ATCC. Grey and pink colored sequences are the primers used to sequence the gene. The mismatches between 5A/11 and ATCC are shown in yellow color.

In 5A/11 several mutations were found by comparing the laccase coding sequence with the sequence of the control strain ATCC 19606. We found amino acid substitutions. They could lead to different properties of the protein.

The obtained results for SM28 shown in Figure 6:

Figure 6: Sequence in blue: ATCC. Grey and pink coloured sequences are the primers used to sequence the gene. The mismatches between SM28 and ATCC are shown in yellow color.

The sequencing experiment showed that the SM28 laccase coding sequence had several mutations compared with the control strain ATCC 19606. These mismatches

between the SM28 strain and the ATCC control strain could mean that the protein from SM28 can have temperature stability, substrate specificity and etc.

The differences in amino acid sequences found between the sequences of ATCC 19606 and the two clinical strains means that the reason why these clones are so persistent could be related to this finding. We mentioned before that these clinical clones are found usually in hospital environment, even in the places that have been treated with disinfectants. The reason of such persistence could be that their laccases are more active and more efficient and in that way they are able to degrade antibacterial substances. More experiments are needed to check the effect of these Single nucleotide polimorphisms (SNPs) on protein activity.

2.2.3 Cloning of the laccase encoding genes

The laccase gene was inserted into the *E. coli* expression vector pBAD/ Mic-His C between *Nco*I and *Sna*BI, resulting in a 4.8 kb plasmid (pBAD/ Mic-His C- *yfiH*). Results shown in Figure 7.This vector has an inducible promoter called araBAD that can be induced by addition of arabinose into the medium. The resultant plasmid was transformed into different strains of *E. coli*.
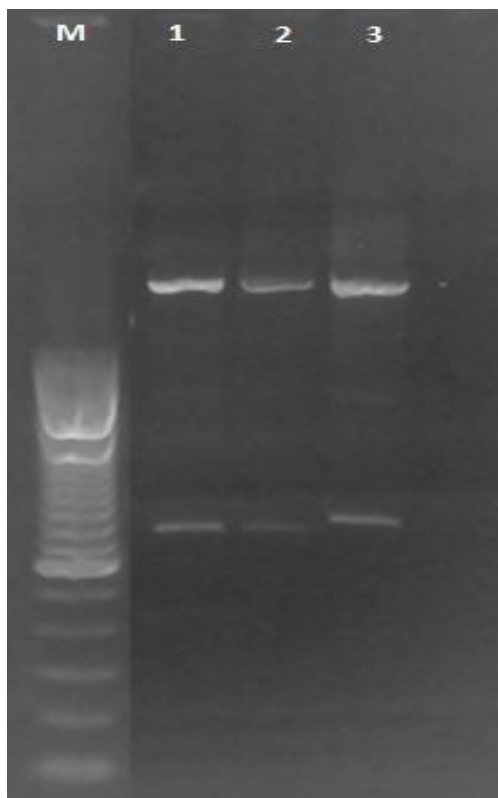
Figure 7: M: 100 bp DNA ladder, Invitrogen. Lanes 1, 2 and 3: each line shows bands of the digested plasmid which is the higher band, and the inserted gene band, which is the lower one

The first strain to be tested was the DH5α. The DH5α was transformed following the protocol mentioned and it was transformed properly because at the next day of the transformation there were some colonies on the agar. A PCR analysis of many different isolates was made and the obtained results as seen in Figure 8 showed that none of the transformants had the contruct of the laccase gene derived from clone SM28, while in the case of the laccase gene from the clone 5A/11 four of the clones had the amplicon in the vector, the clones 4, 5, 6 and 7 to be more specific. More clones were isolated and tested via PCR but none of them had the desired construct.
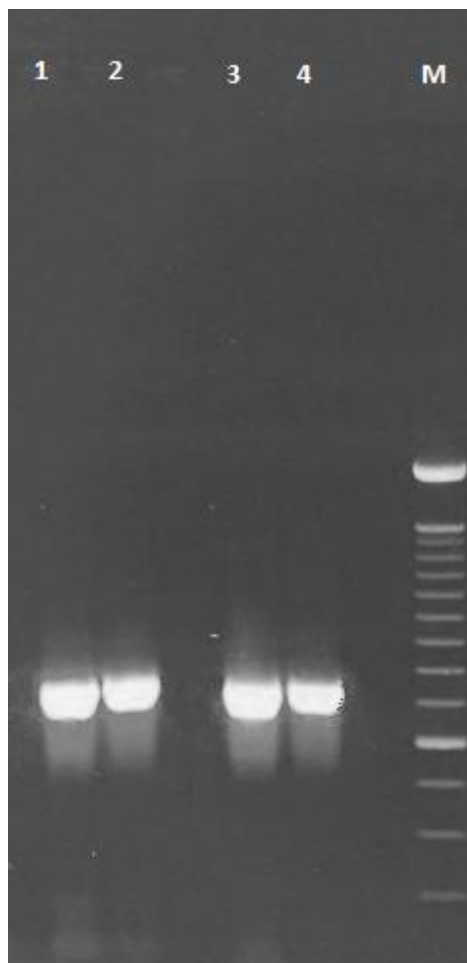
Figure 8: Amplification of DNA extraction of some transformant colonies. Lanes 1, 2, 3 and 4 show that the extracted plasmids of these transformants have the laccase gene inside. M:100 bp DNA ladder, Invitrogen.

With the positive clones 4, 5, 7 and 8 of DH5α lac 5A/11 the overexpression of the recombinant protein was attempted, but with a negative result.

The DH5α was selected because is the host strain frequently used in molecular cloning experiments. But the result was negative, a reason for this could be that the protein from *Acinetobacter baumannii* has some codons that are less frequently used in *E. coli* and for that reason this host organism is not able to express this foreign protein efficiently. Although some authors have managed to express an *Acinetobacter baumannii* protein in this strain, after many trials, the expression of the *yfiH* gene was not possibe.

As the overexpression did not work the plasmid from the positive clones were extracted for re-transformations of other strains. The isolated plasmids were run in an agarose (0.8 %) gel to know if they were isolated properly Results shown in Figure 9:
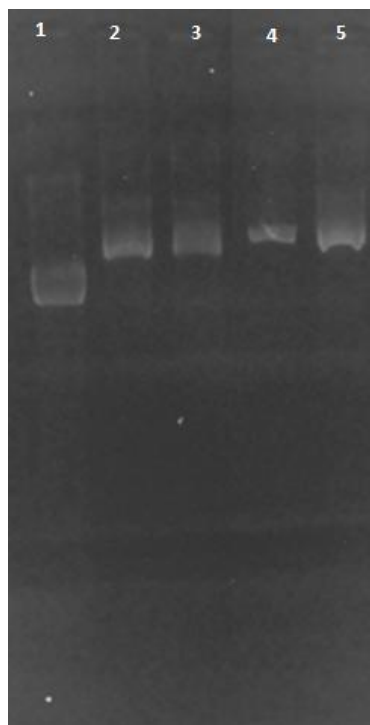

Figure 9: Lane 1, positive control, pBAC/Myc-His C. Lanes 2, 3, 4 and 5 plasmid extractions from positive transformants. The bands of the extracted constructs are a little higher because of the inserted laccase gene.

As the protein overexpression was not possible in the DH5α strain another strain was selected, the strain Rosseta. This strain is used for the expression of recombinant proteins that have codons that are not common in *E.coli* proteins.

The common *E.coli* Rosetta strains were transformed with the construct that had inside the *yfiH* gene (SM28 and 5A/11). The transformation was successful. The overexpression was tried and different samples were obtained. But after running the samples in a 15% polyacrilamide SDS gel, no overexpression was observed.

The next attempt to overexpress the laccase encoding gene was made with another strain, the *E.coli* BL 21.

To transform the BL21 with the plasmids containing the laccase gene from both strains (5A/11 and SM28) were used. The strain BL21 was transformed successfully and different colonies were isolated from each plate and PCR analysis experiments were carried out for detection of the presence of the *yfiH* gene. All the tested transformants had the laccase encoding gene inside except one of the laccase SM28 transformants.   Results shown in Figure 10:
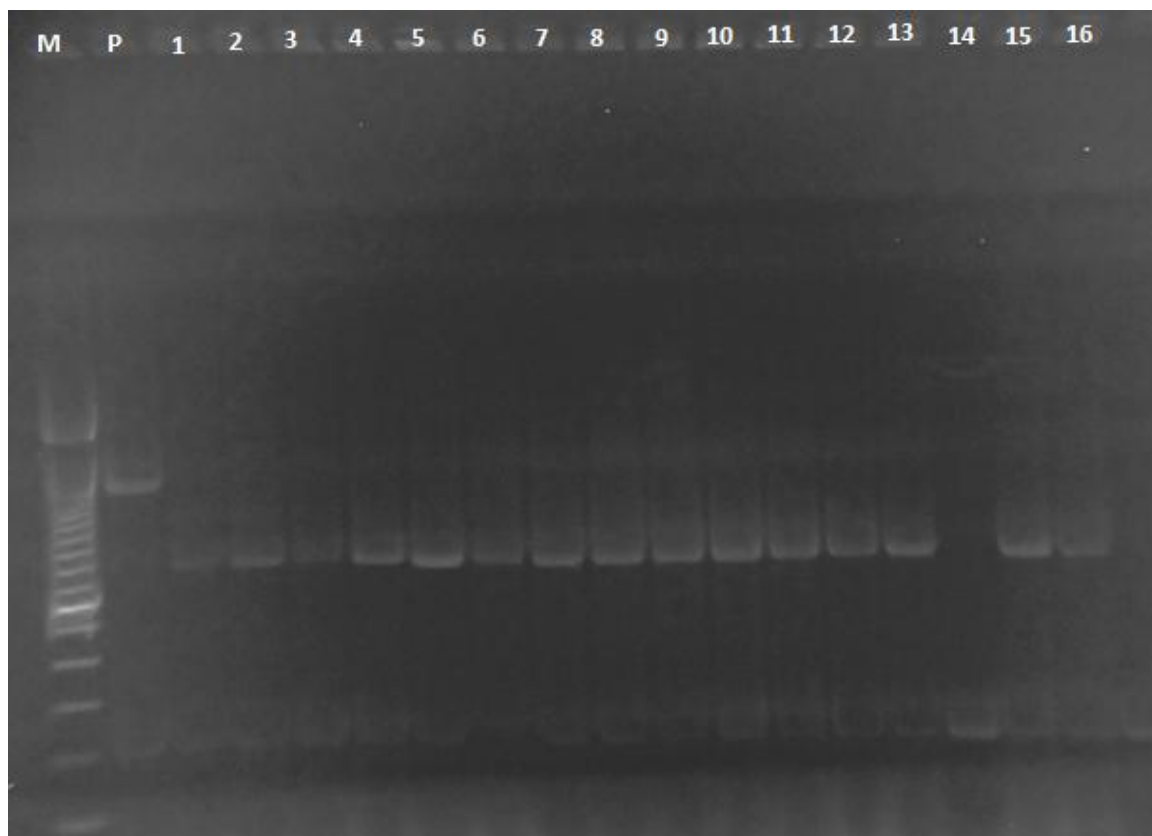

Figure 10: DNA electrophoresis of plasmids extracted from different transformants. **M**:100 bp DNA ladder, Invitrogen. **P:** positive control, pBAD/Myc-His C + enolase. Lanes 1-8: Amplification of extracted DNA from colonies of laccase 5A/11 transformants. Lanes 9-16: Amplification of extracted DNA from colonies of laccase SM28 transformants.

Similar to analysis of transformants DH5α we performed, the overexpression and analysis of BL21 transformants was done. To do the overexpression, positive transformants carrying the *yfiH* gene of *A. baumannii* were used. Protein samples obtained after different induction times were analyzed on polyacrilamide (15%) SDS gel. Results shown in Figure 11:
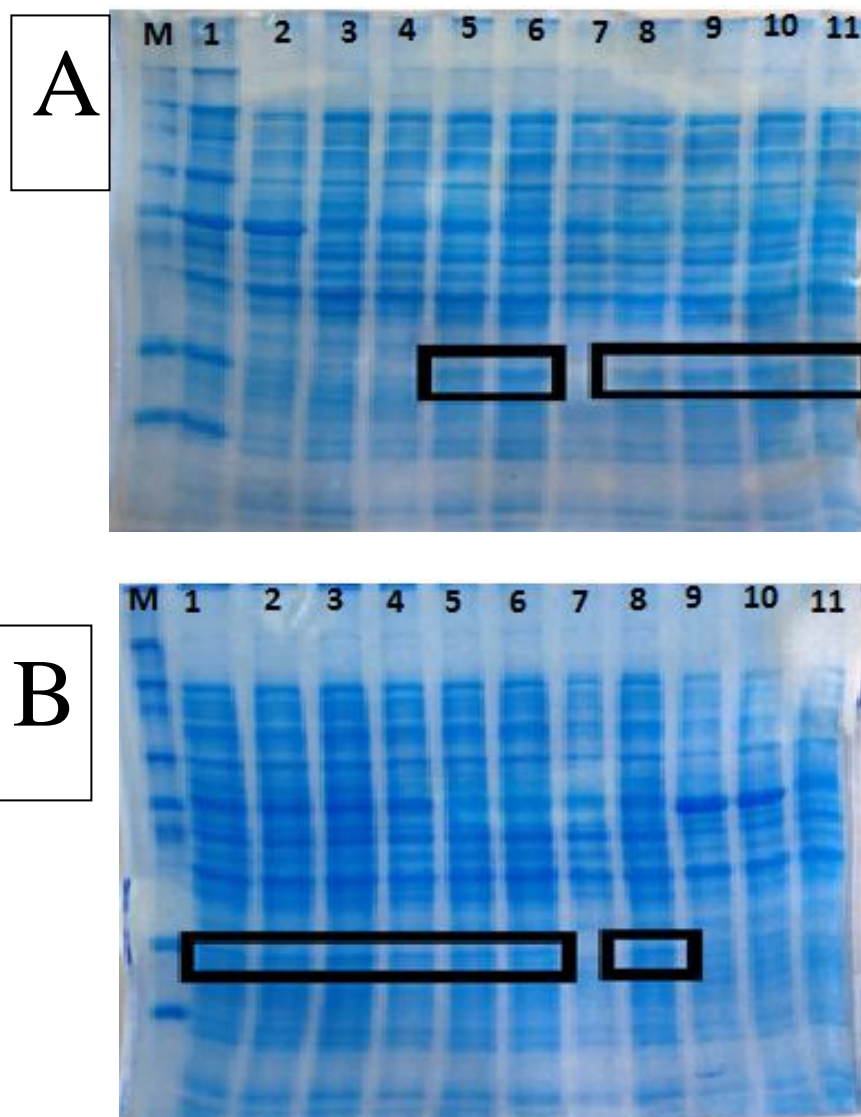
Figure 11: Overexpression analysis of the recombinant protein SDS PAGE. **(A)** M:: Novex sharp pre-stained protein standards, 1. Enolase t_1 , 2. Enolase t_2, 3. BL21 t_2, 4. 5A/11 4 t_0, 5. 5A/11 4 t_1, 6. 5A/11 4 t_2, 7. 5A/11 8 t_0, 8. 5A/11 8 t_1, 9. 5A/11 8 t_2, 10. 5A/11 5 t_2 and 11. 5A/11 7 t_2. **(B)** M: Novex sharp pre-stained protein standards, 1. SM28 4 t_2, 2. SM28 4 t_1, 3. SM28 3 t_2, 4. SM28 3 t_1, 5. SM28 2 t_2,   6. SM28 2 t1, 7. SM28 2 t_0, 8. Enolase t_2, 9. Enolase t_2, 10. Enolase t_1. 11. SM28 1 t_0. The overexpressed proteins bands are inside the black boxes.

In this case, the overpression was successful. The samples obtained at t_0 had no overexpressed recombinant protein while the samples taken at t_1 and t_2 presented a noticeable band of recombinant protein in both cases, in the gel run with strains containing 5A/11 gene samples and in the gel run with the strains containing the SM28 samples. The protein was around 27 kDa.

To have additional evidence, Western Blot was performed. The results of Western Blot showed very clear that the recombinant protein was there. These Results shown in Figure 12:
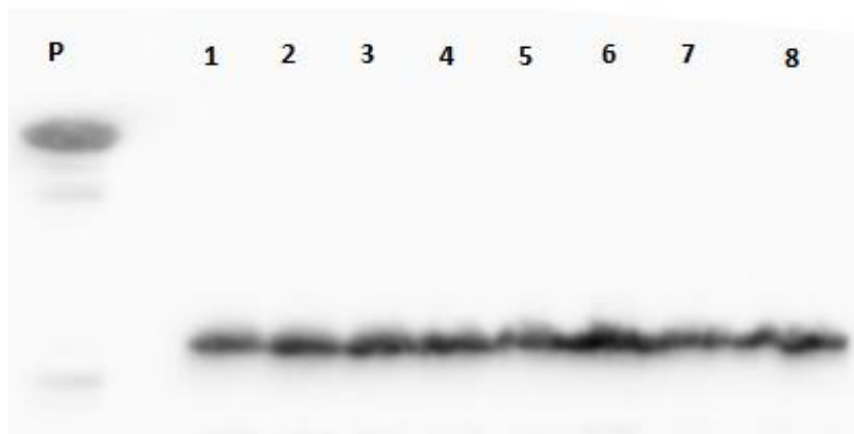

Figure 12: A Western Blot picture. **P:** positive control, enolase. Lanes 1- 4: antibody detection of laccases in 4 protein samples of BL21 laccase 5A/11 transformants. Lanes 5- 8: antibody detection of laccases in 4 protein samples of BL21 laccase SM28 transformants.

The presence of the recombinant protein was confirmed by incubation with His-tag antibody.

A first attempt to measure the laccase activity was made by zymography. Results shown in Figure 13:

Figure 13: The zymography made in a 15% polyacrilamide gel. The positive control took a green color which shows the laccase activity. The protein samples obtained from gave no signal.

The activity assay tried with the recombinant polypeptides overexpressed by the transformants was negative, but the positive control did give signal, so this means that the technique was used correctly. The reason why the positive control worked and the samples not could be the origin of each. The positive control is a fungal laccase and the samples are laccases that come from bacteria. To test the activity of bacterial laccases another assay conditions might be needed. Therefore, more experiments are needed to know if the recombinant protein is active or not.

3. CONCLUSIONS

1. The laccase encoding genes of *Acinetobacter baumannii* of clone I and clone II (clinical isolates 5A/11 and SM28) have been amplified.

2. There are several point mutations found in the laccases from clone I and clone II when compared to the sequence of laccase from the control strain *Acinetobacter baumannii* ATCC19606.

3. The amino acid substitutions found in amino acid sequence of laccases from clone I and clone II are different which is consistent with the different genetic background of clone I and clone II.

4. The cloned laccase polypeptides were successfully overexpressed in the *E. coli* BL21 strain, but not in DH5α and Rosetta strains, which shows the importance of the genetic background for heterologous gene expression.

5. The zymographic detection of *A. baumannii* laccases was tried. Although the control (fungal laccase) yielded positive signal, the result obtained with *A. baumannii* laccase was negative, suggesting that the zymographic assay requires further optimization

.

With all these achievements the three first objectives have been fulfilled, the objectives of characterization, cloning and expression. But still there is one thing left to characterize, the activity of the recombinant protein. The zymography technique was not optimal. So, still more experiments are needed to demonstrate that the laccases of *Acinetobacter baumannii* overexpressed in *E. coli* have the expected activity.

Apart from that there are more experiments that can be done for further characterization of the recombinant enzyme. The stability, pH and substrate binding can be measured with the help of a spectophotometer by exploiting the fluorescence properties of the enzyme.

4. ACKNOWLEDGMENTS

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