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# AN EXHAUSTIVE EXAMINATION AND ASSESSMENT OF THE REPLICATION AND RECOMBINANT PRODUCTION OF A HEAT-RESISTANT LACCASE ENZYME IN ESCHERICHIA COLI

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## ABSTRACT

Laccase is an enzyme known for its ability to expedite chemical reactions. It belongs to a specific class of enzymes that naturally generate reactive radicals, which have applications in various biological processes and find use in commercial applications across chemical and biotechnological industries. Laccases are not limited to bacteria; they are also found in different species of fungi and plants. Their industrial applications are diverse, including the detoxification of industrial effluents, utilization in cosmetic and pulp and paper industries, and medical applications such as diagnostic tools in laboratories and in the development of anti-cancer drugs. They are also used in water purification processes. The objective of this current research is to ascertain the production of recombinant laccase in Escherichia coli and optimize the laccase parameters to achieve the fastest possible expression of the enzyme. Initially, recombinant laccase will be produced on a laboratory scale, and its expression will be analyzed. The laccase gene will be obtained from the thermophilic bacterium Clostridium thermocellum. Escherichia coli and BL21 will be employed for cloning, vector propagation, and observing the expression of the recombinant laccase enzyme in Escherichia coli. Primers will be designed by retrieving the gene sequence from the NCBI database and designing them using the Primer-3 input software (v. 0.4.0) based on the coding region. Restriction enzymes will be chosen using the NEB cutter (V2.0) tool. DNA isolation will be carried out using the DNA chloroform method, and its quality will be verified through 1% agarose gel electrophoresis. DNA isolation will be followed by PCR amplification and confirmation on 1% agarose gel. The purified laccase gene fragments, F1 and F2, will be cloned into the expression vectors pET22b(+) and pET28b(+) through single digestion with NdeI and double digestion of the plasmid with NcoI and XhoI. E. coli BL21 codonPlus cells will be rendered competent using the

CaCl2 method, and the cloned DNA will be introduced into the competent cells via the heat shock method. Shake flask fermentation will be conducted by inducing the cells with lactose and IPTG as inducers in LB media, and their expression will be assessed using 12% SDS-PAGE.During this study, the effects of various influential parameters on the kinetics of the laccase enzyme will also be investigated. Optimization and the study of reaction kinetics will involve using different substrates, such as ABTS, Syringaldazine, or HOBT.

## INTRODUCTION

Laccases are the specific group of enzymes known as multi copper oxidases. These enzymes first discovered a tree which is found in Japan *Rhus vernicifera*, (Alcalde, 2007). Laccases commonly found in the nature many of the different plants, fungal and bacterial species. The enzymes are found in extracellular (fungi and plants) or as well as intracellular (bacteria) (Sharma and Capalash., 2006). Due to their potential use in biotechnological applications laccases play important roles in organisms that use it to perform their different metabolic function. The first bacterial laccase was isolated from *Azospirillum lipoferum* (Diamantidis, *et al*., 1999). Bacterial laccases have been isolated and characterized from different bacterial, plants or fungi species like as the *Bacillus subtilis, Streptomyces griseus, Escherichia coli, Alteromonas sp* and *Ochrobactrum specie* (Liu *et al.,* 2016). Laccases involve in the breakdown of phenolic compounds as well as the non-phenolic compounds with the release of water as by product. Laccases can only oxidize non-phenolic compounds in the presence of starter due to its lower reduction potential in comparison to the non-phenolic compounds (Canas and Camarero., 2010). ABTS, HOBT (1-hydroxybenzotriazole), violuric acid and N-hydroxyacetanilide are examples of such laccase mediators (Sharma and Capalash., 2006). Different article shows the laccase relation with reduction potential and their ability to oxidize the substrates. Laccases are basically oxidizes substrates with lower reduction potential than itself and cannot oxidize substrates with higher redox potential, (Alcalde, 2007). However, with the help of mediators such as those mentioned above, laccases can oxidize compounds with higher redox potential. Bacterial and plants laccase involve in the reduction as compared with fungus (Alcalde, 2007). Laccases contain four copper atoms in their active site, which are distributed around three copper centers namely, copper (T1), copper (T2) and binuclear (T3). Copper T1 accepts electrons from substrates and become reduced in the process and the substrates are in turn oxidized. Guaiacol, syringaldazine and ABTS are specific substrates used to detect and measure the activity of laccases. There are different reagents such as fluoride, azide, thiocyanide, cyanide, halides and hydroxides stops the laccase function by binding to the trinuclear copper centre T2 and T3 (Alcade, 2007). There has been a lot of interest in laccases recently due to their great potential in biotechnological applications. Due to their wide substrate specificity laccases have found applications in the following industrial processes, bioremediation, textile industry, pulp bleaching, organic synthesis, food industry, biosensor technology (Dwivedi *et al*., 2010).There are many procedures and protocol from which we isolate the *clostridium thermocellum* from the stocks and use for our research purposes (McBee, 1948). After making a proper growth medium we grow the bacterium and isolate it from that (Fleming and Quinn., 1971 ; Johnson *et al*., 1981).The discovery of this bacterium showed that *C.thermocellum* is used for the recombinant production and is used as research tool for much genetic purpose. The growth of that bacterium is required not only a simple media but also required many multivitamins like as the biotin, pyridoxamine and *p*-aminobenzoic acid (Johnson *et al.,* 1981). The pH of that media is maintained about7.7 to 8.8. Now it is confirmed that the growth of the *C.thermocellum* is done between the pH 6.7 and 7.0 (Freier *et al.,* 1988). The optimum temperature is about 55°C.The most important product of the fermentation is formation of the ethanol production due to all this effectiveness the researchers are take too much interest in that bacteria. The fermentation end product of *C.thermocellum* is high but the wild type bacteria only tolerate ethanol up to 5 g/L after that it is stopped (Herrero and Gomez., 1980). The factor which involve in fermentation process is due to the inside membrane structure. The C.thermocellum main cell wall is made of lipids. The structure consist of 16 carbon branched and

straight chain fatty acid. The 16 carbon consist of plasmaogens that had a total lipid concentration with other additional components. The lipid concentration is about ~82 μ g/mg of general cell weight while the 28% with the weight of plasmaogens (Timmons *et al.,* 2009). The structure of the membrane had the greatest degree of fluidity that is consist of the moderate level of the ethanol. When the fluidity of the membrane is increased the structure become loses due to which overall negative charge is appeared on the membrane. To increase the level of the ethanol the structure of *C. thermocellum* must changed with its membrane composition. By decreasing the fluidity of the membrane the overall production of the ethanol should be enhanced. They also had ability to detoxify a variety of environmental pollutants. Their property to act as a variety of substrates and moreover to detoxify a variety of pollutants have produces them to make the more effective and important for various biological and chemical industries. Bacterial laccases are fulfills the disadvantages of fungal laccases as a poor stability. Bacterial laccases are much more active or stable on various temperatures and tolerate a higher range of pH. They had various industrial uses like as detoxification of industrial effluents, cosmetic industries, pulp or paper industry as well as medical applications as a diagnostic tool in various laboratories, anti cancer drugs as well as in water purification mechanism. The main hurdle to commercial the bacterial laccase is lack of stock and higher cost of redox mediators. To get the cheap and good production of the enzymes is change the trend of use chemicals as compared to use of biocatalyst (Imran *et al*., 2012).Enzymes exhibit a lot of variety among them like as the Laccases or laccase-like enzymes have been get from the different species of bacteria, plants and fungi. After getting there genomic sequence from bioinformatics they are further used for the analysis like as the gene expression recombinant formation etc. Different enzymes show different expressions in different host like as *E.coli* (Hoegger *et al., 2006,* Ausec *et al., 2011* and Sirim *et al., 2011*). One benefit of laccases and LMCOs is their higher substrate attachment. The quantification of the *B.subtilis, B.pumilus,* Streptomyces *pristinaespiralis, G.forsetii, M.tractuosa* and *S. linguale* LMCOs for oxidation of 91 potential laccase substrates has been discussed (Reiss *et al*., 2003).The expression systems of the enzymes is vary from specie to specie like as yeast shows low expression and production time as compared to filamentous fungus is longer than with yeast (Robert *et al*., 2011). Bacterial Laccase like multicopper oxidases shows expression in *Escherichia coli*. In industrial biocatalyst the *E.coli* is considered to be the best expression system for the recombinant production of proteins. Different procedures and techniques are applied for enhance the production of the active recombinant enzymes in *E.coli* (Soresen *et al*., 2005). The expression levels can be increased by using the same copy number of plasmids as regarding with strong promoters like as the plasmid of the pET series. The host strain for the pET derived plasmid is *E.coli* BL21 (DE3). *E.coli* JM109 (over expressing in $lacI^q$ from the genome) was used as the host strain for pQE-60 derived plasmids. The expression level of protein is determined by the size or thickness of band which is checked by the SDS-PAGE by using culture of high activity. Specific size of the band is detected in all samples of the protein. In summary, this study reviles that *bacillus* LMCOs shows a variety of useful benefits which is not correlated, with the enzymes of other bacterial species. The higher expression level a board substrate specificity high stability, high activity yield in *E.coli* the laccase is considered to be the best candidate for the further research development.

## MATERIALS AND METHODS

### DNA Extraction

In the molecular biology the purification and separation of the DNA from the cells is the basic principle. It should be separated from within the cell and out of the cell. The isolation of the DNA from bacteria is the simple process. The separation of the DNA from bacteria is needed to be grown in suitable medium at optimum temperature to get the maximum yield. The genomic DNA separation needs to be total DNA from RNA, protein, lipids etc. The genomic DNA of *Clostridium thermocellum* had purchased.

**Genomic DNA media components**

Take all the chemicals and measure the weight on weighing balance machine. Put the chemicals into the flask. Put the $dH_2O$ into the flask and reached the volume up to 80ml. Dissolve all the chemicals by using magnetic stirrer. When all chemicals dissolve fill the flask up to mark 100ml by $dH_2O$.

**Phenol-chloroform mixture**

2ml overnight culture is taken and the cells are harvested by centrifugation for 10 minutes. 875 µl of TE buffer is added to the cell pellet and the cells are resuspended in the buffer by gentle mixing.100 µl of 10% SDS and 5 µl of Proteinase K are added to the cells. The above mixture is mixed well and incubated at 60ºC for an hour in an incubator. 1 ml of phenol-chloroform mixture is added to the contents mixed well by inverting and incubated at room temperature for 5 minutes. The contents are centrifuged at 10,000 rpm for 10 minutes at 4ºC. The highly viscous jelly like supernatant is collected using cut tips and is transferred to a fresh tube. The process is repeated once again with phenol-chloroform mixture and the supernatant is collected in a fresh tube.100 µl of 5M sodium acetate is added to the contents and is mixed gently. 2 ml of isopropanol is added and mixed gently by inversion till white strands of DNA precipitates out.

**Qualitative analysis of Genomic DNA of *Clostridium thermocellum***

The agarose gel comprises of particular size pores in which we put the desired sample into particular valves. The valve size controls the speed of the sample. The sample which had the low molecular weight is move effectively when as compared with the high molecular weight. Many other factors which affect the gel are charge, size, and m/z ratio, suitable conditions, gel thickness, and voltage.

**PCR amplification of laccase gene**

PCR amplification was done as described by sajjad *et al.,* 2012. For amplification, Applied Biosystems™ Veriti™ 96-Well Fast Thermal Cycler was used. 25 µl reaction mixtures were prepared according to the mixture composition and thermal profile. Scheme used for the construction of recombinant expression DNA, primers and their restriction sites, used for PCR are given in table (1)

**Table 1: PCR Amplification condition for laccase enzyme**

| **Reagents** | **Stock conc.** | **Working conc.** |
|---|---|---|
| Template DNA | | Approx. 100 ng |
| F. Primer | 50 µM | 1 µM |
| R. Primer | 50 mM | 1 µM |
| dNTPs | 2.5 mM | 0.4 mM |
| $MgCl_2$ | 25 mM | 3 mM |
| Taq DNA polymerase | 5 $U\mu l^{-1}$ | 2U |
| PCR buffer(without $MgCl_2$) | 10X | 1X |

**PCR product analysis**

Agarose gel electrophoresis was done for analyzing the PCR product using Bio-Rad Mini-Sub® Cell. Samples were prepared by mixing PCR product with 6X DNA sample loading dye in a final concentration of 2X. 1% agarose gel was prepared containing EtBr approximately 0.2-0.5 µg/ml. After the gel being solidified, samples were loaded into the slots of submerged gel and run in 1x TAE buffer for 30 min. at the constant voltage of 100V using Bio-Rad Power Pac HC High Current Power Supply. After electrophoresis, the gel was visualized under UV-Transilluminator or analyzed in Bio-Rad Gel DocTM XR+ Gel Imaging System for imaging and analyzing DNA fragments.

**F1 Restriction and cloning in expression vector pET22b (+)**

Cloning in pET22b (+), purified DNA was restricted with NDI restriction enzyme. Total volume of single digestion reaction mixture 20 µl was prepared containing 5 µl template DNA , restriction buffer 2 µl ,10X NEB Buffer, 0.5 µl Nd1 and 12.5 µl sterile $dH_2O$. The ligation mixture was incubated on ice for 1 h and then at 20°C for overnight. This ligation mixture containing desired recombinant plasmid DNA was then transformed in competent cells of *E.coli* BL21 CodonPlus (RIPL).

**Cloning in expression vector pET28b (+)**

Prior to clone in pET28b (+), purified DNA was restricted with XhoI and NcoI restriction enzymes (Zero cutters). Total volume 40µl of reaction mixture was prepared containing 5µl DNA plasmid, 5µl 10X NEB Buffer, 1µl NcoI, 1µl XhoI and 38µl sterile $dH_2O$. The reaction mixtures were then incubated at 37℃ overnight. Restriction of the plasmid was checked with the help of 1% agarose gel electrophoresis and required DNA fragments were purified using MONARCH® DNA Gel Extraction Kit Protocol according to the manufacturer's instructions. Purified restricted plasmid was then ligated into linearized pET28b (+). The reaction mixture (20µl) contained 5µl restricted plasmid, 2µl linearized pET28b (+), 2µl 10X T4 DNA ligase buffer, 1µl T4 DNA ligase ($1U\mu l^{-1}$) (Invitrogen™ T4 DNA Ligase 10124752) and10µl $dH_2O$. The ligation mixture was incubated on ice for 1 h and then at 20°C for overnight. This ligation mixture containing desired recombinant plasmid DNA was then transformed in competent cells of E.coli BL21 Codonplus (RIPL).

**Competent *E. coli* BL21 CodonPlus cells transformation with plasmid DNA**

2.5µl of *E. coli* BL21 CodonPlus (RIPL) competent cells were taken in chilled sterile 17x100 mm polypropylene tube and 1.5µl or less ligation mixture containing plasmid DNA was added to the tube (the volume of DNA solution was kept 5% of the volume of the competent cells or no more than 50ng in a volume of 10mL or less). The tube was gently swirled for a few seconds to mix by finger flicking tube and incubated on ice for 30 min. The mixture was subjected to heat shock by swiftly transferring to 42℃ water bath for 45 seconds and returning back to ice for 5 min. 900µL of S.O.C. medium was then added and incubated at 37℃ for 1 hour without shaking. 100µl of transformed cells were spread on pre-warmed LB agar plate's 1.5% agar containing 50µg/ml with Ampicillin by evenly spreading cells over the plate using a "hockey puck" spreader dipped in ethanol and flamed before spreading. The plates were then incubated at 37℃ overnight.

**Blue and white colony transformation**

By using ampicillin in your growth medium should prevent bacteria that did not take up the plasmid during the transformation from growing. By using these methods we confirm that the white colonies see on your screening plate contain our plasmid. It is always a good idea to run controls with your cloning experiment.

**Expression analysis of recombinant laccase by SDS-PAGE**

Comb was carefully removed and the wells were washed with 1X Tris-glycine running buffer. 4 µl of protein marker, 20 µl of each uninduced and induced samples were loaded in the wells. The gel was electrophoresed at low voltage for stacking gel and at constant voltage of 120V while running through the resolving gel, using Bio-Rad Power Pac HC High Current Power Supply. The gel was electrophoresed for about two and a half hrs till the dye reached at the bottom of the gel. After electrophoresis, the gel was stained with coomassie brilliant blue R-250 solution for 15 min and then destained with the destaining solution till the background of the gel became transparent. The gel was placed on the orbital shaker in order to ensure even staining and destaining of the gel.

## RESULTS

### Agarose gel electrophoresis of laccase gene F1 analysis

The gel is shown with the loading wells at the top. Lanes are clearly marked. In this figure 1 the lanes are numbered. The PCR product used for each reaction described in the legend. Conclusions are included in the legend. The size of the DNA ladder is 1KB is indicated.

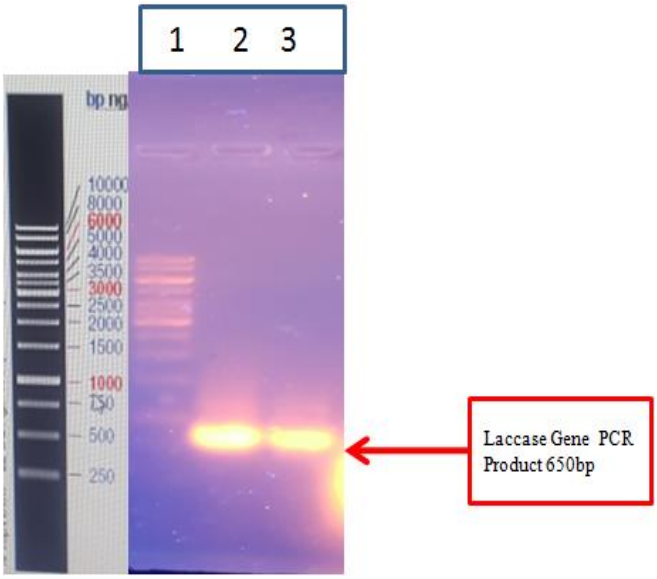


**Figure 1:** ***Agarose gel electrophoresis PCR F1 Laccase enzyme Lane 1. DNA Ladder 1KB base pair. Lane 2. PCR product of laccase agarose gel F1 Lane 3. Duplicate PCR product of laccase agarose gel F1***

### Gene Cleaning and agarose Gel

DNA fragment was excised from the agarose gel and transferred to 15 ml microfuge tube. 400µl of gel dissolving buffer to the gel slice. Sample was incubated at 37-55°C with the periodic vortexing until the gel slice was completely dissolved about 5-10 min. Sample was then loaded onto the column inserted into the collection tube and centrifuged at 13,000 rpm for about a min. The flow-through was discarded and column was re-inserted into the collection tube. DNA was washed twice with the 200µl of DNA wash buffer followed by spin for about a min. The column was transferred to a clean microfuge tube and DNA was eluted with 6 µl of DNA elution buffer added to the center of the matrix, centrifuged for a min to elute DNA and stored at -20°C for later use in cloning.


**Figure 2:** F1 gene cleaning Agarose gel electrophoresis Lane 1. Gene cleaning by Kit method. Lane 2. DNA Ladder against F1 laccase clean gene.

**Single digestion by *Nde I* restriction enzyme**

The purified DNA was restricted with NdeI and XhoI restriction enzyme. Total volume of the reaction mixture is 20µl. After the double restriction reaction mixtures then incubated at 37℃ overnight. Restriction of the plasmid was checked with the help of 1% agarose gel electrophoresis. First band shows the successful restriction with NdI second band also same but the quantity of the sample in lesser concentration

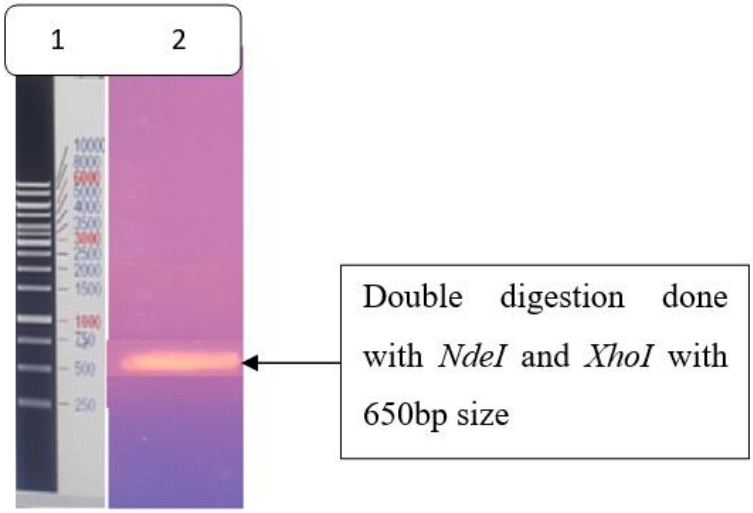


***Figure 3: F1 laccase enzyme restriction by double digestion with Nd1 and XhoI. Lane 1 DNA Ladder and 2. Successful restriction by NdeI and XhoI.***

**Ligation of F1 in pET 22b (+) plasmid**

Purified restricted plasmid was then ligated into linearized pET22b (+). The reaction mixture (20µl) restricted plasmid, Linearized pET22b (+) vector, T4 DNA ligase buffer, T4 DNA ligase enzyme and $dH_2O$. The ligation mixture was incubated on ice for 1 hour and then at 20°C for overnight. This ligation mixture containing desired recombinant plasmid DNA was then transformed in competent cells of *E.coli* BL21 Cells.

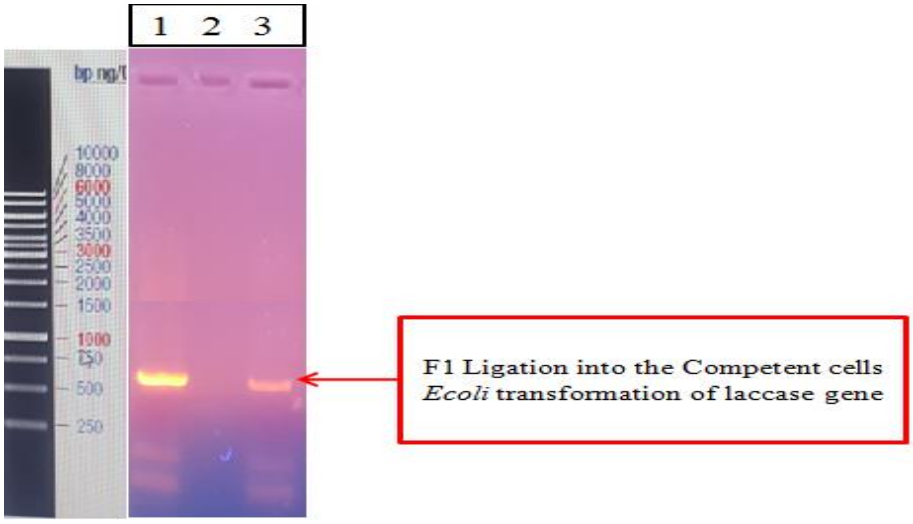


***Figure 4: ligation of F1 laccase gene into the competent cells. Lane 1 and 3. Expression of laccase gene into competent cells against ladder***

**Colony PCR against F1**

Colony PCR was done to confirm the positive ligation of desired sample. Healthy colonies from the agar plate of LB media with ampicillinwas inoculated with fresh LB media.

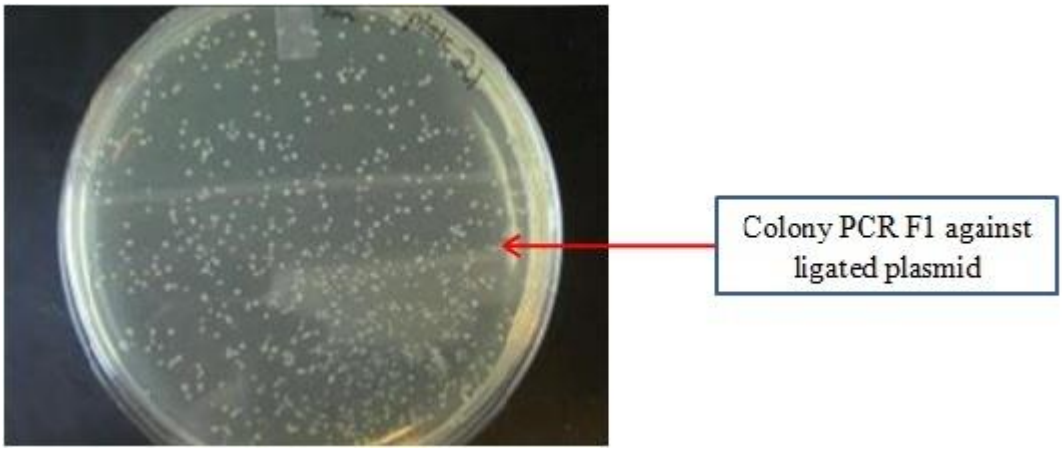


***Figure 5: F1 laccase enzyme Colony PCR) (F1) showing the successful ligation of plasmid into the vector pET22 b (+) E. coliBL21.***

**Graphical representation of F1 laccase with IPTG**

I was growing the cells on the different concentrations of IPTG to check that how the different concentrations of IPTG effects the expression level. The concentrations are given in mM. The decreased concentration had good effects on expression. When concentration is increased more than 1.5mM the expression level is low. The reason is that high levels of IPTG concentration cause the toxicity into the cells which decreased the expression level. The concentration effects growth of *E.coli* cells as well as the expression level. I was observed that 1mM is the best concentration for the growth of *E. coli* cells as well as the expression level.

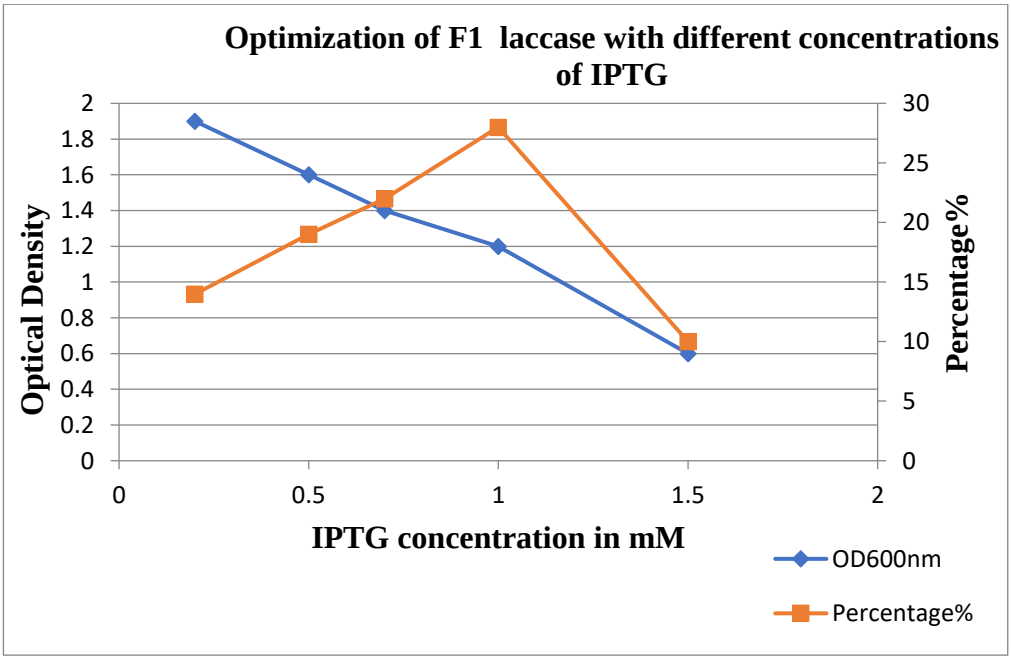


**Figure 6 Graphical representation of F1 laccase with IPTG**

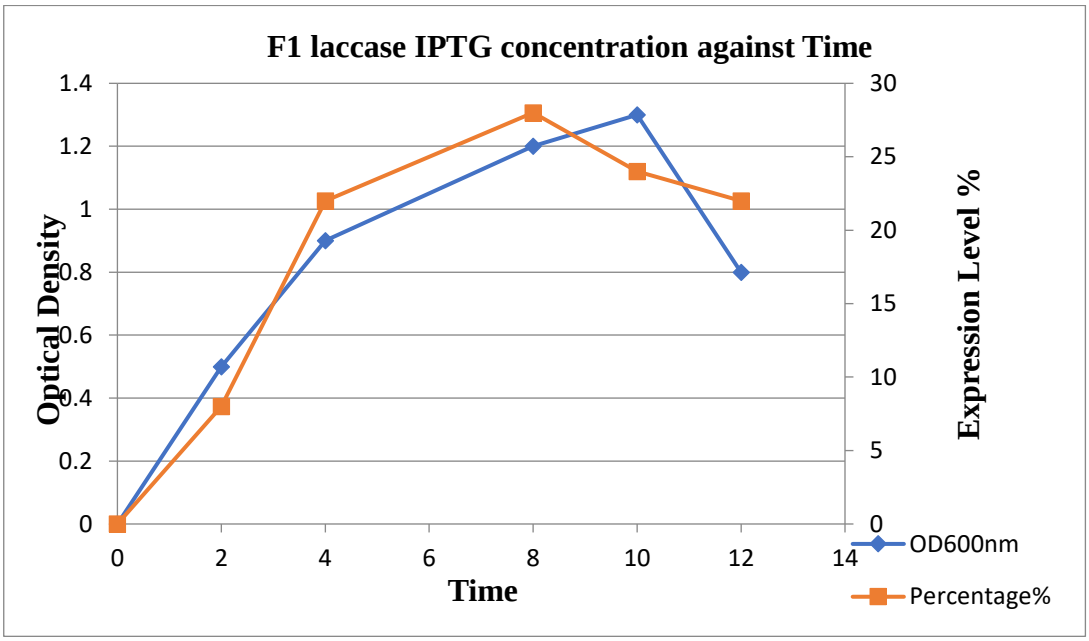


**Figure 7 Graphical representation of F1 laccase IPTG against time**

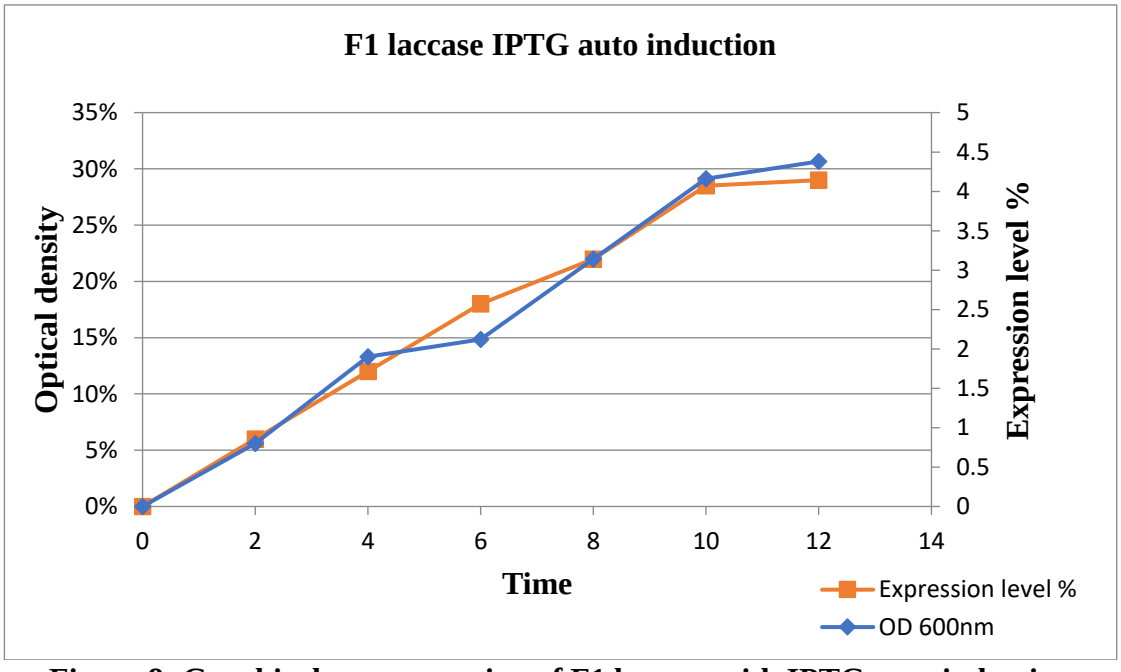


**Figure 8; Graphical representation of F1 laccase with IPTG auto induction**

## DISCUSSION

Different strains like as the BL21 (DE3) and K12 are commonly used for the expression of recombinant proteins. The BL21 (DE3) strains correlate with the *E.coli* B strains. BL21 (DE3) strains had not presented the outer membrane proteins (OmpT) and Lon proteases. The absence of these 2 proteases it is confirm that other protein which are produced in these strains are not be effected (Terpe., 2006). The proteins which are over expressed in E.coli some time showed the formation of the inclusion bodies as a result. This over expression of heterologous proteins in E. coli showed that the protein is not properly folded and that proteins are biologically non active or insoluble. By providing the combination of molecular chaperons, mixture of two different proteins and low temperature the E. coli host strains may resolve this issue (Baneyx., 1999). The recombinant proteins from the inclusion bodies can be recovered but this method is too much difficult and expensive or time consuming. On the other hand besides the formation of inclusion

bodies the E.coli as expression host had disadvantage like unable to secret the protein into the extracellular medium. This causes the problem to perform the posttranslational modifications into the expression of different proteins (Glick, Pasternak and Patten, *et al.,* 2010). The qualities of good promoter are that it is easy available low cost level and induce easily or shows the low basal expression (Perpet,., 2006). To produce a host protein expression the promoter is induced by an inducer such isopropyl-β-D-galactoside (IPTG). The IPTG attached with the repressor protein and changes it configuration. The changed repressor is dispatches from the operator and this move the RNA polymerase to attaches with the promoter to starts the transcription of the gene of interest. The promoter is worked positively by activators or negatively by repressors. The control of expression from the Pm promoter by XylS protein is an example of a positive regulation, while repressor acting on a promoter to prevent transcription is an example of negative regulation. Bacteriophage T7 genome produces the T7 promoter and T7 RNA polymerase (T7 RNAP). T7 promoter controls the expression of gene plasmid vector. For the expression system to perform function correctly an E.coli host strain BL21 must be present, because it also consist the genetic things like as T7 RNAP, lacUV5, lac repressor which is needed for the expression of the target gene. By using the expression vector target gene is clones which show the expression downstream. The T7 RNAP is put into the E. coli BL21 chromosome by a λDE3 phage lysogen gene (Sorensen and Mortensen.,2005). T7 promoter had greater binding capacity with the T7 RNAP that control the expression of our cloned gene put into the E.coli. RNA polymerase starts the transcription and affects the target gene. (Wagner*et al*,.,2008). For T7 RNAP copied from the bacteriophage T7 gene 1 which encodes it. The quantification of the B. subtilis, B. pumilus, S. pristinaespiralis, G. forsetii, M. tractuosa and S. linguale LMCOs for oxidation of 91 potential laccase substrates has been discussed (Reiss, *et al*.,2003).The expression systems of the enzymes is vary from specie to specie like as yeast shows low expression and production time as compared to filamentous fungus is longer than with yeast (Robert, *et al*.,2011). Bacterial Laccase like multicopper oxidases shows expression in Escherichia coli. In industrial biocatalyst the *E.coli* is considered to be the best expression system for the recombinant production of proteins. Different procedures and techniques are applied for enhance the production of the active recombinant enzymes in *E.coli*. The expression levels can be increased by using the same copy number of plasmids as regarding with strong promoters like as the plasmid of the pET series. The host strain for the pET derived plasmid is *E. coli* BL21. *E.coli* JM109 (over expressing in $lacI^q$ from the genome) was used as the host strain for pQE-60 derived plasmids. The expression level of the protein is determined by the size or thickness of the band which is confirmed by the SDS-PAGE by using the culture of high activity. Specific size of the band is detected in all samples of the protein. In summary, this study reviles that bacillus LMCOs shows a variety of useful benefits which is not correlated, with the enzymes of other bacterial species. The higher expression level, a board substrate specificity, high stability, high activity yield in *E.coli* the laccase is considered to be the best candidate for the further research development.The laccase gene is cloned from the Pycnoporus sanguineus specie and inserted between the two strong promoters Pcbh1 or Tcbh1 terminator from Trichoderma reesei. In this way it forms the recombinant plasmid pCH-lac. By using Agrobacterium mediated technique, the pCH-lac was combined into the chromosomes of T. reesei. Twenty positive transformants were obtained from employing the hygromycin B as a selective agent.PCR was used to ensure that the laccase gene was properly inserted into the chromosomal DNA of T.reesei. To check the activity of laccase production by recombinant transformants was performed in the shaking flask, and the production of laccase reached up to 8.8 IU/mL. The SDS-PAGE analysis of the fermentation broth shows that the molecular mass of the protein was about 68 kDa. The molecular mass is the same as that of the laccase produced by P. sanguineus. This analysis shows that laccase was successfully cloned or shows its expression in the T. reesei. The protein excretes out of from the cells or shows its expression. The laccase produced by the recombinant T. reesei showed good temperature stability. It also helps in the breakdown of the toxic material of bisphenol A efficiently. To check the degradation level of waste material after 1 hour reaction with 0.06 IU/mL laccase and 0.5 mmol/L

ABTS used as a starter at 60°C and the pH is maintained up to 4.5. The breakdown rate is reached up to 95% which shows that it had huge potential value in treating the household waste material.

## CONCLUSION

The production of recombinant laccase isolated from thermostable anaerobic bacterium *Clostridium thermocellum* in *E. coli* BL21 CodonPlus (RIPL). The recombinant formation and optimization was the ultimate objective of the present research work. The successful recombinant formation is useful as a source various products like as industrial scale production of the recombinant enzyme which is future used for living and non living. Some crop plants have been genetically modified so they produce and contain proteins normally found only in bacteria. These proteins make crop plants more resistant to certain pests or tolerant of particular types of herbicides. Before rDNA technology, hepatitis B vaccines used weakened or killed hepatitis viruses to stimulate a response from the human immune system. Newer vaccines use hepatitis-B proteins produced with rDNA technology. Substances can be produced in bacteria by using rDNA technology which makes them more affordable and easily available. rDNA used to produced Samtotrophin and insulin.

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