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Cloning and Expression of Four Laccase genes isolated from Arctic marine Psychrobacter strains in Escherichia coli and psychrophilic Pseudomonas

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Biotechnology
Submission date: June 2017
Supervisor: Martin Frank Hohmann-Marriott, IBT
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Abstract

A group of diverse bacterial isolates were discovered in Arctic marine habitat during a research expedition in May 2009. These isolates were identified and sequenced based on 16S rRNA sequencing. *Psychrobacter* strains discovered among the isolates were investigated further for their laccase-like multicopper oxidase activity. Four of the strains P11f6, P11G3, P11G5 and P2G3 were found to show laccase activity. The genes responsible for the laccase-like multicopper oxidase activity were identified and sequenced. The laccase genes were designated P11f6, P11G3, P11G5, P2G3 and their genomic DNA was isolated. The discovery of these *Psychrobacter* strains and subsequent laccase activity shown by the strains, form the basis of this project.

In this project, the four laccase genes, P11f6, P11G3, P11G5 and P2G3 were cloned and expressed in two expression hosts, *E. coli* BL21 (DE3) and psychrophilic *Pseudomonas* Psm26D7. The expression hosts harbouring the cloned gene were induced for protein expression and cultivated at varying temperatures and incubation periods.

Protein expression levels in *E. coli* BL21 (DE3) carrying the laccase genes were analysed by SDS-PAGE, results showed that P11f6 and P11G5 laccase genes were expressed. While, P11G3 and P2G3 were not expressed in *E. coli* BL21 (DE3).

The four laccase genes were then introduced into psychrophilic *Pseudomonas* Psm26D7 by conjugation, followed by induction for protein expression. SDS-PAGE analysis of protein expression levels indicates P11G3, P11G5, and P2G3 laccase genes were expressed in psychrophilic *Pseudomonas* Psm26D7. Expression of P11f6 in *Pseudomonas* Psm26D7 was not successful.

The solubility of the recombinant laccase proteins obtained from both expression hosts were tested and the expressed proteins were found to be insoluble.

Copper have been shown in previous studies to enhance production, activity and proper folding of laccases from fungi and bacterial sources. This was investigated in this project. Results showed copper supplementation of expression cultures was more successful in *E. coli* BL21 (DE3) harbouring the laccase genes than *Pseudomonas* Psm26D7, as small amount of soluble proteins were obtained from expressions in *E. coli* BL21 (DE3) and none in *Pseudomonas* Psm26D7.

The laccase assay activity of soluble proteins obtained from copper supplemented cultures was determined by using ABTS as substrate and its oxidation was monitored by measuring absorbance with a spectrophotometer. The laccases were observed to show activity.

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Abbreviations

ABTS	=	Azinobis (3-ethylbenzothiazoline-6-sulphonic acid)
Bp	=	Base pairs
$Cucl_2$	=	Copper (11) chloride
dNTP	=	Deoxynucleoside Triphosphate
E. coli	=	*Escherichia coli*
IPTG	=	Isopropyl-β-D-thiogalactoside
kDa	=	Kilodaltons
LA	=	Lysogeny agar
LB	=	Lysogeny broth
NEB	=	New England Biolabs
OD	=	Optical density
PCR	=	Polymerase chain reaction
SDS-PAGE	=	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SOC	=	Super optimal broth with catabolite repression
Tm	=	Melting temperature
T7 RNAP	=	T7 RNA Polymerase

1 Introduction

Background information

This thesis is based on the discovery and isolation of a group of diverse bacterial isolates in Arctic marine habitat during a research expedition. Several works, including identification and 16S rRNA gene sequencing of these isolates have previously been carried out. Here a brief overview of events that led to the discovery of these bacterial isolates will be presented.

The work was done in collaboration with the Arctic University of Norway, UiT, Tromsø. Between 14th and 26th May, 2009 on a research cruise of R/V Jan Mayen, Northern Norway, within the Arctic, biota, water and sediments were sampled. The sampling was carried out in ten different location, spanning a region between and around the Svalbard archipelago and the bear island in the Barents Sea. The collections led to the establishment of a library of 1448 single bacterial isolates originating from biota (773), sediments (418) and water (257) samples. The strain library consists of at least 31 genera based on 16S rRNA gene sequences of 550 isolates which include, *Alteromonas, bacillus, Flavobacterium, Pseudoalteromonas, Pseudomonas, Psychrobacter* and others. The *Psychrobacter* isolates were further investigated for their laccase-like multicopper oxidase activity. Four strains P11f6, P11G3, P11G5 and P2G3 were found to show laccase activity and their genomic DNA was isolated.

The work described above was previously carried out prior to this project.

1.1 *Psychrobacter*

The four laccase genes (P11f6, P11G3, P11G5 & P2G3) cloned and expressed in this thesis, were isolated from Arctic marine *Psychrobacter* strains. Hence a brief description of *Psychrobacter* was necessary. *Psychrobacter* are gram-negative bacteria. They belong to the family known as Moraxellaceae. They are capable of growth at low or medium temperatures. *Psychrobacter* are psychrophilic, psychrotolerant, osmotolerant and halotolerant (Bowman, 2006). *Psychrobacter* were first described by Juni and Heym, as being aerobic, rod shaped, oxidase positive and non- motile, (Bozal et al., 2003). Members of the genus can be isolated from a variety of sources which include deep sea water, Antarctic sea ice, Antarctic ornithogenic soil, coastal marine sediments, Arctic permafrost, (Bowman, 2006).

Due to their ability to survive at cold environments, *Psychrobacter* strains are therefore cold adapted, which makes them important sources of cold active or cold adapted enzymes. Such cold active enzymes harboured by *Psychrobacter* strains, which are relevant in

biotechnological applications include, glutamate dehydrogenases, anti-freeze proteins, lipases and β-lactamase (Bowman, 2006).

1.2 Cold-adapted enzymes

Psychrophilic genes isolated from psychrophiles encodes cold active or cold adapted enzymes. Cold adapted enzymes are produced by psychrophilic or cold adapted microorganisms. Psychrophiles are microorganisms that are capable of growth at low temperatures. They can be obligate psychrophiles or facultative psychrophiles (psychrotolerant or psychrotrophs). Cold environments such as the Arctic, Antarctic and deep sea water support the growth of these organisms because these environments are permanently cold (Feller, 2013). Some examples of psychrophilic microorganisms are Gram-negative bacteria such as *Pseudoalteromonas, Psychrobacter, Pseudomonas, Moraxella* and *Vibrio* species, Gram-positive bacteria such as *Arthrobacter, Micrococcus* and *Bacillus species* and Archaea such as *Methanococcoides, Halorubrum* and *Methanogenium* species, (Feller & Gerday, 2003).

Cold adapted microorganisms adapt to growth at low temperatures through a variety of cellular processes, which includes but not limited to the synthesis of cold shock and cold acclimation proteins, (Scherer & Neuhaus, 2006). Cold adapted enzymes have high potentials in biotechnological applications and this is due to their high enzymatic activity at low temperatures and low thermal stability compared to enzymes from their mesophilic and thermophilic counterpart, (Cavicchioli et al., 2011). The use of cold adapted enzymes ensures energy conservation by performing reactions at low temperatures which would otherwise consume a lot of energy if performed at high-temperature (Feller et al., 1998).

Cold adapted enzymes are beginning to garner much interest among researchers, due to their perceived potential in biotechnological applications, which for example include use, as additives in detergent, bioremediation, textile industry, food industry and molecular biology, (Cavicchioli et al., 2011).

1.3 Laccases

General overview

Laccases (EC 1.10.3.2) belong to a group of enzymes known as multi copper oxidases. Laccase was first discovered in the Japanese lacquer tree, *Rhus vernicifera*, (Alcalde, 2007). Laccases are widely distributed in nature, where they are found in fungi, plants and bacteria. The enzymes are thought to be localized extracellularly (fungi & plants) or intracellularly (bacteria), (Sharma & Capalash, 2006). Besides their potential use in biotechnological applications, laccases play several physiological roles in organisms that harbours it. The first bacterial laccase was isolated from *Azospirillum lipoferum*, (Diamantidis et al., 1999). But subsequently, bacterial laccases have been isolated and characterised from *Bacillus subtilis, Streptomyces griseus, Escherichia coli, Alteromonas* sp., and *Ochrobactrum* sp., (Liu et al., 2016).

Laccases catalyses the oxidation of phenolic and non-phenolic compounds coupled with the reduction of molecular oxygen to water as by product. However, laccases can only oxidize non-phenolic compounds in the presence of a mediator due to its lower reduction potential in comparison to the non-phenolic compounds, (Canas & Camarero, 2010). 2,2'-azinobis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), 1-hydroxybenzotriazole, violuric acid and *N*-hydroxyacetanilide are examples of such laccase mediators, (Sharma & Capalash, 2006).

It has been reported that, there is a correlation between the reduction potential of laccases and their ability to oxidize substrates. Laccases typically oxidises substrates with lower reduction potential than itself and cannot oxidise substrates with higher redox potential, (Alcade, 2007). However, with the help of mediators such as those mentioned above, laccases can oxidise compounds with higher redox potential. Bacterial and plants laccases have a lower redox potential than fungal laccases, (Alcalde, 2007).

Laccases contain four copper atoms in their active site, which are distributed around three copper centers namely, copper type 1 (T1), copper type 2 (T2) and binuclear copper type 3 (T3). The copper T1 gives laccase its blue colour. The copper T1 accepts electrons from substrates and become reduced in the process and the substrates are in turn oxidised. Copper T2 and T3 combine to form a trinuclear center and electrons are transferred from copper T1 to the trinuclear center. The reduction of molecular oxygen to water occurs at the copper T2 and T3 trinuclear centers, (Reiss et al., 2013). An example of copper centers found in the active site of a laccase is presented in Fig.1.

Guaiacol, syringaldazine and ABTS are specific substrates used to detect and measure the activity of laccases. Reagents such as fluoride, azide, thiocyanide, cyanide, halides and

hydroxides inhibits laccase activity by binding to the trinuclear copper centre (T2 & T3), (Alcade, 2007).

There have been a lot of interest in laccases recently due to their presumed 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).

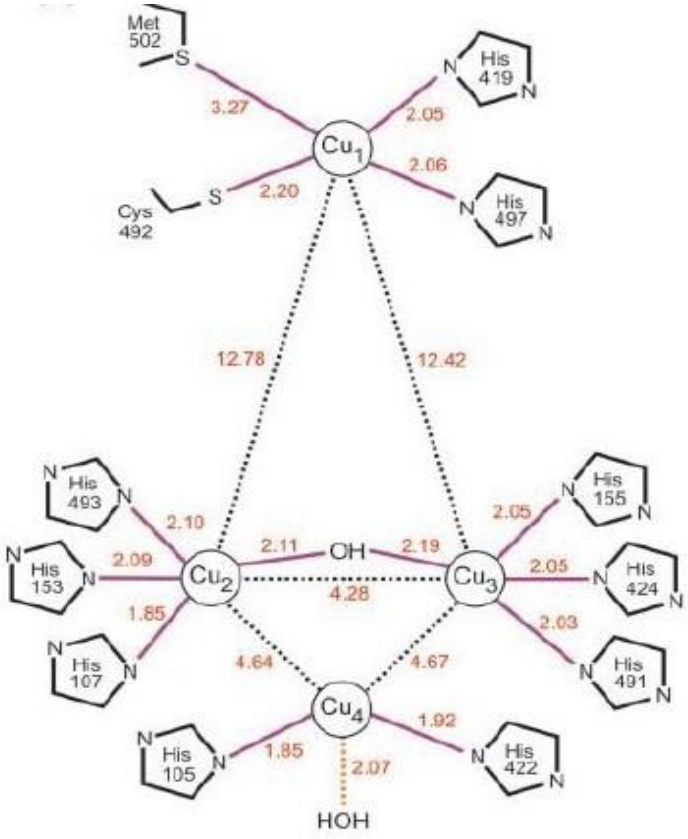

Fig. 1. Schematic representation of copper centers in a laccase structure, (taken from Enguita et al., 2010).

1.4 Expression hosts

1.4.1 *Escherichia coli* expression host

Escherichia coli are gram-negative bacteria, belonging to the family known as enterobacteriaceae and class, called gammaproteobacteria. They normally inhabit the intestines of humans and animals.

E. coli are routinely used for most molecular cloning and expression procedures for the production or overexpression of heterologous protein. Due to vast amount of information regarding its genetics, biochemistry and physiology, *E. coli* has become the organism of choice as host organism for recombinant protein production, (Glick et al., 2010). The advantages of using *E. coli* as host strain for recombinant protein expression include ease of genetic manipulation, transformation, short generation time, and ability to reach high cell density while growing on simple media.

E. coli BL21 and K12 strains are mostly used in the expression of most recombinant proteins. The derivative BL21(DE3) strains belongs to the *E. coli*, B strains. BL21 (DE3) strains lacks outer membrane proteins (OmpT) and Lon proteases. Absence of these two proteases, ensures heterologous proteins produced in these strains are not degraded, (Terpe, 2006).

The overexpression of heterologous proteins in *E. coli* often results in the production of proteins in inclusion bodies. Inclusion bodies formation, are caused by the overproduction and improper folding of the expressed proteins, which are therefore biologically inactive and insoluble. However, the co-expression of molecular chaperons, fusion proteins and low temperature cultivation of *E. coli* host strains may overcome this problem, (Baneyx, 1999). Although, recombinant proteins can be recovered from inclusion bodies, but the extraction and recovery process is often laborious and expensive (Glick et al., 2010)

Apart from inclusion bodies formation, drawbacks of *E. coli* as expression host also include inability to secrete proteins into extracellular medium and difficulty to perform post-translational modification of heterologous proteins, (Glick et al., 2010).

1.4.2 Psychrophilic *Pseudomonas* expression host

As explained in the background information section above, *psychrophilic Pseudomonas* was among the bacterial isolates discovered during the research cruise in collaboration with the Arctic University of Norway, UiT, Tromsø, Norway. A brief detail of how it evolved into an alternative expression host used in this thesis is described. In total 39 psychrophilic *Pseudomonas* strains were assessed for their ability to show inducible phenotype for the XylS/Pm system. Four psychrophilic *Pseudomonas* that demonstrated high induction rate were chosen for further study. Transposon Tn5 system was used to introduce the XylS/Pm expression cassette into the host chromosome to regulate expression of T7 RNA polymerase. Among the resulting transposase mutant strains, two strains (Psm 26D7 & Psm 32D7) that gave the highest level of marker gene expression for mCherry was selected for future studies, (personal communication with Rahmi). The experiment described above were previously carried out.

Pseudomonas are gram negative rod-shaped bacteria that inhabits soil and water environments. Psychrophilic *Pseudomonas*, Psm26D7 was used as an alternative expression host strain in this project. The psychrophilic *Pseudomonas* was isolated from Arctic marine environment. The heterologous expression of psychrophilic proteins in mesophilic host such as *E. coli* with optimum growth temperature of 37^{o}C can lead to the production of improperly folded and insoluble proteins. These insoluble proteins normally accumulate as inclusion bodies in the host cytoplasm. As stated earlier, low temperature cultivation of *E. coli* can help overcome inclusion bodies formation. However, low temperature cultivation of *E. coli* results in reduced growth rate as temperature decreases, (Margesin et al., 2008). Cold adapted enzymes are heat labile and high- temperature cultivation of the expression host will affect the proper folding of the proteins. Production of properly folded proteins, may require a psychrophilic expression host that can be cultivated at low temperature. The use of a psychrophilic host would be one of the alternatives to circumvent insolubility or inclusion bodies formation of cold adapted proteins expressed in *E. coli.*

The psychrophilic *Pseudomonas* psm26D7 expression strain is capable of growth at low temperatures and therefore can be utilised in expression of heterologous proteins at lower temperatures, which are challenging for the cultivation of *E. coli.*

The psm26D7 have been constructed to include the integration of a XylS/Pm regulator promoter system in its chromosome which controls the expression of T7 RNA polymerase.

1.5 Expression systems

Expression systems are required for production of recombinant proteins. The expression vector and a suitable host strain both harbours important genetic elements that makes up these systems. The systems depend on expression vectors which harbours the cloned gene, that have been inserted downstream of an inducible promoter. The plasmid encoding the gene of interest are transformed into a suitable host strain for the expression of heterologous proteins. A plasmid vector used for production of recombinant proteins should have the following genetic elements: an origin of replication, promoter, cloning site, antibiotic selective marker, translational start site, ribosome binding site, and transcription and translation terminators.

The expression of heterologous proteins requires the presence of a strong and inducible promoter. A good promoter system is one that is easy and cheap to induce and have low basal expression levels, (Terpe, 2006). To produce a heterologous protein the promoter is induced by an inducer such isopropyl-β-D-galactoside (IPTG). The IPTG binds to the repressor protein and alter its conformation. The altered repressor is displaced from the operator and this allow the RNA polymerase to bind to the promoter to stimulate transcription of the gene of interest. The promoter is either regulated 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 a repressor acting on a promoter to prevent transcription is an example of negative regulation.

1.5.1 T7 expression system

The T7 promoter and T7 RNA polymerase (T7 RNAP) originates from bacteriophage T7 genome. A plasmid vector harbours the T7 promoter where it controls expression of the gene placed downstream. An *E. coli* host strain BL21 must be present for this expression system to function effectively, because it also contains genetic elements (T7 RNAP, lacUV5, lac repressor) required for the expression of the target gene. The target gene to be expressed, is cloned downstream of the T7 promoter encoded by the expression vector. The gene (bacteriophage T7 gene 1) encoding T7 RNAP is integrated into the *E. coli* BL21 chromosome by a λDE3 phage lysogen (Sørensen & Mortensen, 2005).

T7 RNAP has a strong recognition for the T7 promoter, it controls the expression of the cloned gene of interest and its transcription of the target gene is much faster than the *E. coli* RNA polymerase, (Wagner et al., 2008). For T7 RNAP to be active, it must first be transcribed from

bacteriophage T7 gene 1 which encodes it. The bacteriophage T7 gene 1 encoding T7 RNAP is placed under the control of lacUV5 promoter, which is repressed by a lac repressor. The lac repressor also represses the T7 promoter. But in the presence of an inducer such as isopropyl-β-D-thiogalactoside (IPTG), the lac repressor is displaced from the promoter and T7 RNAP is expressed, leading to the transcription of the cloned gene, (Wagner et al., 2008).

The T7 expression system requires a tight control (T7 promoter) over expression of the cloned gene (prior to induction) to avoid leaky expression. Leaky expressions results in expression of the cloned gene prior to induction and are caused by basal expression of T7 RNAP from uninduced host cells. However, most expression vectors that use the T7 expression system have been designed to minimise leaky expressions. The use of BL21 (DE3) strains which harbours pLysS or pLysE plasmids helps to reduce basal expression to minimal levels or eliminated from the system completely. These plasmids encode T7 lysozyme which inhibits T7 RNAP, (Dubendorff & Studier, 1991).

The pET expression vectors are examples of T7 based expression systems, mostly used in the production of recombinant proteins in *E. coli* expression host strain.

1.5.2 XylS/pm expression system

The Pm promoter and its regulator XylS originate from *Pseudomonas putida* TOL plasmid pWW0. The TOL plasmid encode enzymes that catabolises toluene, xylenes and its derivatives. There are many genes involved in this degradative function of the TOL plasmid. These genes are arranged into two catabolic pathway operons: upper pathway operon and meta pathway operon. The upper and meta operon are positively regulated by xylR and xylS proteins respectively. The product of the genes of the upper operon catabolise initial substrates to benzoate/akylbenzoate, which is regulated by XylR proteins, under the transcriptional control of σ^{54}- RNA dependent Pu promoter. The benzoate/akylbenzoates are further oxidised to Krebs cycle intermediates by enzymes of the meta operon: regulated by the XylS protein under control of σ^{70} or σ^{s}-dependent Pm promoter, (Gallegos et al., 1996; Winther-Larsen et al., 1999).

XylS is a transcriptional regulator that belongs to the AraC/XylS family. XylS is expressed from Ps1 and Ps2 promoters on the TOL plasmid pWW0. The Ps1 promoter is inducible, while the Ps2 promoter is constitutive and produces low level expression of XylS, (Gallegos et al, 1997).

The genes encoded in the meta pathway operon catabolism of benzoates and its derivatives are under the transcriptional control of the Pm promoter. In the presence of a benzoate, XylS become activated and initiates expression from the Pm promoter, (Gonzalez-Perez et al., 2002). The Pm promoter and its cognate regulator xylS has been used in constructing plasmid vectors for production of recombinant proteins in gram negative bacteria. Specifically, Pm promoter and xylS have been inserted in the replicon of a broad host range RK2 vector for the construction of a broad host expression vector that can function in gram-negative bacteria, (Blatny et al., 1997b). These expressions vectors have been used for low and high-level production of recombinant proteins in gram-negative bacteria; The process involves induction of the expression system (XylS/Pm) with an effector (Benzoic acid derivates). The effector binds to XylS and activates it. The activated XylS then bind to the Pm promoter and initiates transcription of the target gene, as shown in Fig.2.

It has been reported that XylS undergo dimerization in the presence or absence of an inducer and therefore binds to Pm as a dimer, (Dominguez-Cuevas et al., 2008).

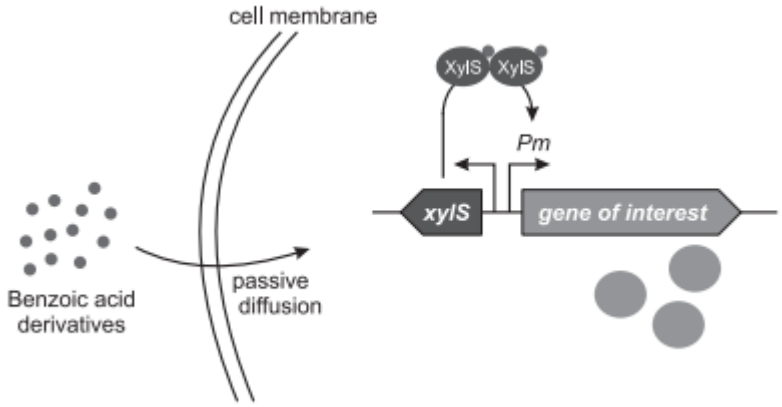

Fig.2. Induction of the XylS/Pm expression system (taken from Brautaset et al., 2008).

1.6 Aim of study

Psychrobacter were among a group of bacterial isolates discovered in collaboration with the Arctic University of Norway, Tromsø during a research expedition. The *Psychrobacter* strains were screened for their laccase activity. Four of the strains, P11f6, P11G3, P11G5 and P2G3 were discovered to harbour candidate laccase genes which were responsible for the laccase activity. The screening for laccase activity, identification of genes responsible for laccase activity and sequencing of the strains have previously been carried out.

The goal of this thesis involves cloning and expression of the four candidate laccase genes designated P11f6, P11G3, P11G5 and P2G3. *E. coli* and psychrophilic *Pseudomonas* will be utilized as expression hosts.

Furthermore, the solubility of the recombinant proteins generated will be evaluated and subsequently the activity of laccase will be tested by using a specific laccase substrate.

2. Materials and Methods

2.1. Medium and Solutions

Lysogeny Broth

Lysogeny broth medium (LB) was used for the cultivation of bacterial host strains used in this study. The medium will be referred to as lysogeny agar (LA), where agar has been added to the medium. Antibiotics (Apramycin & Kanamycin) were added to the medium when needed, for selective purposes only.

The various components of LB medium preparation are presented as follows;

10.0 g/l Tryptone
10.0 g/l NaCl
5.0 g/l Yeast extract
15.0 g/l Bacteriological Agar*
* only for LA (solid medium).

Dissolve the components in water and autoclave at 121^{o}C for 20 minutes.

Antibiotic stock solution:

100mg/ml stock solution (50μg/ml final concentration) of Kanamycin and Apramycin was prepared respectively and filter sterilized with a syringe filter. Solutions were aliquoted and stored at -20^{o}C.

2.1.2 Media and solutions for preparation of Competent cells

The Psi broth, TFB1 and TFB11 (Transformation buffer 1 & 11) buffers were used in the preparation of competent cells. While super optimal broth with catabolite repression (SOC), medium was used for both competent cell preparation and heat shock transformation experiment.

Psi broth: components/amount per litre
20.0 g/l Tryptone
5.0 g/l Yeast extract
5.0 g/l $MgCl_2$
Adjust pH to 7.6 with KOH, dissolve in 1 litre of water and autoclave.

TfB1 Buffer: component/amount per litre

2.94 g/l Potassium acetate

9.90 g/l $MnCl_2$

1.48 g/l $CaCl_2$

12.1 g/l RbCl

150 ml Glycerol

Adjust to pH 5.8 with acetic acid. Dissolve in water and filter sterilize.

TfB11 Buffer: component/amount per litre

2.10 g/l MOPS

11.0 g/l $CaCl_2$

1.20 g/l RbCl

150 ml Glycerol

Adjust to pH 6.5 with NaOH. Dissolve in water and filter sterilize.

SOC Media: component/amount per litre

20 g/l Tryptone

5 g/l Yeast extract

0.584 g/l NaCl

0.186 g/l KCl

10 ml/l $MgCl_2$ *

10 ml/l $MgSO_4$*

20 ml/l Glucose*

*1M stock solution was prepared respectively.

Dissolve components in water and autoclave at 121^oC for 20 minutes.

2.2. Other Solutions utilised

60% Glycerol: prepared by adding 60 ml of glycerol to water and autoclaved at 121^oC for 20 minutes.

IPTG: IPTG to a final concentration of 1 mM was used in this project (pre-made).

m- Toluic acid: 1M stock solution of m-Toluic acid (10 ml) was prepared by dissolving 1.3615g of m- Toluic acid into 99% ethanol.

2 X SDS gel- loading Buffer:

100mM Tris-HCl (pH 6.5)

4% SDS

0.2% bromophenol blue

20% glycerol

200mM dithiothreitol

SDS-Running Buffer: Clear Page SDS Running Buffer, 500 ml, 20x (CBS Scientific).

Lysis Buffer: 50mM NaH_2PO_4, 300mM NaCl. 0.12g of NaH_2PO_4 and 0.35g of NaCl were dissolved in 20 ml of sterilized water. Adjust pH to 8.0 and filter sterilize.

Enzyme Assay Activity Test: 5mM ABTS, 50mM Tris-HCl (pH7.0) and 0.25mM $CuCl_2$ were used in preparing the reaction mixture.

Copper (11) chloride dihydrate ($CuCl_2$. $2H_2O$): 5 ml of 1M $CuCl_2$. $2H_2O$ was prepared by dissolving 0.85g of copper (11) salt in sterilized water and filtered.

Agarose Gel Electrophoresis

50 x TAE Buffer: 242g Tris base, 57.1 ml Acetic acid, 0.5 mM EDTA adjust to pH 8

0.8% Agarose gel: 1.6g Agarose, 1 x TAE buffer (200ml).

Mix Agarose and 1x TAE buffer. Heat in a microwave till agarose dissolves. Add 20 µl GelRed and store at 65^{o}C.

2.3 Bacterial strains and Cloning vectors used in this study

Table1. Shows the various bacterial strains utilised in this study.

Bacterial strains	Use	Source
E. coli DH5α	Cloning host strain	This study
E. coli BL21 (DE3)	Expression host strain	-80^{o}C freezer
E. coli S17.1	Donor strain	-80^{o}C freezer
Psychrophilic *Pseudomonas* Psm26D7	Alternative expression host	Rahmi

2.3.1 Cloning vector

Cloning vectors are small pieces of DNA that are capable of self-replication in a host cell. They are used in carrying a cloned gene of interest into a host cell for maintenance and propagation. In this project, the laccase genes were cloned into pETite cloning vector for expression in *E. coli* BL21 (DE3), while plasmid pHH100 was used to introduce the laccase genes into Psychrophilic *Pseudomonas* Psm26D7 for expression.

Table 2. Shows plasmids utilised in this study.

Vectors	Features/use	Source
pETite C-His kan	T7-lac promoter, T7 terminator, Carboxyl terminal 6xHis tag, lacks lac repressor, kanamycin resistance gene, ribosome binding site. cloning and expression vector utilised in *E. coli.*	Lucigen
pHH100	Designed based on pHH100_mcherry. Includes origin of transfer (oriT), origin of replication (oriv), mini-RK2 replicon, kanamycin resistance gene, replication initiation protein (trfA). Mobilizable plasmid for transfer of recombinant plasmid to psychrophilic *Pseudomonas* by conjugation.	Rahmi

2.4 Plasmid constructs and cloning strategy

The four laccase genes, P11f6, P11G3, P11G5 and P2G3 were cloned into pETite cloning vector respectively, yielding four recombinant plasmid constructs.

The pETite cloning vector used in this study enable efficient simple cloning of the gene of interest. It does not require the use of restriction digestion, enzymatic treatment or ligation for recombination. It's basically a ligation-independent cloning system. The target genes are placed under the transcriptional control of T7 promoter, followed by amplification with primers containing flanking sequences from the pETite vectors joined to sequences from the target gene. The pre-linearized vector and PCR product containing the target gene are heat-transformed into competent *E. coli* cells. Recombination between the target gene and the vector occur during transformation of the competent *E. coli* host strain. The recombination events result in joining of the target gene to the vector (Lucigen).

The pETite vector-insert DNA constructs (recombinant DNA) were designed based on the expression kit manufacturer's instructions (Lucigen). The constructs were designed using Benchling (www.Benchling.com). In total four different recombinant plasmids were constructed and designated: pETP11f6 (fig.3), pETP11G3 (fig.4), pETP11G5 (Fig.5) & pETP2G3 (fig.6). The laccase gene P11G5 shares a 99% similarity with the P2G3 gene according to both gene sequences. P11G5 gene sequence is only three nucleotides different at the middle of its sequence in comparison with P2G3 (Appendix B).

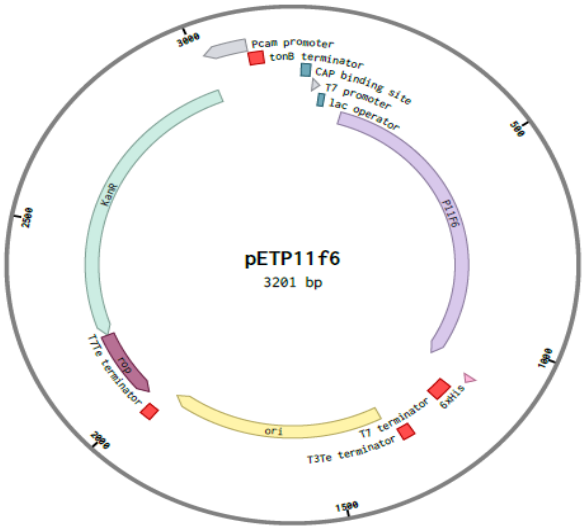

Fig.3 Plasmid map of pETP11f6 construct showing the cloned P11f6 laccase gene.

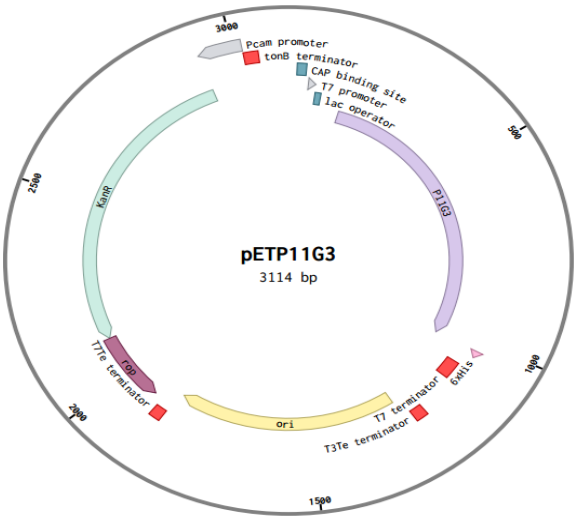

Fig.4 Plasmid map of pETP11G3 construct. P11G laccase gene cloned under control of T7 promoter.

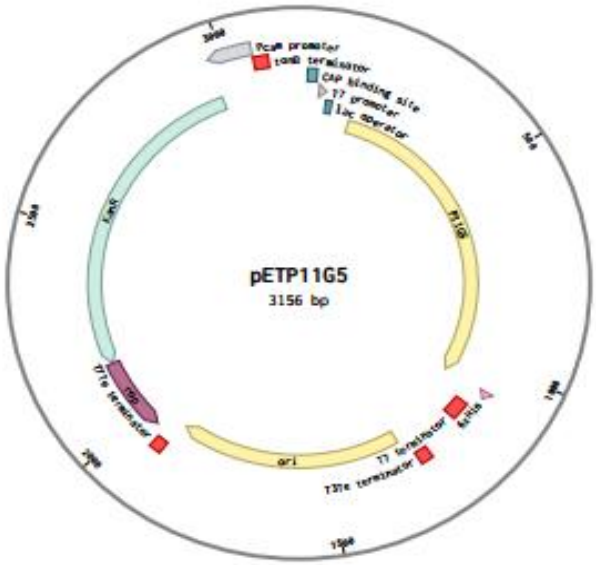

Fig. 5: Plasmid map pETP11G5 construct. Shows P11G5 laccase gene cloned under control of T7 promoter.

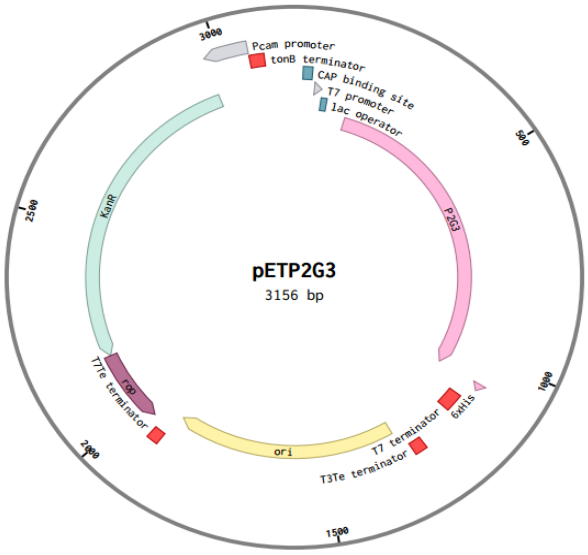

Fig. 6: Plasmid map of pETP2G3 construct. P2G3 laccase gene cloned under control of T7 promoter.

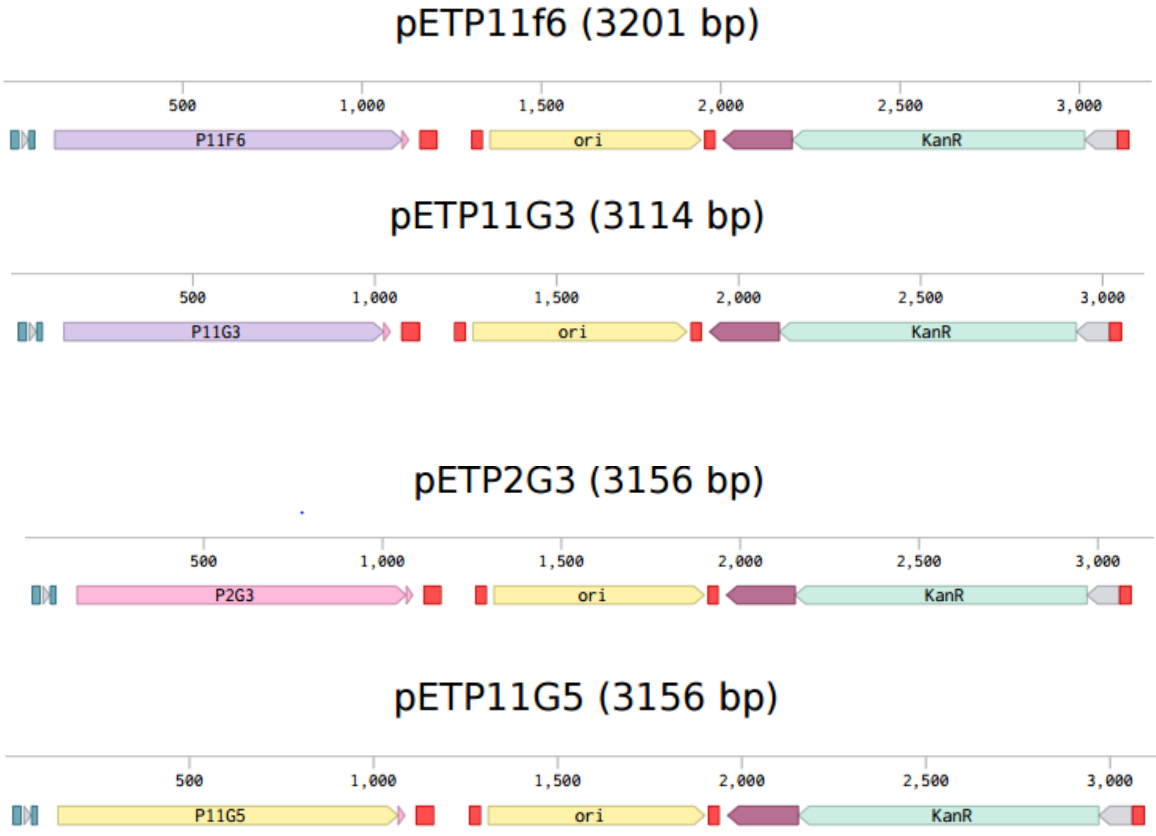

Fig.7 Linear maps of the three constructs pETP11f6, pETP11G3, pETP11G5 & pETP2G3.

2.5 Construction of plasmid for the introduction of laccase genes into Psychrophilic *Pseudomonas* Psm26D7

The pETite cloning vector utilised for cloning the laccase genes cannot replicate in *Pseudomonas*, it also lacks conjugative functions and therefore cannot be used for the transfer of the laccase genes to psychrophilic *Pseudomonas* Psm26D7 expression host.

A plasmid (pHH100) based on pHH100_mcherry, which has the broad-host range mini-RK2 replicon was specifically designed to replicate and facilitate the transfer of recombinant DNA created in *E. coli* DH5α to psychrophilic *Pseudomonas* Psm26D7. Plasmid pHH100 can replicate in broad host organisms, it has conjugative functions and therefore, can be mobilised from a donor cell to a recipient cell.

Plasmid pHH100 was constructed by sub-cloning the region surrounding the cloning site in the pETite vector into pHH100. These regions include, CAP binding site, T7 promoter and lac operator, 6 x His tag and T7 terminator of the pETite vector. Therefore, the laccase genes were under the transcriptional control of T7 promoter. Sequences, such as origin of transfer oriT, origin of replication (oriv), replication initiation protein (trfA) and kanamycin resistance were retained and other sequences from the pHH100_mcherry backbone not needed, were deleted.

One plasmid construct was designed. An example of plasmid pHH100 construct harbouring P11G5 laccase gene is shown in fig.8;

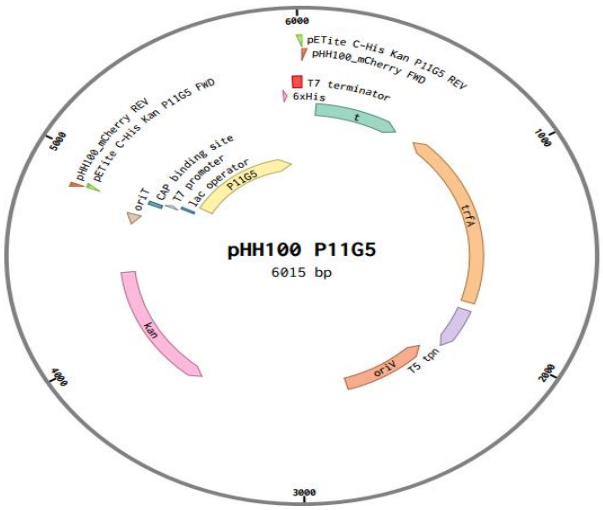

Fig.8 Plasmid map of plasmid pHH100 showing the cloned P11G5 laccase gene under control of T7 promoter.

2.6 General Experimental procedures used in cloning and expression of laccase genes

Preparation of competent *E. coli* cells

A cell is said to be competent, if it has ability to take up free DNA. For *E. coli* which is the organism of choice for most molecular cloning procedures, competence is mainly achieved by treatment with calcium chloride.

The experiment for the preparation of competent *E. coli* cells was performed as follows.

20 ml of SOC medium was inoculated with *E. coli* DH5α and incubated overnight at 37^{o}C with shaking at 225 rpm. 3000 µl of the overnight culture was transferred to 300 ml of psi medium and incubated at 37^{o}C until the optical density at 600 nm (OD_{600}) reach 0.3-0.4. The cell culture was chilled on ice for 5 minutes and transferred to 6 pre-chilled 50 ml falcon tubes. The tubes with the cell culture were then centrifuged for 10 minutes at 4000 rpm. The supernatant was discarded and the pelleted cells was resuspended in 15 ml TFB1 buffer. The tubes were again centrifuged for 10 minutes at 4000 rpm. The supernatant was discarded and the pellets resuspended in 1 ml TFB11 buffer.

The cell solution was transferred in aliquots of 100 µl into sterile Eppendorf tubes, snap-freeze with liquid nitrogen and stored at -80^{o}C. Thereafter, the transformation efficiency was calculated.

Agarose gel electrophoresis

Agarose gel electrophoresis is a method used for the separation of DNA molecules by size. It was used to analyse PCR products in this project. The experiment was performed as follows;

0.8% agarose was prepared as described in section 2.2. The gel was cast by pouring 0.8% agarose into a casting tray and a comb was placed at one end of the tray to form wells. The gel was left to solidify for about 20-30 minutes. After solidification, the gel was placed in gel chamber and 1x TAE buffer was added to the chamber, which has a positive electrode on one end and a negative electrode on the other end.

The samples to be analysed were prepared by adding 1 µl loading dye and 7 µl of sterilised water to 2 µl of PCR products. 1 kb GeneRuler DNA ladder was used as reference to estimate the sizes of the PCR products. The samples and DNA ladder were loaded into wells of the gel submerged in the buffer. The gel chamber was covered with a lid and connected to a power source 100V and allowed to run for 35-40 minutes.

The negatively charged DNA molecules will move from the negative electrode towards the positive electrode when electric current is applied to the gel chamber. After completion of the separation procedure, the gel was visualised by exposure to ultraviolet light and the images were taken with a Gel doc.

The sizes of the DNA molecules were determined by comparison with 1 kb GeneRuler DNA ladder with known sizes.

PCR Product Purification

The PCR products were purified using QIAquick PCR purification kit (Qiagen). The purification procedures were performed according to the instructions of the kit's manufacturer and are presented as follows;

5 volumes of PB buffer was added to 1 volume of the PCR product. The total volume of the PCR reaction was 25 µl, therefore 125 µl of PB buffer was added to 25 µl PCR product, followed by transfer to a spin column placed in a 2 ml collection tube and centrifuged for 60 seconds. The flow through was discarded and the column was returned to the same tube. 750 µl of PE buffer was added to the column and centrifuged for 60 seconds and the flow through was discarded. The column was returned to same tube and further centrifuged for 1 minute. The column was then transferred to a sterile 1.5 ml Eppendorf tube, followed by addition of 50 µl EB buffer to the centre of the column for the elution of the DNA. Additional, centrifugation was carried out for 1 minute. The column was discarded and the eluted DNA was stored at -20^{o}C.

Isolation of plasmid

Plasmids were isolated with Wizard Plus Miniprep DNA Purification kit. The procedures were performed according to the manufacturer's instructions;

5 ml of LB medium containing kanamycin to a final concentration of 50 µg/ml was inoculated with a single colony of transformed cells. The culture was incubated overnight at 37^{o}C with shaking at 225 rpm.

The overnight culture was centrifuged at 10,000 x g. The supernatant was discarded and the pellets was resuspended in 250 µl cell resuspension solution, followed by transfer to a 1.5 ml Eppendorf tube. 250 µl cell lysis solution was added and thoroughly mixed by inverting the tube 4 times. 10 µl alkaline protease was added and the mixture was incubated at room temperature for 5 minutes. This was followed by addition of 350 µl neutralisation solution and the tube was inverted 4 times for proper mixing. The solution was centrifuged at 13,000 x g for

10 minutes at room temperature. The supernatant was decanted into a spin column placed in a collection tube. The tube was centrifuged at 13,000 x g for 1 minute. The flow through was discarded and the spin column returned to the collected tube. 750 µl wash solution was added to the spin column and centrifuged at 13,000 x g for 1 minute. The flow through was discarded and the spin column reinserted into the collection tube. The washing step was repeated with 250 µl wash solution, followed by centrifugation at 13,000 x g for 2 minutes at room temperature. The spin column was transferred to a sterile 1.5 ml Eppendorf tube. Care was taken to prevent the transfer of the column wash solution along with the spin column.

50 µl nuclease free water was added to the spin column and centrifuged at maximum speed for 1 minute at room temperature to elute the plasmid DNA. Thereafter, the spin column was discarded and the DNA was stored at -20^{o}C.

2.6.1 Cloning of Laccase genes (creation of recombinant DNA)

As explained earlier, the pETite cloning strategy does not require restriction digestion and ligation. The recombinant DNA (vector-insert DNA constructs) was created by homologous recombination between the pETite vector and insert PCR product which have ends complementary to the vector through transformation. The manipulation and propagation was done in *E. coli* DH5α.

2.6.2 Amplification of laccase genes by Polymerase chain reaction (PCR)

The four laccase genes P11f6, P11G3, P11G5 & P2G3 were amplified from genomic DNA by PCR, using primers designed by following instructions of the expression kit's manufacturer (Lucigen). Three primer sets were designed and one set of primers was used for the amplification of P11G5 and P2G3 laccase genes due to reasons stated in section 2.4.

The PCR reaction was performed with Q5 High-fidelity DNA polymerase (NEB) and the melting temperatures was calculated with NEB T_m calculator (NEB.com). At the completion of the PCR reaction, the products were verified by agarose gel electrophoresis and purified with QIAquick purification kit (Qiagen) as described in section 2.6.

The reaction mixture set up and thermocycling conditions upon which the PCR was performed are presented as follows:

Table 3: Components of the reaction mixture for Q5 High-fidelity DNA polymerase PCR. The PCR reaction was performed on ice. The primers used are presented in Appendix A.

Component	Amount
5x Q5 Reaction Buffer	5 µl
10 mM dNTPs	0.5 µl
10 µM Forward Primer	1.25 µl
10 µM Reverse Primer	1.25 µl
Q5 High-fidelity DNA polymerase	0.25 µl
Template DNA	Variable
Nuclease free water	To 25 µl

Table 4: Thermocycling conditions used in performing Q5 High-fidelity DNA polymerase PCR.

Step	Temperature	Time	Cycle
Initiation denaturation	98^{o}C	30 seconds	
Denaturation	98^{o}C	10 seconds	
Annealing	50-72^{o}C	20 seconds	25x
Extension	72^{o}C	20-30 seconds/kb	
Final Extension	72^{o}C	2 minutes	
Hold	4^{o}C	∞	

2.6.3 Heat-shock Transformation

Transformation is the process by which competent cells take up free DNA. The expression kit manufacturer's transformation protocol, was utilized in this study and was performed as follows;

The *E. coli* DH5α was recovered from the -80^{o}C freezer and thawed on ice for 10-15 minutes. 2 µl of pETite vector DNA and 3 µl of insert PCR products were added to tubes containing *E. coli* DH5α cells (4 tubes). The solution was stirred carefully and briefly with pipette tip.

The culture tubes containing cells and DNA were incubated on ice for 30 minutes and heat-shocked by placing the tubes in a water bath at 42^{o}C for 45 seconds. The tubes were then placed on ice for 2 minutes. 960 µl of SOC medium was added to the cells in the culture tube and placed in a shaking incubator at 37^{o}C for 1 hour at 225 rpm.

At the end of the incubation period, the cells were pelleted at 10,000 rpm for 30 seconds in a centrifuge. The supernatant was discarded and the cells resuspended in 100 µl SOC medium. The cells were plated on LA plates supplemented with kanamycin to a final concentration of 50 µg/ml for selection of transformed cells. The plates were incubated overnight at 37ºC.

2.6.4 Colony PCR

E. coli DH5α transformants were screened for the presence of insert DNA by colony PCR. A single colony (in total 4 colonies: one for each construct) from four different transformed cells were picked with a sterile pipette and dissolved in four different tubes containing 30 µl nuclease free water and the solution was used as a source of template DNA. The colony PCR products were analysed by agarose gel electrophoresis. Colony PCR screening with Taq DNA polymerase is presented as follows:

Table 5: Components of Colony PCR reaction setup for screening of transformed *E. coli* cells for presence of recombinant DNA with Taq DNA polymerase. Primers used are shown in Appendix A.

Component	**Amount**
10x standard Taq buffer	2.5 µl
10 mM dNTPs	0.5 µl
50 µM Forward Primers	0.5 µl
50 µM Reverse Primers	0.5 µl
Taq DNA polymerase	0.125 µl
Template solution	1 µl
Nuclease free water	Add to 25 µl

Table 6: Thermocycling conditions setup of Colony PCR with Taq DNA polymerase

Step	**Temperature**	**Time**	**Cycles**
Initial denaturation	95ºC	6 minutes	
Denaturation	95ºC	30 seconds	
Annealing	55ºC	30 seconds	30x
Extension	68ºC	1 minute/kb	
Final Extension	68ºC	5 minutes	
Hold	4ºC	∞	

2.6.5 Glycerol stock

Having confirm the presence of the insert DNA in *E. coli* DH5α transformants by colony PCR, glycerol stocks of the transformed *E. coli* cells were made to preserve the cells for subsequent use and long term storage. The procedure was performed as follows;

Pre-cultures were made in a 13-ml culture tube containing 3 ml of LB medium plus kanamycin to a final concentration of 50 μg/ml. The medium was inoculated with single colony of *E. coli* transformant and incubated overnight at 37^{o}C with shaking at 225 rpm. After overnight incubation, 800 μl of 60% glycerol was added to 400 μl of overnight liquid culture in a cryotube and stored at -80^{o}C.

2.6.6 Plasmid Isolation

The isolation of recombinant plasmids harbouring the laccase genes was performed as follows: 13-ml culture tubes containing 5 ml LB medium supplemented with kanamycin to a final concentration of 50 μg/ml was inoculated with a single colony of *E. coli* cells carrying the recombinant plasmids. The cells were incubated overnight at 37^{o}C with 225 rpm shaking in a shaking incubator. After overnight incubation, the plasmids were isolated and purified according to the protocol (described in section 2.6) of the Wizard *Plus* SV Miniprep DNA Purification kit (Promega) and the solution was stored at -20^{o}C.

The plasmid concentration was measured with a Nanodrop.

2.6.7 Sequencing

The sequencing of the DNA constructs was performed by GATC Biotech Company (LightRun sequencing). The reaction solution for the sequencing was set up as follows: 10 μl each of solution was required: 5 μl plasmid DNA and 5 μl primers (forward or reverse). Two reactions for each construct was prepared and sequenced, one primer per reaction. The primers used were included with the expression kit (Appendix A). The plasmid DNA and the primer (forward or reverse) was mixed in a 1.5 mL Eppendorf tube, labelled and sent for sequencing. The sequencing results were analysed with multiple sequence alignment by hierarchical clustering (MultAlin). The sequencing results are presented in Appendix B.

2.7 Expression of laccase genes in *E. coli* BL21 (DE3)

2.7.1 Transformation of recombinant DNA into *E. coli* BL21 (DE3) cells

Plasmid DNA miniprep of the four laccase genes P11f6, P11G3, P11G5 & P2G3 were transformed into *E. coli* BL21 (DE3) prior to induction, for protein expression. The transformation was performed according to instructions of the expression kit manufacturer (Lucigen).

E. coli BL21 (DE3) cells (4 tubes) were recovered from the -80^{o}C freezer and thawed on ice. 1 µl of plasmid DNA miniprep of the four laccase genes were added to the thawed *E. coli* BL21 (DE3) cells on ice respectively. The culture tubes with the cells and plasmid DNA miniprep were incubated on ice for 30 minutes. The culture tubes were placed in a water bath and heat shocked at 42^{o}C for 45 seconds. Afterwards, the tubes were placed on ice for 2 minutes and 960 µl of SOC medium was added to the cells in the tubes. The culture tubes were incubated at 37^{o}C for 1 hour at 225 rpm in a shaking incubator. 100 µl of the cells were plated on LA plates containing kanamycin to a final concentration of 50 µg/ml. The plates were then incubated overnight at 37^{o}C. Colonies of the transformed cells were used to make glycerol stocks as previously described.

2.7.2 Induction of protein expression and solubility test in *E. coli* BL21 (DE3)

E. coli BL21 (DE3) harbouring the recombinant plasmids were cultivated at growth temperatures of 37^{o}C and 10^{o}C to induce protein expression respectively. SDS-PAGE analysis was performed to verify protein expression and solubility of the expressed proteins was also evaluated. The experiments were performed as follows:

Protein expression in transformed *E. coli* BL21 (DE3) cells cultivated at 37^{o}C

E. coli BL21 (DE3) cells containing the recombinant plasmid were recovered from glycerol stocks by using a toothpick to scrape the top of the frozen bacteria. The toothpick containing some cells were used to inoculate 5 ml of LB medium containing kanamycin to a final concentration of 50 µg/ml. The 13-ml culture tubes containing the cells were incubated overnight at 37^{o}C with shaking at 225 rpm in a shaking incubator. *E. coli* BL21 (DE3) without the recombinant plasmid was used as negative control.

500 µl of overnight cultures were inoculated into 50 ml of LB medium containing kanamycin to a final concentration of 50 µg/ml and incubated at 37^{o}C with shaking at 225 rpm, until the cultures reached OD_{600} of 0.5-1.0. Once the cultures reached the specified $OD_{600,}$ 1-ml each of

cultures (uninduced cells) were collected, aliquoted into Eppendorf tubes and pelleted in a centrifuge at 12,000 rpm for 1 minute. The pelleted cells were resuspended in 50 µl SDS-PAGE loading buffer. The uninduced cells plus SDS-PAGE loading buffer were stored at -20^{o}C until analysis by SDS-PAGE.

The remaining cultures were induced for protein expression by adding IPTG to a final concentration of 1 mM and incubated further at 37^{o}C for 4 hours at 225 rpm. The OD_{600} of the induced cells were recorded. At the completion of incubation period, 1-ml each of the induced cells were collected, aliquoted into Eppendorf tubes and pelleted by centrifugation at 12,000 rpm for 1 minute. The pelleted cells were resuspended by adding 100 µl SDS-PAGE loading buffer and stored at -20^{o}C until SDS-PAGE analysis to verify protein expression.

Evaluation of Solubility of the Recombinant proteins

To determine the solubility of the recombinant proteins, the remaining induced overnight cultures were collected and centrifuged at 4,000 x g for 15 minutes. The supernatant liquid was discarded and the pelleted cells were resuspended in 1-ml lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, pH 8.0). 1 mg/ml lysozyme was added and the cells were kept on ice for 30 minutes. Afterwards the cells were lysed on ice by sonication. The cell lysate was centrifuged at 10,000 x g for 20 minutes. Cell free supernatants were collected as the soluble portion of the protein and kept on ice. The remaining cell pellets which contains insoluble proteins were resuspended in 1-ml lysis buffer and kept on ice. The soluble and insoluble portions of the recombinant proteins were processed further for use in SDS-PAGE analysis.

SDS-PAGE analysis

To evaluate protein expression using SDS-PAGE, the samples were recovered from -20^{o}C. The samples containing SDS-PAGE loading buffer were heated for 10 minutes at 95^{o}C in a heating block and centrifuged at 12,000 x g for 1 minute. 5- 10 µl each of the samples were loaded onto wells of a Clear PAGE Precast Gels (cbs scientific) placed in a Dual Cool Mini-Vertical PAGE system containing 1x SDS-PAGE running buffer. Protein ladder was included to determine the molecular weights of the proteins. The Dual Cool Mini-Vertical PAGE system was connected to an electric source and run at 170V for 45-60 minutes. The gels were visualised by staining with Coomassie G-250 stain (Biorad).

The gels were first washed in water for 5 minutes and this was repeated three times. The water was removed and 50 ml Coomassie stain was added to each gel. The gels were placed on a shaker and allowed to shake for 1 hour. The stain was discarded and the gels were rinsed in

water for 30 minutes. Thereafter, the gels were stored in water and the images captured with a scanner or Gel doc.

Protein expression in transformed *E. coli* BL21 (DE3) cells at 10°C

The growth temperature of *E. coli* BL21 (DE3) harbouring the recombinant plasmid was reduced to evaluate protein expression levels and solubility of the recombinant proteins at lower temperature.

Glycerol stocks of transformed *E. coli* BL21 (DE3) cells were used to inoculate 5 ml LB medium supplemented with kanamycin (50 µg/ml final concentration) with the aid of sterile toothpick. Untransformed *E. coli* BL21 (DE3) was used as control. The cultures were incubated at 37°C until it reached an OD_{600} of 0.5-1.0. 1-ml culture of uninduced cells were collected and centrifuged at 12,000 rpm for 1 minute. The pelleted cells were resuspended in 50 µl SDS-PAGE loading buffer and stored in -20°C for SDS-PAGE analysis. IPTG to a final concentration of 1 mM was added to the remaining culture to induce protein expression. The cultures were then transferred to a shaking incubator set at 10°C. The induced and uninduced cultures were incubated at 10°C overnight with 225 rpm shaking. Both cultures were incubated simultaneously unlike in the previous experiment earlier described. 1 ml of overnight cultures were collected and centrifuged at 12,000 rpm for 1 minute. The cell pellets were resuspended in 100 µl SDS-PAGE loading buffer and stored in -20°C until SDS-PAGE analysis.

SDS-PAGE analysis and solubility of the recombinant proteins was determined and performed as earlier described.

2.7.3 Addition of Copper to the culture medium of *E. coli* BL21 (DE3) harbouring laccase genes

This experiment was performed similarly to earlier described procedures for the induction of protein expressions at 10°C, albeit with the addition of copper chloride ($CuCl_2$) and cultivation temperature of 15°C. when the cultures reached OD_{600} of 0.5-1.0, copper chloride to a final concentration of 0.5 mM was added to the culture containing transformed *E. coil* BL21 (DE3). This was followed by addition of IPTG to a final concentration of 1 mM, to induce protein expression. The cells were incubated overnight at 15°C and shaking at 225 rpm. The OD_{600} of the induced cultures were recorded. After overnight incubation, 1ml of the copper supplemented induced cells were harvested and centrifuged at 12,000 x g for 1 minute. The cell pellets were resuspended in 100 µl of SDS-PAGE loading buffer and stored in -20°C. The

remaining induced overnight cultures were processed further to determine the solubility of the copper supplemented recombinant proteins.
SDS-PAGE analysis and the solubility of the copper supplemented recombinant proteins were performed as described previously.

2.7.4 Laccase assay activity measurement

The laccase activity of the proteins expressed from copper supplemented cultures were analysed at room temperature. The assay mixture contained 50 mM Tris-HCl buffer (pH 7.0), 5 mM ABTS as substrate, and 0.25 mM copper chloride. The reaction was performed in a transparent 96-well plates (Nunc). 190 µl of assay mixture was added to the wells of transparent 96-well plates (Nunc) and reaction was initiated by adding 10 µl of cell free extract which contains soluble portions of the recombinant proteins. The experiment was performed at room temperature and overnight incubation. Oxidation of ABTS was monitored by absorbance at 420 nm with a spectrophotometer. The experiment was carried out in triplicates.

2.8 Expression of laccase genes in Psychrophilic *Pseudomonas* Psm26D7

Cloning of laccase genes into plasmid pHH100

The four recombinant plasmids containing the laccase genes P11f6, P11G3, P11G5 & P2G3 obtained from cloning laccase genes into the pETite vector were amplified by PCR. The plasmid pHH100 was also amplified by PCR. One set of primers was used for the amplification of the recombinant plasmids and another set for amplifying plasmid pHH100. The PCR reactions were performed with Q5 High-fidelity DNA polymerase. NEB T_m calculator was used in calculating the melting temperature. Agarose gel electrophoresis was used to verify the products of the PCR. After product verification and purification, recombinant plasmids were sub-cloned into plasmid pHH100 through transformation into *E. coli* DH5α.

Components of the PCR reaction and the cycling conditions used are presented as follow:

Table 7: PCR reaction set up using Q5 High fidelity DNA polymerase. Primers used are presented in Appendix A.

Components	Amount
5 x Q5 reaction buffer	5 µl
10 mM dNTPs	0.5 µl
10 µM Forward Primer	1.25 µl
10 µM Reverse Primer	1.25 µl
Template DNA	Variable
Q5 High-fidelity DNA polymerase	0.25 µl
Nuclease free water	Add to 25 µl

Table 8: Cycling conditions used in performing Q5 High-fidelity DNA polymerase PCR.

Step	**Temperature**	**Time**	**Cycle**
Initiation denaturation	98^{o}C	30 seconds	
Denaturation	98^{o}C	10 seconds	
Annealing	50-72^{o}C	20 seconds	25x
Extension	72^{o}C	20-30 seconds/kb	
Final Extension	72^{o}C	2 minutes	
Hold	4^{o}C	∞	

Enzyme treatment of PCR products

The PCR products were digested with the enzyme Dpn1. Dpn1 digests only methylated DNA and unmethylated PCR products are not affected.

1 µl of Dpn1enzyme was added to the PCR products and the mixture was placed on a 37^{o}C water bath for 1 hour. Afterwards, the PCR products were purified with QIAquick purification kit (Qiagen) as described in section 2.6.

Transformation of *E. coli* DH5α and *E. coli* S17.1

The PCR products of the recombinant plasmids containing the laccase genes, P11f6, P11G3, P11G5, P2G3 and plasmid pHH100 were transformed into *E. coli* DH5α. The transformation procedures were performed as earlier described in section 2.6.3. The resulting recombinant

DNA obtained from the heat shock transformation of *E. coli* DH5α were isolated using Wizard Plus Miniprep DNA Purification Kit (promega). The concentration of miniprep DNA was measured with a Nanodrop, followed by storage at -20ºC.

The miniprep recombinant DNA obtained from the transformation of *E. coli* DH5α were transformed into *E. coli* S17.1(donor strain) by heat shock. Heat shock transformation protocol was discussed in section 2.6.3

Colony PCR was performed as described in section 2.6.4 to determine the presence and correct sequences of insert DNA in *E. coli* S17.1 transformants.

2.8.1 Introduction of laccase genes into psychrophilic *Pseudomonas* Psm26D7 by Conjugation

Conjugation is the transfer of DNA from a donor cell to a recipient cell. The bacterial cells involved in conjugation need to be in direct contact for conjugation to occur. The introduction of recombinant plasmids harbouring the laccase genes into *Pseudomonas* Psm26D7 by conjugation was performed as follows:

A single colony of transformed *E. coli* S17.1 containing the recombinant DNA was picked with the aid of a toothpick and used to inoculate 5 ml LB medium supplemented with kanamycin to a final concentration of 50 μg/ml. Glycerol stock of *Pseudomonas* Psm26D7 was used to inoculate 5 ml LB medium supplemented with apramycin (50 μg/ml). The transformed *E. coli* S17.1 cells were incubated overnight at 37ºC and shaking at 225 rpm, while the *Pseudomonas* Psm26D7 was incubated overnight at 30ºC and shaking at 225 rpm in a shaking incubator. 13-ml culture tubes were used in both cases.

After overnight incubation, 1-ml overnight culture of *Pseudomonas* Psm26D7 was transferred to 50 ml falcon tube containing 10 ml LB medium and the culture was incubated at 30ºC for 4 hours at 225 rpm. Also, 1-ml overnight culture of transformed *E. coli* S17.1 harbouring laccase genes was subculture to 13 ml culture tubes containing 5 ml each of LB medium and incubated at 37ºC for 2 hours at 225 rpm until the OD_{600} reached 0.3-0.5.

At the completion of the incubation period for both cultures, 2-ml each of *Pseudomonas* Psm26D7 and transformed *E. coli* S17.1 containing recombinant DNA were harvested respectively and transferred to 1.5 ml sterile Eppendorf tubes. The Eppendorf tubes containing the mixed cells were centrifuged at 5,000 rpm for 5 minutes. The supernatants were discarded until approximately 100 μl of culture medium was retained in the tubes for resuspension of the

cell pellets. The cell solution was transferred with a pipette to an LA agar plate (without antibiotics) and allowed to stand for 15 minutes for the cell solution to settle into the agar plates. The LA plates containing mixed cells were incubated overnight at 30^{o}C. After overnight incubation, the plates were transferred to the lab working bench for overnight room temperature incubation.

After room temperature incubation, colonies were scooped and resuspended in 100 µl LB medium. Serial dilutions were carried out and 10^{-6} dilution were transferred to selective LA agar plate containing both kanamycin and apramycin to a final concentration of 50 µg/ml and incubated at room temperature.

Colonies of *Pseudomonas* Psm26D7 transconjugants were used to prepare glycerol stocks and stored at -80^{o}C for subsequent use.

2.8.2 Induction of protein expression in psychrophilic *Pseudomonas* Psm26D7

Pseudomonas Psm26D7 harbouring laccase genes (hereafter called transconjugants) were induced for protein expression by adding m-Toluic acid and IPTG to expression culture medium. The *Pseudomonas* transconjugants were cultivated at different temperatures to determine protein expression levels and solubility of recombinant proteins.

Protein expression in *Pseudomonas* Psm26D7 cultivated at 10^{o}C

Glycerol stocks of *Pseudomonas* Psm26D7 transconjugants containing laccase genes were used to inoculate 13-ml culture tubes containing 5 ml of LB medium supplemented with kanamycin to a final concentration of 50 µg/ml. The cultures were incubated overnight at 30^{o}C at 225 rpm in a shaking incubator. The overnight cultures were subculture to 250 ml flask containing 20 ml LB medium supplemented with kanamycin (50 µg/ml) and incubated at 30^{o}C with 225 rpm shaking until OD_{600} reach ~ 0.1. At the specified $OD_{600,}$ the liquid cultures were split into two (induced and uninduced culture). The induced cultures were supplemented with both m-Toluic acid to a final concentration of 0.5 mM and IPTG to a final concentration of 1 mM to induce protein expression. Both cultures were first incubated for 24 hours at 10^{o}C and shaking at 225 rpm. After incubation for 24 hours, OD_{600} of both cultures were recorded. The induced and uninduced cultures were incubated further for 72 hours and then 4 days respectively at 225 rpm in a shaking incubator. OD_{600} of both cultures were measured at intervals. Thereafter, 1-ml induced and uninduced samples were harvested by centrifugation at

12,000 rpm for 1 minute. The pelleted cells of both samples were resuspended in 100 µl SDS-PAGE loading buffer and stored at -20^{o}C until SDS-PAGE analysis.

SDS-PAGE analysis and determination of solubility of the expressed proteins were performed as described in previous section.

Induction of protein expression in *Pseudomonas* Psm26D7 at room temperature

The psychrophilic *Pseudomonas* Psm26D7 transconjugants were induced for expression at room temperature. The experiments were performed as follows:

Pre-cultures of *Pseudomonas* transconjugants were made in a 13-ml culture tubes containing 5 ml LB medium supplemented with kanamycin to a final concentration of 50 µg/ml. *Pseudomonas* Psm26D7 was used as a control. The cultures were incubated overnight at 30^{o}C with 225 rpm in a shaking incubator. 1 ml of the overnight cultures were subculture into 20ml LB medium supplemented with kanamycin to a final concentration of 50 µg/ml. The cultures were incubated at 30^{o}C with 225 rpm until it reaches OD_{600} of ~ 0.1. Afterwards, the cultures were split into two (induced and uninduced culture). The induced cultures were induced for expression by adding 1 mM final concentration of IPTG and 0.5 mM final concentration of m-Toluic acid. Both cultures were incubated at room temperature by placing on a benchtop shaker. The cultures were incubated first for 24 hours and followed by another 24 hours incubation.

The induced and uninduced cells were harvested by centrifugation and further processed for SDS-PAGE analysis as previously described. The SDS-PAGE analysis and evaluation of solubility of the express proteins were also performed as described in the previous sections.

2.8.3 Copper supplemented culture medium of *Pseudomonas* Psm26D7 transconjugants grown at room temperature

The experimental setup was the same as the one described in the previous section, except for the addition of copper chloride to the induced cultures.

The induced cultures were supplemented with $CuCl_2$ to a final concentration of 0.5 mM. The induced and uninduced cultures were incubated for 24 hours at room temperature. OD_{600} were recorded for both samples. The samples were further processed as described in the previous section.

SDS-PAGE analysis and solubility of expressed proteins were also determined as described in section 2.7.3.

3 Results

The results will be presented in three sections. Section 3.1 will include results of the cloning and expression of laccase genes in *E. coli* BL21 (DE3). Section 3.1.4 will present results of the copper supplemented cultures of *E. coli* BL21 (DE3) harbouring laccase genes. Laccase assay activity test results will also be described (section 3.1.5). Section 3.2 will focus on the results of the experiments performed by introducing the recombinant laccase genes into psychrophilic *Pseudomonas* Psm26D7.

3.1 Expression of laccase genes in *E. coli* BL21 (DE3)

Verification of sequences by Colony PCR

The four laccase genes, P11f6, P11G3, P11G5 & P2G3 were amplified by PCR as discussed in section 2.6.2. The PCR products were verified by agarose gel electrophoresis. The PCR products and pre-processed linearized pETite vector were transformed into *E. coli* DH5α. Colony PCR was performed to confirm and verify the presence of the insert DNA in *E. coli* transformants. (Fig.9 & 10).

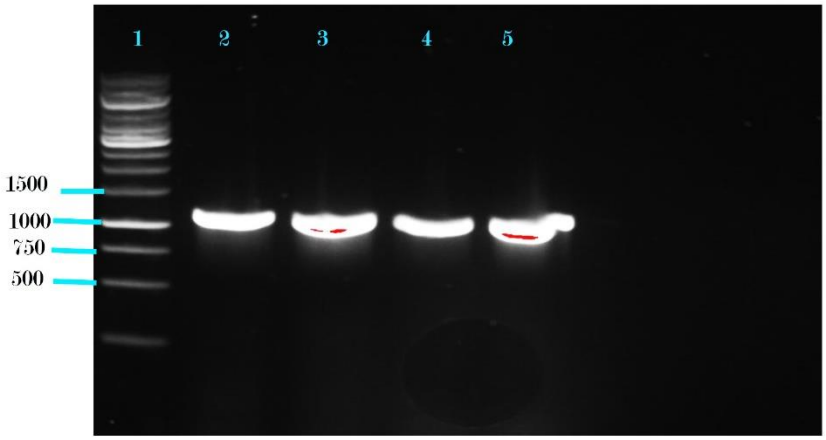

Fig.9 Agarose gel electrophoresis of colony PCR, lane 1 shows GeneRuler 1kb DNA ladder.
Lane 2 & 3 indicates P11f6 with a predicted size of 966 bp, lane 4 & 5 shows P11G5 with an expected size of 921 bp.

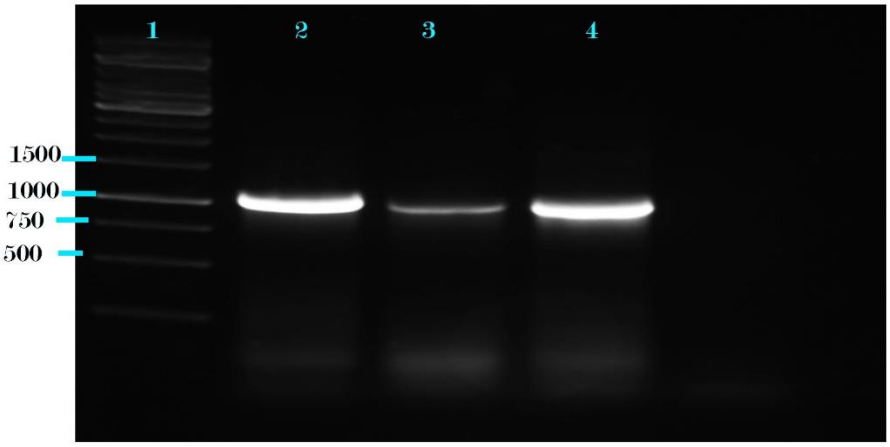

Fig.10: Agarose gel electrophoresis of colony PCR of P2G3 & P11G3. lane 1 shows GeneRuler 1kb DNA ladder, lane 2 indicates P2G3 with an expected size of 921bp, lane 4 shows P11G3 with an expected size of 879 bp.

3.1.2 Protein expression and solubility test in transformed *E. coli* BL21 (DE3) at 37°C

E. coli BL21 (DE3) harbouring recombinant DNA were induced for protein expression by adding IPTG to a final concentration of 1 mM. The transformed and induced *E. coli* BL21 (DE3) containing recombinant DNA were cultivated at 37°C for 4 hours and checked for protein expression by SDS-PAGE using samples from the induced cultures (fig.11). There were detectable specific bands of the expected sizes in lane 2 and 4 (induced samples) (fig.11), which represents P11f6 and P11G5 respectively. The presence of specific bands in lane 2 and 4 indicates that laccase genes P11f6 and P11G5 were expressed and there was, no similar thick bands in *E. coli* BL21 (DE3) without recombinant DNA used as control.

The induced cultures were harvested by centrifugation and solubility of the recombinant proteins were determined by SDS-PAGE analysis of samples from insoluble and soluble fractions (Fig.12). The recombinant laccase proteins P11f6 and P11G5 produced at *E. coli* optimum growth temperature of 37°C was found to be in the insoluble fraction as shown in (Fig.12). This is not surprising, as most heterologous proteins expressed in *E. coli* are produced in aggregates form known as inclusion bodies, which are insoluble.

To improve solubility of the recombinant protein, it was decided that the growth temperature of the *E. coli* expression host be reduced to 10°C. This form the basis of the next experiment.

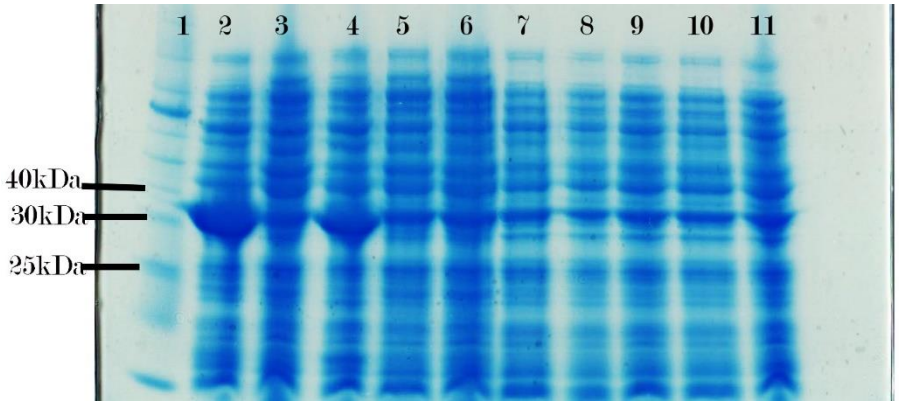

Fig.11: Expression levels of laccase genes expressed in *E. coli* BL21 (DE3) at 37ºC. SDS-PAGE of induced and uninduced samples prepared from cultures of transformed *E. coli* BL21 (DE3). lane 1 shows protein ladder 10-250 kDa (NEB), lane 2 shows P11f6 (induced), lane 3 indicates P11G3 (induced), lane 4 shows P11G5 (induced), lane 5 indicates P2G3 (induced), lane 6 untransformed *E. coli* BL21 (DE3) as negative control (induced), lane 7 shows P11f6 (uninduced), lane 8 shows P11G3 (uninduced), lane 9 shows P11G5 (uninduced), lane 10 shows P2G3 (uninduced) and lane 11 shows untransformed *E. coli* BL21 (DE3) (uninduced). The expected molecular weights of the proteins are as follows: P11f6: 35.07 kDa, P11G3: 32.01 kDa, P11G5: 33.62 kDa, P2G3: 33.62 kDa. 10% Precast gel was used.

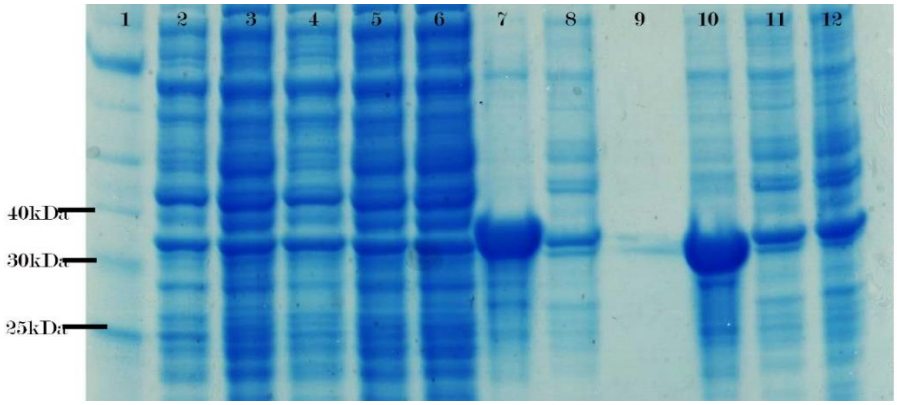

Fig.12: SDS-PAGE gel image. Solubility test of expressed proteins in *E. coli* BL21 (DE3) at 37ºC
Lane 1 shows protein ladder 10-250 kDa (NEB biolabs), lane 2: P11f6 (soluble), lane 3: P11G3 (soluble), lane 4: P11G5 (soluble), lane 5: P2G3 (soluble), lane 7 indicate P11f6 (insoluble), lane 8: P11G3 (insoluble), lane 10: P11G5 (insoluble), lane 11: P2G3 (insoluble). 10% Precast gel was used.

3.1.3 Induction of protein expression and solubility test in *E. coli* BL21 (DE3) at 10ºC

The growth temperature of transformed *E. coli* BL21 (DE3) containing recombinant DNA was lowered to 10ºC to enhance protein expression and solubility of the expressed proteins. The induced cultures were incubated at 10ºC concurrently with the uninduced cultures. Unlike in the previous experiment the uninduced samples were harvested and processed before inducing the remaining culture. SDS-PAGE analysis of samples from both cultures after overnight incubation shows specific bands of the predicted sizes in lane 2 (P11f6) fig.13. The specific band suggest protein overexpression in lane 2 corresponding to laccase genes P11f6. However, the expression levels of P11f6 in lane 2 (Fig.13) seems to be higher than the corresponding P11f6 of expression cultures cultivated at 37ºC.

The solubility of the expressed recombinant protein P11f6 was checked by SDS-PAGE of samples from soluble and insoluble fractions obtained by centrifugation. Again, the protein was found to be insoluble Fig.14: lane 8.

In fig.15, the sizes of the bands in the region of the protein of interest appear to be of the same sizes in comparison with bands of *E. coli* BL21 (DE3) without recombinant plasmid used as control. The sizes of the bands in the induced and uninduced samples appear to be the same size which may suggest, there was no expression of P11G3 and P2G3 laccase genes. The bands may be due to background proteins from *E. coli* BL21 (DE3).

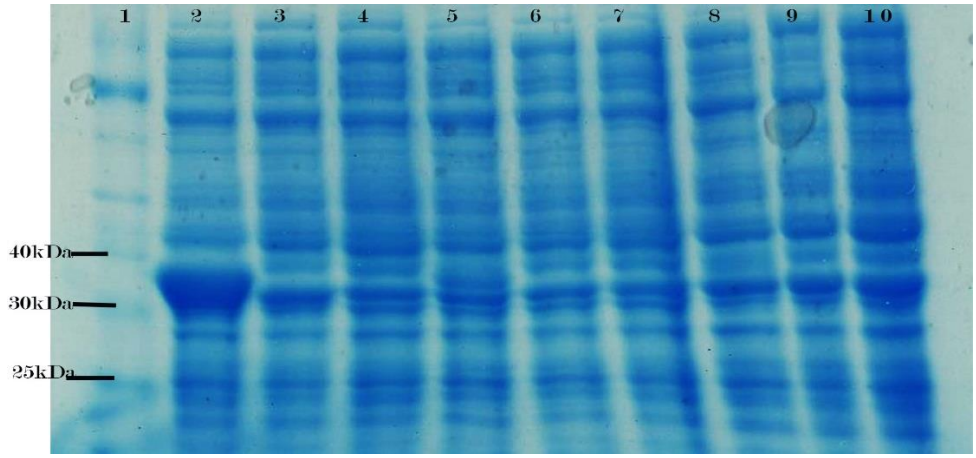

Fig.13: Expression levels of laccase genes expressed in *E. coli* BL21 (DE3) at 10ºC. SDS-PAGE of induced and uninduced samples prepared from cultures of transformed *E. coli* BL21 (DE3). lane 1 shows protein ladder 10-250 kDa, lane 2: P11f6 (induced), lane 3: P11G5 (induced), lane 4: untransformed *E. coli* BL21 (DE3) negative control, lane 5: P11f6 (uninduced after overnight incubation), lane 6: P11G5 (uninduced after overnight incubation), lane 7: control, lane 8: P11f6 (uninduced prior to overnight incubation), lane 9: P11G5 (uninduced prior to overnight incubation), lane 10: control. Expected protein molecular weights, P11f6: 35.07 kDa P11G5: 33.62 kDa. 10% precast gel was used.

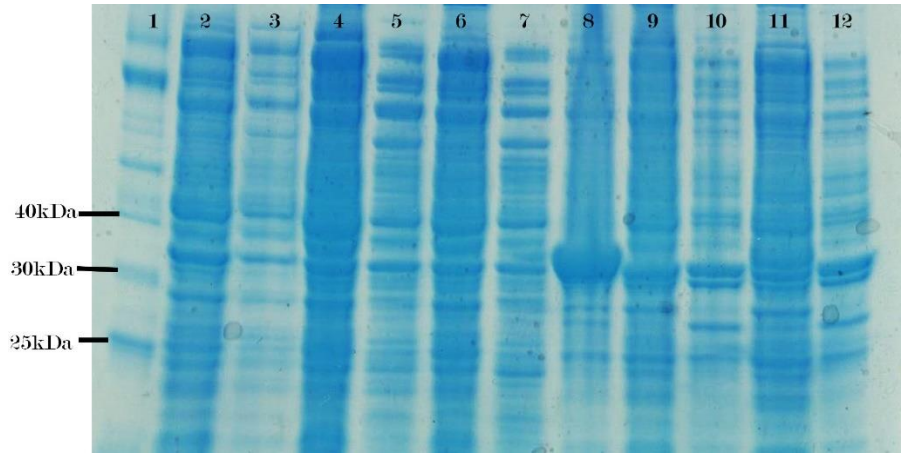

Fig.14: determination of solubility of recombinant proteins expressed in *E. coli* at 10ºC. SDS-PAGE of soluble and insoluble fraction obtained from induced culture of transformed *E. coli* BL21 (DE3). Lane 1: protein ladder 10-250 kDa, lane 2: P11f6 (soluble), lane 3: P11G5 (soluble), lane 8: P11f6 (insoluble), lane 9: P11G5 (insoluble), Expected protein molecular weights, P11f6: 35.07 kDa, P11G5: 33.62 kDa. 10% precast gel was used.

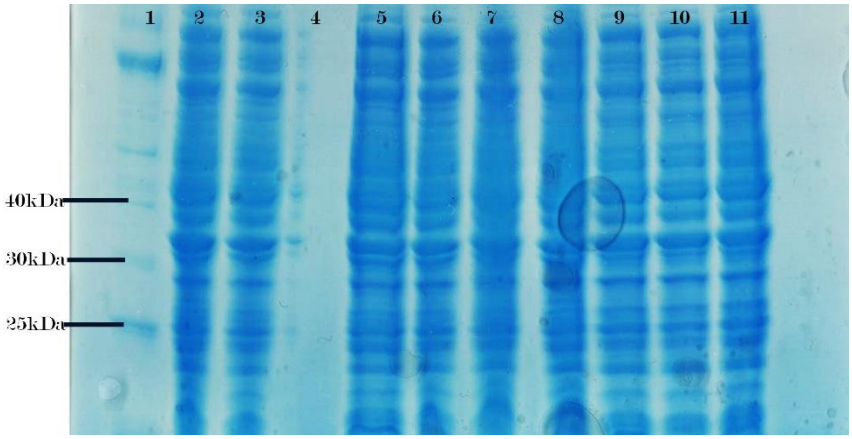

Fig.15: verification of protein expression in *E. coli* BL21 (DE3) at 10ºC by SDS-PAGE. Lane 1: protein ladder 10-250 kDa, lane 2: P11G3 (induced), lane 3: P2G3 (induced), lane 5: untransformed *E. coli* BL21 (DE3) (induced) as control, lane 6: P11G3 (uninduced after overnight incubation), lane 7: P2G3 (uninduced after overnight incubation), lane 8: control (uninduced), lane 9: P11G3 (uninduced before overnight incubation), lane 10: P2G3 (uninduced before overnight incubation), lane 11: control. 10% precast gel was used. Expected protein molecular weights, P11G3: 32.01 kDa, P2G3: 33.62 kDa.

3.1.4 Protein expression of copper supplemented cultures of *E. coli* BL21 (DE3)

Previous studies have shown that, copper ions enhance production and proper folding of laccases obtained from fungi and other bacterial sources (Collins & Dobson, 1997, Durao et al., 2007, Ihssen et al., 2015, Malhotra et al., 2004).

Copper chloride to a final concentration of 0.5 mM was added to induced cultures of transformed *E. coli* BL21 (DE3) harbouring the laccase genes. The cultures were incubated overnight at 15^{o}C. The induced and uninduced samples were analysed for overexpression of protein by SDS-PAGE. There were specific bands of expected sizes of the laccase recombinant proteins in lane 6-9 of the induced samples (Fig.16). However, there was corresponding bands in the uninduced samples, lane 1-4 (Fig.16). Lane 2 (Fig.16) which corresponds to the laccase gene P11G3 had a very thick band in the uninduced sample, this may be caused by leaky expression in the expression system.

Cell free extracts from induced cultures were harvested by centrifugation. Cell free extract which contains soluble fractions and unlysed cells which contains insoluble fractions of the laccase recombinant proteins were analysed by SDS-PAGE. It was observed that, laccase recombinant proteins expressed from copper supplemented cultures of *E. coli* BL21 (DE3) were expressed in both soluble and insoluble fractions (fig.17).

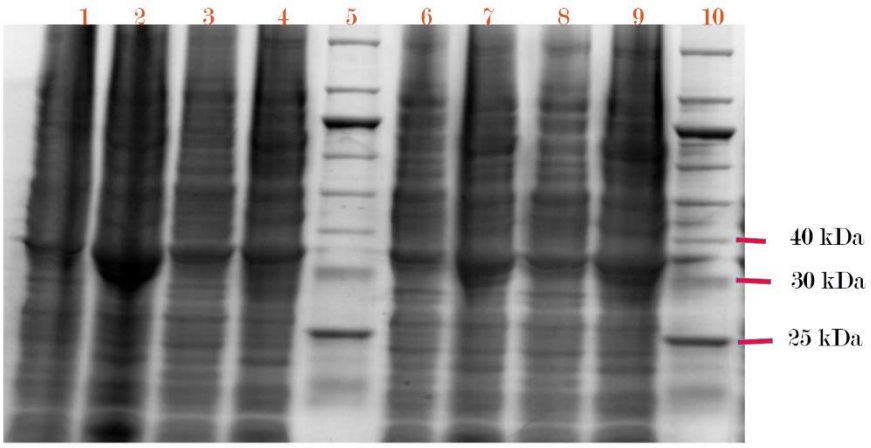

Fig.16: SDS-PAGE analysis of copper chloride supplemented cultures of transformed *E. coli* BL21 (DE3) at 15^{o}C. lane 1: P11f6 (uninduced), lane 2: P11G3 (induced), lane 3: P11G5 (uninduced), lane 4: P2G3 (uninduced), lane 5: protein standard 10-250 kDa, lane 6: P11f6 (induced), lane 7: P11G3 (induced), lane 8: P11G5 (induced), lane 9: P2G3 (induced), lane 10: protein ladder 10-250 kDa. Expected molecular weight of proteins, P11f6: 35.07 kDa, P11G3: 32.01 kDa, P11G5: 33.62 kDa, P2G3: 33.62 kDa. 8% precast gel was used.

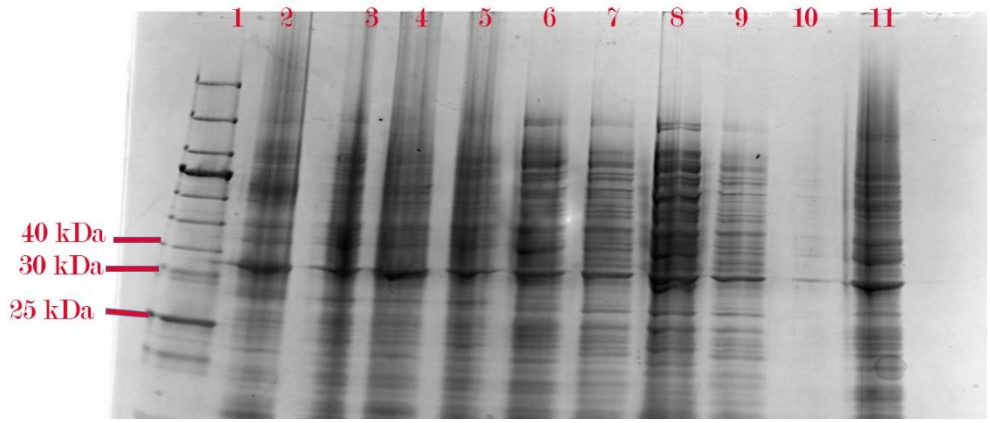

Fig.17: verification of solubility of laccase recombinant proteins obtained from copper supplemented cultures of transformed *E. coli* BL21 (DE3). SDS-PAGE of soluble and insoluble fractions.
Lane 1: protein ladder 10-250 kDa (NEB), lane 2: P11f6 (insoluble), lane 3: P11G3 (insoluble), lane 4: P11G5 (insoluble), lane 5: P2G3 (insoluble), lane 6: P11f6 (soluble), lane 7: P11G3 (soluble), lane 8: P11G5 (soluble), lane 9: P2G3 (soluble).

3.1.5 Laccase assay activity measurement

The recombinant laccase proteins P11f6, P11G3, P11G5 and P2G3 produced in *E. coli* BL21 (DE3) from copper supplemented expression cultures were tested for the enzyme activity encoded by the laccase genes.

The laccase activity was tested as described in subsection 2.7.4. ABTS was used as substrate. The 200 µl assay mixture included 10 µl soluble or cell free extract of laccase recombinant proteins from the four laccase genes respectively. *E.* coli BL21 (DE3) without recombinant plasmid was used as control. The reaction was initiated by adding the cell free extract containing soluble protein fraction to the reaction mixture. The reaction was monitored at specific time intervals and absorbance at 420 nm was measured with a spectrophotometer. The experiment was carried out in triplicates in a transparent 96-well plate (Nunc). The results of the absorbance measurements were the mean values.

Fig. 19 shows the colour change which indicates the oxidation of ABTS substrate. According to Fig.18, P11G5 had the highest intensity with absorbance increasing gradually with time, followed by P11f6 and P11G3 respectively. P2G3 had a lower colour intensity in comparison with others. *E. coli* BL21 (DE3) without recombinant plasmid used as control did not give any colour change and the absorbance remained constant after overnight incubation at room temperature Fig.18 & 19.

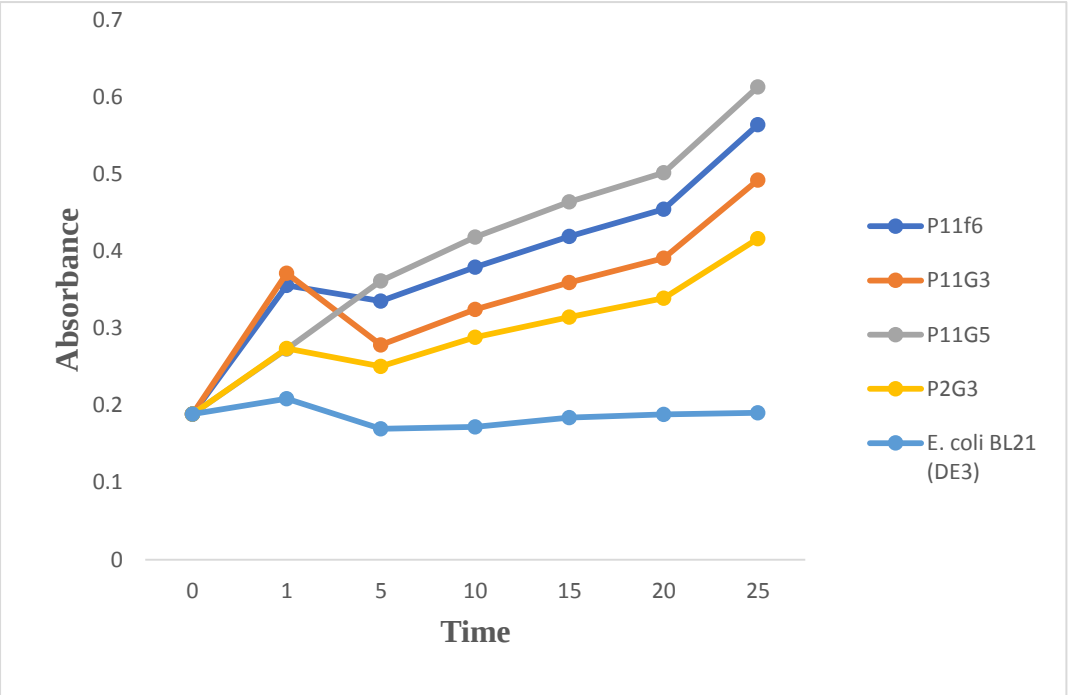

Fig.18: Graphical representation of absorbance measurement of each reaction mixture with a spectrophotometer.

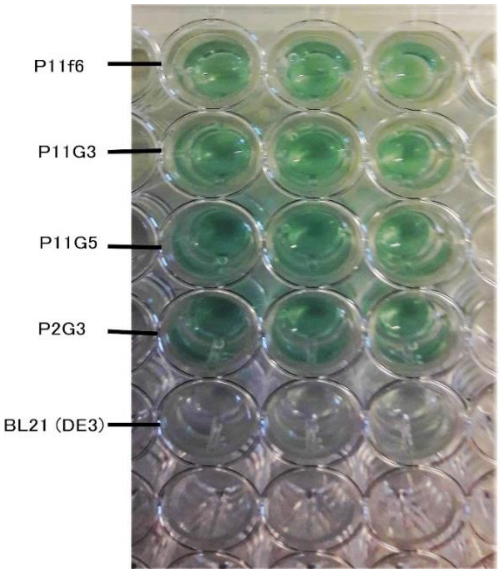

Fig.19: Laccase activity test. assay mixture in a transparent 96-well plate. The colour change is due to oxidation of ABTS substrate by laccases.

3.2 Expression of laccase genes in psychrophilic *Pseudomonas* Psm26D7

Cloning of laccase genes into plasmid pHH100

The laccase genes P11f6, P11G3, P11G5 & P2G3 were amplified by PCR from their corresponding recombinant DNA constructs earlier created in *E. coli* DH5α and plasmid pHH100 was also amplified by PCR. The PCR products were verified by agarose gel electrophoresis. Both PCR products were transformed into *E. coli* S17.1 and subsequently introduced into psychrophilic *Pseudomonas* Psm26D7 by conjugation. Colony PCR was performed to determine the presence and sizes of insert DNA in *E. coli* transformants (fig.20).

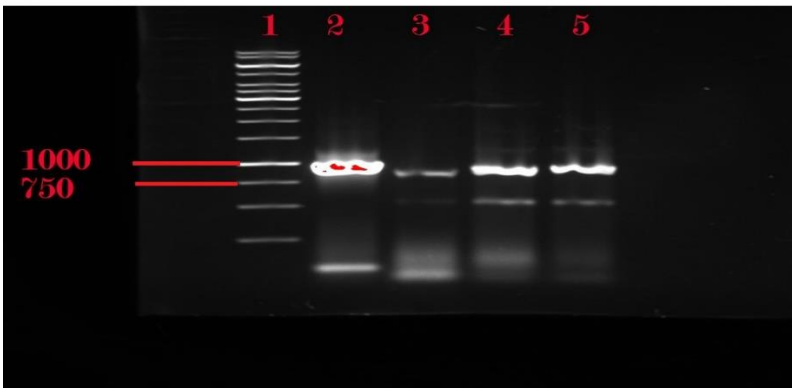

Fig.20: Agarose gel electrophoresis of Colony PCR products. Lane 1 shows GeneRuler 1kb DNA ladder, lane 2: P11f6 with a predicted size of 966 bp, lane 3: P11G3 with a predicted size of 879 bp, lane 4: P11G5 with an expected size of 921 bp, lane 5: P2G3 with a predicted size of 921 bp.

3.2.1 Induction of protein expression and solubility test in psychrophilic *Pseudomonas* Psm26D7

Psychrophilic *Pseudomonas* Psm26D7 was used as the alternative expression host. The psychrophilic *Pseudomonas* Psm26D7 containing recombinant DNA (hereafter, called transconjugants) were induced for protein expression by adding IPTG and m-Toluic acid to a final concentration of 1 mM and 0.5 mM respectively. The experiment was performed at varying growth temperatures and expression conditions, to determine protein expressions levels and solubility of the expressed proteins.

3.2.2 Protein expression and solubility test in *Pseudomonas* transconjugants cultivated at 10°C

The *Pseudomonas* Psm26D7 transconjugants harbouring recombinant DNA were cultivated at 10°C after induction by adding 1 mM IPTG and 0.5 mM m-Toluic acid. The expression cultures were incubated overnight. After overnight incubation, OD_{600} was recorded. Samples from the induced cultures were harvested and evaluated for protein expression by SDS-PAGE analysis (fig.21). Weak bands were detected in lane 8 and 9 (fig.21), but not visible enough to ascertain whether there was protein overexpression or not. The weak bands may be due to low cell density as shown by the OD_{600} values of the induced culture. The experiment was repeated and the incubation period extended to 72 hours. Samples from the induced cultures were harvested and evaluated for protein expression by SDS-PAGE analysis (fig.22). lane 9 and 10, fig.22 show a thicker band, which may suggest overexpression of P11G5 and P2G3 laccase genes. The experiment continued by extending incubation period of the expression culture to 4 days. There was, noticeable bands in lanes 9 and 10 (fig.23) which may also suggest overexpression of P11G5 and P2G3. The images of Fig. 22 & 23 are unclear and analysis of the result may not be as accurate.

The solubility of the expressed proteins was determined by methods described earlier. Soluble cell free extract and insoluble unlysed cells of the proteins obtained by centrifugation were processed and analysed by SDS-PAGE (fig.26). The expressed recombinant proteins were insoluble.

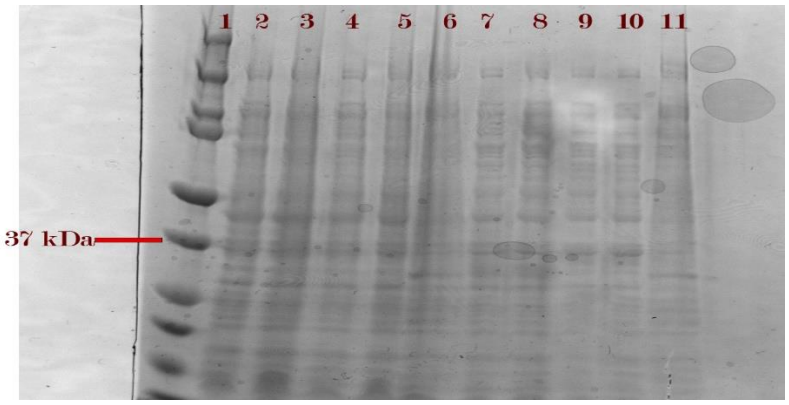

Fig.21: SDS-PAGE analysis of protein expression by transconjugants cultivated at 10°C (overnight incubation). Lane 1 shows protein ladder 10-250 kDa (Biorad), lane 2 shows P1f6 (uninduced), lane 3 shows P11G3 (uninduced), lane 4 shows P11G5 (uninduced), lane 5: P2G3 (uninduced), lane 6: Psm26D7 control (uninduced), lane 7: P11f6 (induced), lane 8: P11G3 (induced), lane 9: P11G5 (induced), lane 10: P2G3 (induced), lane 11: Psm26D7 control (induced). Expected molecular weights of protein, P11f6:35.07 kDa, P11G3: 32.01 kDa, P11G5: 33.62 kDa, P2G3: 33.62 kDa.

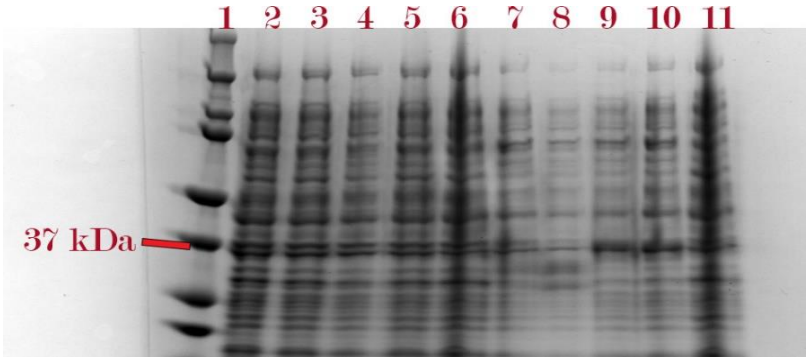

Fig 22: SDS-PAGE verification of protein expression by transconjugants cultivated at 10^{o}C (72 hours incubation), lane 1 shows protein ladder 10-250 kDa (Biorad), lane 2: P11f6 (uninduced), lane3: P11G3 (uninduced), lane 4: P11G5 (uninduced), lane 5: P2G3 (uninduced), lane 6: Psm26D7 control (uninduced), lane 7: P11f6 (induced), lane 8: P11G3 (induced), lane 9: P11G5 (induced), lane 10: P2G3 (induced), lane 11: Psm26D7 control (induced). Expected molecular weights of protein, P11f6:35.07 kDa, P11G3: 32.01 kDa, P11G5: 33.62 kDa, P2G3: 33.62 kDa.

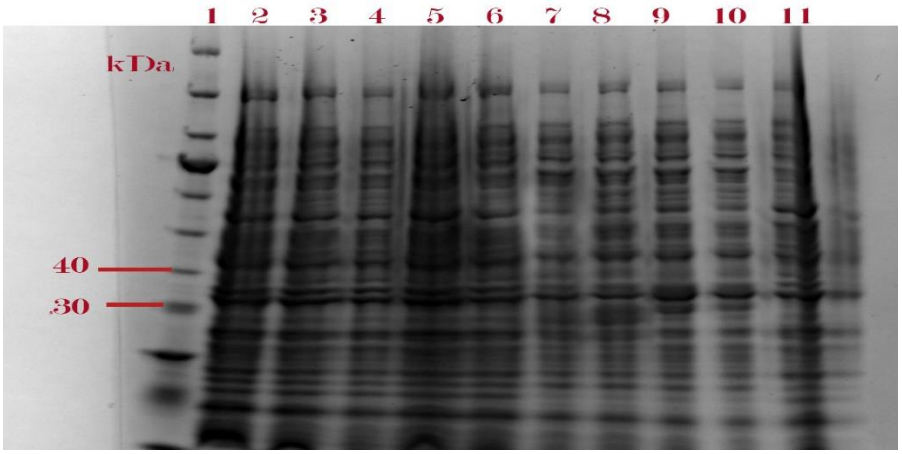

Fig 23: SDS-PAGE verification of protein expression by transconjugants cultivated at 10^{o}C (4 days incubation), lane 1 shows protein ladder 10-250 kDa (NEB), lane 2: P11f6 (uninduced), lane3: P11G3 (uninduced), lane 4: P11G5 (uninduced), lane 5: P2G3 (uninduced), lane 6: Psm26D7 control (uninduced), lane 7: P11f6 (induced), lane 8: P11G3 (induced), lane 9: P11G5 (induced), lane 10: P2G3 (induced), lane 11: Psm26D7 control (induced). Expected molecular weights of protein, P11f6:35.07 kDa, P11G3: 32.01 kDa, P11G5: 33.62 kDa, P2G3: 33.62 kDa.

3.2.3 Induction of protein expression in *Pseudomonas* Psm26D7 transconjugants cultivated at room temperature and 30°C

The cultivation of *Pseudomonas* Psm26D7 transconjugants harbouring recombinant DNA at 10°C resulted in slow growth rate and longer generation time of the transconjugant cells. This study observed that the psychrophilic *Pseudomonas* Psm26D7 attained higher growth rate and shorter generation time when cultivated at 30°C in comparison with room temperature, 10°C and 5°C cultivation respectively. This suggests that the psychrophilic *Pseudomonas* Psm26D7 has an optimum growth temperature of 30°C.

The *pseudomonas* Psm26D7 transconjugants were then cultivated and induced for protein expression at 30°C. The induced cultures were incubated for 4 hours and OD_{600} was recorded before and after induction. Samples from the induced cultures were collected and processed for SDS-PAGE analysis (fig.25: lanes 2-9). Despite the higher cell densities reached by *Pseudomonas* Psm26D7 transconjugants grown at 30°C, there was no specific bands in the region of the expected proteins to suggest protein overexpression.

The *Pseudomonas* Psm26D7 transconjugants were further cultivated at room temperature and incubated overnight and 48 hours respectively, in a bid to improve expression and solubility of the proteins. The transconjugants were first induced and incubated overnight at room temperature. The samples were processed and analysed by SDS-PAGE. There were specific bands of the expected protein sizes in lane 7, 8 and 9, which represents P11G3, P11G5 and P2G3 (Fig.24) respectively. The experiment continued by extending the incubation period of the *Pseudomonas* Psm26D7 transconjugants to 48 hours at room temperature. Samples from the induced cultures were harvested and processed for SDS-PAGE analysis. There were visibly thicker bands in lanes 15, 16 and 17, which represents P11G3, P11G5 and P2G3 respectively (fig.25). The bands observed in fig.25 were much thicker than those observed in fig.24 for the same proteins. The induced culture was centrifuged to obtain cell free supernatant and insoluble unlysed cells portions. The soluble cell free extract and insoluble unlysed fractions of the proteins were processed and analysed by SDS-PAGE (Fig.26: lane 1-8). The expressed proteins were observed to be in the insoluble fractions, fig. 26.

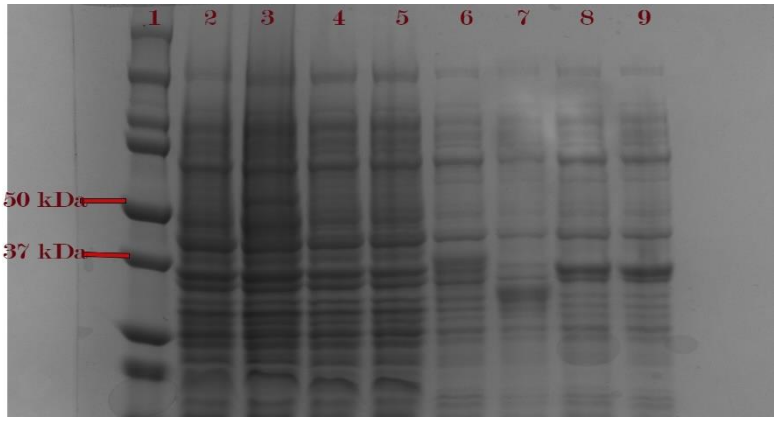

Fig.24: Protein expression levels of laccase genes expressed from *Pseudomonas* transconjugants, SDS-PAGE of induced and uninduced samples of cultures incubated overnight at room temperature. Lane 1: protein ladder 10-250 kDa, lane 2: P11f6 (uninduced), lane 3: P11G3 (uninduced), lane 4: P11G5 (uninduced), lane 5: P2G3 (uninduced), lane 6: P11f6 (induced), lane 7: P11G3 (induced), lane 8: P11G5 (induced), lane 9: P2G3 (induced). Expected protein molecular weights are, P11f6: 35.07 kDa, P11G3: 32.01 kDa, P11G5: 33.62 kDa, P2G3: 33.62 kDa.

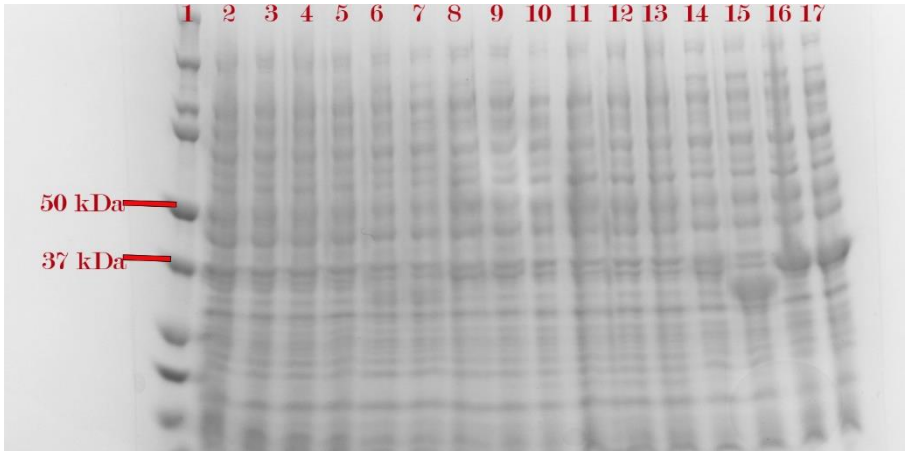

Fig.25: SDS-PAGE verification of laccase gene expression in *Pseudomonas* transconjugants cultivated at room temperature (48 hours incubation) and 30^{o}C respectively. Lane 1 shows protein ladder 10-250 kDa (Biorad), lane 2: P11f6 (induced), lane 3: P11G3 (induced), lane 4: P11G5 (induced), lane 5: P2G3 (induced), lane 6: P11f6 (uninduced), lane 7: P11G3 (uninduced), lane 8: P11G5 (uninduced), lane 9: P2G3 (uninduced), lanes 2-9 contain samples of transconjugants cultivated at 30^{o}C. lane 10 shows P11f6 (uninduced), lane 11: P11G3 (uninduced), lane 12: P11G5 (uninduced), lane 13: P2G3 (uninduced), lane 14: P11f6 (induced), lane 15: P11G3 (induced), lane 16: P11G5 (induced), lane 17: P2G3 (induced). Lanes 10-17 contain samples of transconjugants cultivated and expressed at room temperature. 8% precast gel was utilised. Expected protein molecular weights are, P11f6: 35.07 kDa, P11G3: 32.01 kDa, P11G5: 33.62 kDa, P2G3: 33.62 kDa.

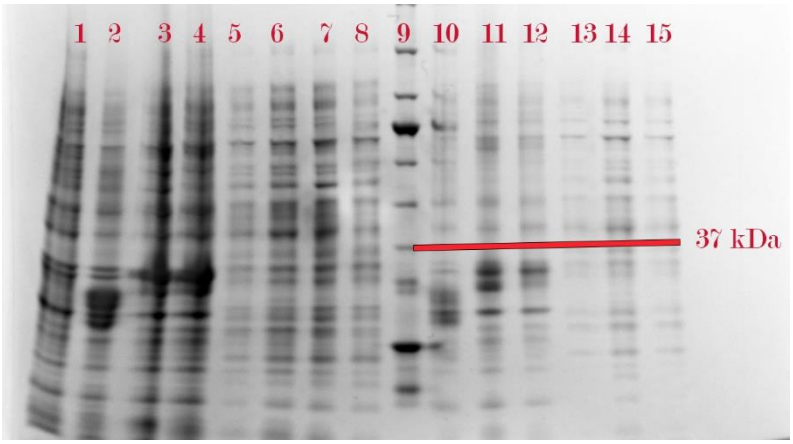

Fig.26: solubility of expressed proteins by transconjugants cultivated at 20ºC and 10ºC respectively. Lane 1 shows P11f6 insoluble portion, lane 2: P11G3 (insoluble), lane 3: P11G5 (insoluble), lane 4: P2G3 (insoluble), lane 5: P11f6 (soluble), lane 6: P11G3 (soluble), lane 7: P11G5 (soluble), lane 8: P2G3 (soluble), lanes 1-8 are samples of transconjugants expressed at room temperature, lane 9: protein ladder 10-250 kDa (Biorad), lane 10: P11G3 (insoluble), lane 11: P11G5 (insoluble), lane 12: P2G3 (insoluble), lane 13: P11G3 (soluble), lane 14: P11G5 (soluble), lane 15: P2G3 (soluble), lanes 10-15 contain samples of transconjugants expressed at 10ºC. 8% precast gel was used and 5 µl samples loaded to lanes of gel.

3.2.4 Addition of Copper chloride to induced cultures of *Pseudomonas* Psm26D7 transconjugants.

The experiment was performed similar, to expression of the laccase genes in *E. coli* BL21 (DE3), copper chloride to a final concentration of 0.5 mM was added to induced cultures of *Pseudomonas* Psm26D7 transconjugants. The cells were incubated overnight at room temperature. Samples from the induced cultures were analysed for protein expression by SDS-PAGE. There were visible thick bands in lane 9-10 which represents P11G5 and P2G3 respectively (Fig.27).

Solubility of the expressed proteins was tested, but most of the proteins were in the insoluble fraction (Fig.28).

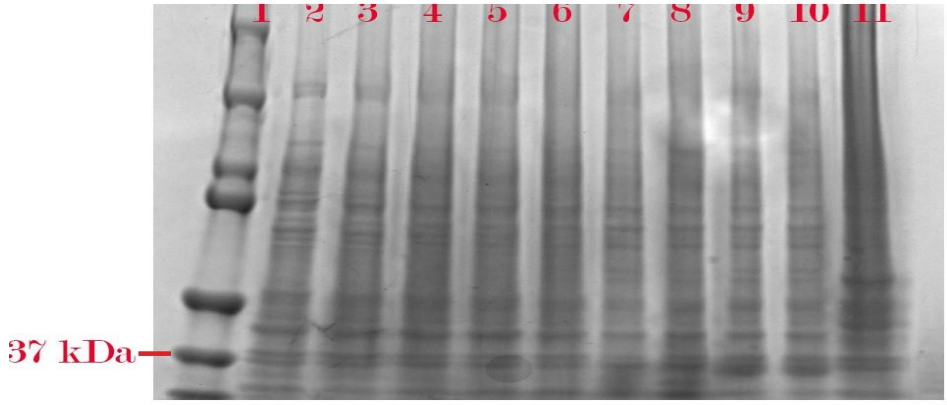

Fig.27: shows the expression of copper supplemented cultures of transconjugants cultivated at room temperature. Lane 1 shows protein ladder 10-250 kDa (Biorad), lane 2: P11f6 (uninduced), lane 3: P11G3 (uninduced), lane 4: P11G5 (uninduced), lane 5: P2G3 (uinduced), lane 6: Psm26D7 control (uninduced), lane 7: p11f6 (induced), lane 8: P11G3 (induced), lane 9; P11G5 (induced), lane 10: P2G3 (induced), lane 11: Psm26D7 control (induced). Expected protein molecular weights are, P11f6: 35.07 kDa, P11G3: 32.01 kDa, P11G5: 33.62 kDa, P2G3: 33.62 kDa. 10% precast gel was used.

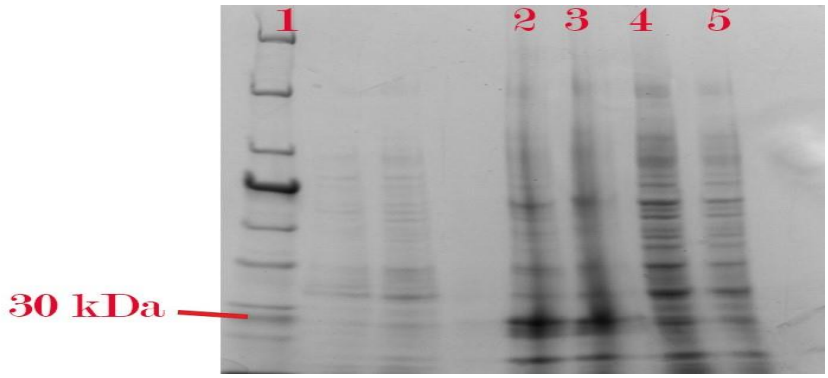

Fig.28: solubility of expressed proteins by transconjugants supplemented with copper chloride and cultivated at room temperature. Lane 1 shows protein ladder 10-250 kDa (NEB Biolabs), lane 2 shows P11G5 (insoluble) with an expected molecular weight of 33.62 kDa, lane 3 shows P2G3 (insoluble) with an expected molecular weight of 33.62 kDa, lane 4: P11G5 (soluble), lane 5: P2G3 (soluble) with molecular weight of 33.62 kDa. 10% precast gel was used.

4 Discussion

4.1 Cloning of laccase genes

pETite cloning and expression vector was utilised in the cloning and expression of the laccase genes in *E. coli* expression strain. The T7 promoter controls the transcription of the cloned laccase genes. As previously explained, no ligation or use of restriction enzyme is required for cloning the insert into the vector. Cloning and recombination between the target genes and the vector occurs *in vivo* in a competent *E. coli* DH5α cells.

The laccase genes (P11f6, P11G3, P11G5, P2G3) were amplified by PCR from genomic DNA isolated from four different *Psychrobacter* strains. The respective PCR products and pETite vector were co-transformed into competent *E. coli* DH5α, resulting in a recombinant plasmid containing individual laccase genes.

4.2 Expression of laccase genes in *E. coli*

The four laccase genes P11f6, P11G3, P11G5 and P2G3 isolated from four different *Psychrobacter* strains were expressed in *E. coli* BL21 (DE3) expression strain.

Expression cultures of transformed *E. coli* BL21 DE3 containing recombinant plasmid were grown at varying temperatures and expression conditions. The induced culture of *E. coli* BL21 DE3 harbouring the laccase genes grown at 37^{o}C yielded expression of P11f6 and P11G5 (lane 2 and 4 respectively), fig.11. The P11f6 had a thicker band which suggests higher expression levels compared to the P11G5. There was presence of bands of almost identical sizes from the induced samples of P11G3 (lane 3), P2G3 (lane 5) and uninduced samples of expression cultures lane 7-10 (Fig.11), these bands may be background proteins from *E. coli* cells, as the same bands were detected in untransformed *E. coli* BL21 (DE3) used as control. Another line of thought was the bands were a result of leaky expression in the expression system. leaky expressions of gene of interest are not uncommon with expression of proteins in *E. coli* BL21 (DE3) strain. This may be particularly deleterious to the cells especially if the expressed proteins are toxic. leaky expression of proteins prior to induction can lead to reduced expression levels of the proteins after induction if the proteins are toxic (Makrides, 1996). To troubleshoot leaky expressions in the expression system, 0.5% glucose was added to the expression culture medium. However, it didn't have any effect as the bands were still detected in the uninduced samples. Having repeated the experiment to check if there would be a different outcome regarding the expression of the laccase genes from cultures incubated at 37^{o}C, the outcome

seems to be consistent as only P11f6 and P11G5 had more specific bands. The next step was to check for the solubility of the proteins expressed.

Protein expression in *E. coli* may lead to the formation of aggregates of insoluble proteins called inclusion bodies. This was particularly evident in the proteins expressed from P11f6 and P11G5 laccase genes when checked for its solubility. The proteins were found to be expressed in insoluble fractions, lane 7 and 10 (Fig.12).

The expression cultures were incubated at 10^{o}C after induction to enhance solubility of the expressed proteins in conformity with previous reports (Sørensen & Mortensen, 2005). SDS-PAGE gel image of the expressed proteins showed that there was specific thick band in lane 2 (P11f6) Fig.13. However, the expressed protein was found to be in the insoluble form, as shown in lane 8 (P11f6) Fig.14. Again, there was no detectable specific bands in Fig.15, which suggest there was no overexpression of P11G3 and P2G3 laccase genes at 10^{o}C cultivation of *E. coli* BL21 (DE3).

Previous studies have shown that copper enhances the proper folding and activity of recombinant laccases from bacterial sources (Durao et al., 2007). The induced expression cultures were supplemented with Copper chloride to a final concentration of 0.5 mM and incubated overnight at 15^{o}C. SDS-PAGE analysis shows the presence of detectable bands in the induced samples (Fig. 16), suggesting low expression levels. In lane 2 which contains P11G3 sample (uninduced), there was a very thick band, which may be due to basal expression prior to induction. However, the corresponding band in the induced sample of P11G3 was much weaker, lane 7 (Fig.16). The detection of specific thick band in the uninduced sample of P11G3 (lane 2) Fig.16, may suggests leakiness in the expression system.

The solubility of the proteins from copper supplemented expression cultures were analysed by SDS-PAGE as presented in Fig.17. The proteins were found to be expressed in soluble and insoluble fractions, albeit in small proportions as seen in the bands present in Fig.17.

A comparison of expressions at the different temperatures shows expression of P11f6 and P11G5 (lane 2 and 4 respectively, fig.11) by cultivating transformed *E. coli* expression strain at 37^{o}C, but P11f6 had a thicker band than P11G5. At 10^{o}C cultivation of transformed *E. coli* expression strain, only P11f6 (lane 2 fig.13) seem to be expressed. Based on observable Phenotype, expression levels of P11f6 recombinant laccase proteins expressed at 10^{o}C was higher than P11f6 recombinant proteins expressed at 37^{o}C cultivation of *E. coli* expression strain.

Copper supplemented expression culture incubated at 15^{o}C yielded low amount of proteins as shown in the bands present in the induced samples of the four recombinant laccase proteins, lane 6-9, fig.16.

The comparison would have been more accurate if the proteins were quantified with the aid of a software for protein quantification. However, this was attempted with image lab software (Biorad) found in the chemidoc in the laboratory. It was not possible because most of the SDS-PAGE images were initially taken with a scanner except fig.16 and fig.17 (image lab format) and the software could not analyse the scanned images and the images could not be converted to image lab format.

4.3 Laccase assay activity measurement

The soluble recombinant proteins obtained from copper supplemented expression cultures of *E. coli* BL21 (DE3) harbouring the laccase genes were tested for activity as described in section 2.7.4. ABTS was used as the substrate and its oxidation was monitored by measuring absorbance at 420 nm with a spectrophotometer at specific time intervals. The intensity of the colour change of the reaction mixture was monitored. The reaction was carried out in triplicates and the result was calculated as the mean values. There was colour change in the reaction mixture containing recombinant proteins from P11f6, P11G3, P11G5 and P2G3 (Fig.19) from a colourless solution to a green colour, which suggests the recombinant laccase proteins has activity. The absorbance mean values of the reaction mixture containing P11G5 was slightly higher than P11f6 and that of the other reaction mixture Fig. 18, suggesting reaction mixture containing P11G5 had more colour intensity. Reaction mixture containing P2G3 had a slightly lower absorbance than P11G3 reaction mixture, suggesting lower colour intensity in comparison with P11G3. However, *E. coli* BL21 DE3 without recombinant plasmid did not show any colour change and the absorbance values remained constant as shown in Fig.18 and Fig.19 respectively.

4.4 Expression of laccase genes in psychrophilic *Pseudomonas*

4.4.1 Sub-cloning of laccase genes into pHH100

As stated earlier the pETite cloning vector utilised in cloning and expression of the laccase genes in *E. coli* lacks conjugative functions and cannot also replicate in *Pseudomonas*. Therefore, the pETite vector cannot be used to introduce the laccase genes into psychrophilic *Pseudomonas* Psm26D7 by conjugation. Hence a mobilizable plasmid, based on plasmid pHH100_mcherry was constructed to allow introduction and expression of laccase genes in psychrophilic *Pseudomonas* Psm26D7 strain.

The recombinant DNA constructs of P11f6, P11G3, P11G5 and P2G3 earlier created in *E. coli* DH5α were amplified by PCR by same set of primers (Appendix A). The plasmid pHH100 was also PCR amplified by different primers (Appendix A). The PCR products of recombinant DNA constructs were sub-cloned into pHH100 resulting in a mobilizable plasmid harbouring the laccase genes. The plasmid pHH100 construct containing the laccase gene P11G5 is shown in Fig.8.

4.4.2 Induction of protein expression in psychrophilic *Pseudomonas* Psm26D7 transconjugants

The miniprep DNA of pHH100 containing laccase genes P11f6, P11G3, P11G5 and P2G3 were transformed into *E. coli* S17.1, followed by transfer of the recombinant DNA constructs to psychrophilic *Pseudomonas* Psm26D7 by conjugation. The *Pseudomonas* Psm26D7 transconjugants containing the laccase genes were then induced for protein expression by adding to a final concentration, 0.5 mM m-Toluic acid and 1 mM IPTG respectively.

Psychrophilic *Pseudomonas* Psm26D7 strain was designed to include a XylS/Pm system. The gene which encodes the T7 RNAP is under transcriptional control of Pm promoter. The XylS/Pm system requires the presence of an inducer such as a Benzoic acid derivative (m-Toluic acid) to transcribe genes placed under the control of Pm promoter. The laccase genes were cloned under the transcriptional control of inducible T7 promoter of pETite cloning and expression vector, which requires IPTG for induction. Hence it was necessary to induce psychrophilic *Pseudomonas* Psm26D7 transconjugants with both m-Toluic acid and IPTG to activate the expression of genes under the control of Pm and T7 promoters respectively.

The psychrophilic *Pseudomonas* Psm26D7 transconjugants harbouring laccase genes were induced for protein expression by cultivating expression cultures at different temperatures and

incubation periods. SDS-PAGE analysis of the samples harvested from the induced expression cultures showed that cultivation of induced psychrophilic *Pseudomonas* Psm26D7 transconjugants at room temperature and incubated for 48 hours had high protein expression levels for three of the laccase genes (Fig.25). This is evident in specific thick bands detected in lane 15 (P11G3), lane 16 (P11G5), lane 17 (P2G3) Fig.25. Corresponding expression cultures induced at room temperature and incubated overnight showed lower expression levels as shown by weaker bands in lane 7: P11G3, lane 8: P11G5, lane 9: P2G3, Fig.24.

Evaluation of the solubility of the expressed recombinant laccase proteins indicates that, the proteins were insoluble as shown in Fig. 26. As stated earlier, the psychrophilic *Pseudomonas* Psm26D7 had the shortest generation time when cultivated at 30^{o}C compared with cultivation at 5^{o}C, 10^{o}C & room temperature respectively. However, induction of the expression cultures at 30^{o}C did not yield any detectable expression levels as shown in lane 2-9, Fig.25. Expression cultures induced at 10^{o}C were accompanied with low expressions levels, even when the cultures were incubated for extended periods. Cultivation of the induced expression cultures at 10^{o}C resulted in very low cell density when OD_{600} was measured after overnight incubation. Hence the incubation period of the expression cultures was extended to 72 hours and then 4 days respectively. However extended incubation period did not improve the expressions levels. Extended incubation periods may have its own disadvantage in that, if the cells enter a stationary phase during growth, already expressed proteins maybe degraded.

SDS-PAGE analysis of samples harvested from expression cultures induced at 10^{o}C (overnight incubation) indicates very weak and indistinct bands in lane 9 (P11G5) & 10 (P2G3) Fig.21. Expression cultures induced at 10^{o}C and incubated for 72 hours and 4 days respectively shows the presence of specific bands as shown by the SDS-PAGE gel image in Fig.22 & 23 respectively. In each case, the expressed recombinant proteins were found to be insoluble.

Analysis of the copper supplemented induced expression cultures of psychrophilic *Pseudomonas* Psm26D7 transconjugants indicates expression of P11G3 and P2G3 as shown by the presence of thick bands in lane 9 & 10 of Fig.27. However, the expressed proteins were observed to be in the insoluble fraction as shown by SDS-PAGE gel image in Fig.28.

A phenotypic comparison of protein expression levels in psychrophilic *Pseudomonas* Psm26D7 transconjugants induced and cultivated at different temperatures shows that, expression cultures induced at room temperature with 48 hrs incubation had the best or highest expression levels compared with incubation at other temperatures utilised in this study.

4.5 Comparison of protein expression levels in *E. coli* and psychrophilic *Pseudomonas* Psm26D7

A comparison of protein expression levels in both expression hosts would have been more accurate by quantification of the expressed proteins. As stated previously it was not possible to quantify protein expressions in *E. coli* BL21 (DE3) with image lab software, because the SDS-PAGE gel images were taken with a scanner. However, relative quantification of protein was possible with recombinant proteins expressed in *Pseudomonas* Psm26D7 with the image lab software (Biorad) (data not shown). Hence the comparison would be based on observable phenotype.

Expression of the four laccase genes in *E. coli* BL21 (DE3) cultivated at 37^{o}C and 10^{o}C respectively yielded expression of recombinant laccase proteins P11f6 and P11G5. There was no detectable expression of P11G3 and P2G3 in *E. coli* BL21 (DE3).

Expression of the laccase genes in psychrophilic *Pseudomonas* Psm26D7 incubated at room temperature yielded overexpression of P11G3, P11G5 and P2G3 recombinant laccase proteins. There was no visible overexpression of P11f6 laccase gene in *Pseudomonas* Psm26D7. Low level expression of the laccase genes P11G5 and P2G3 were seen in *pseudomonas* Psm26D7 cultivated at 10^{o}C. Cultivation of the psychrophilic *Pseudomonas* Psm26D7 transconjugants at 30^{o}C did not yield protein expression, despite having a high growth rate at this temperature.

It seems the different induction system utilised in both expression host strains favours the expression of individual laccase genes. IPTG induction of protein expression in *E. coli* BL21 (DE3) yielded only P11f6 and P11G5. While IPTG and m-Toluic acid induction of protein expression in psychrophilic *Pseudomonas* Psm26D7 which has the XylS/Pm system integrated in its chromosome yielded P11G5, P11G3 and P2G3. Both P11G3 and P2G3 laccase genes were not previously expressed in IPTG induced *E. coli* BL21 (DE3). The P11f6 laccase gene was not expressed in *Pseudomonas* Psm26D7.

But with the addition of copper chloride to expression cultures of *E. coli* BL21 (DE3) laccase genes P11G3 and P2G3 that were not previously expressed seem to have been expressed, albeit at low levels. However, the same cannot be said of copper supplemented cultures of *Pseudomonas* Psm26D7 as P11f6 laccase gene was not expressed.

Expression of the laccase genes in both hosts also seem to be temperature dependent, cultivation of both expression hosts at 10^{o}C after induction yielded high expression levels of only P11f6 in *E. coli* BL21 (DE3), while *Pseudomonas* Psm26D7 yielded low levels of P11G5, P2G3 and P11f6 was not expressed.

P11f6 laccase gene showed a higher protein expression levels when *E. coli* BL21 (DE3) was cultivated at 10^{o}C, while P11G5 had a high expression levels at 37^{o}C cultivation of *E. coli* BL21 (DE3). Cultivation of *Pseudomonas* Psm26D7 transconjugants at room temperature with 48 hours incubation yielded higher expression levels of P11G3, P11G5 and P2G3 recombinant laccase proteins in comparison with expressions at other temperatures.

5 Conclusion

Laccases are multicopper oxidase enzymes which have a lot of potential applications in the biotechnological industry. These biotechnological applications include in industries, such as the food, textile, bioremediation, pulp and paper industries and others.

This thesis was aimed at cloning and expression of four laccase genes designated, P11f6, P11G3, P11G5, P2G3 isolated from four different *Psychrobacter* strains. *E. coli* BL21 (DE3) and psychrophilic *Pseudomonas* Psm26D7 were utilised as expression hosts.

Expression of proteins from recombinant DNA ensures large quantity of the proteins are produced in the expression host strain compared with small amount found in the original source organism.

The four laccase genes were expressed in both expression hosts at varying temperatures and incubation periods, albeit in different combinations. P11f6 and P11G5 laccase genes were expressed in *E. coli* BL21 (DE3), while P11G3, P11G5 and P2G3 were expressed in psychrophilic *Pseudomonas* Psm26D7. Though the laccase genes were expressed in both expression host strains in different proportions, solubility of the expressed proteins seem to be an unresolved issue even with low temperature cultivation of both expression strains. However, copper supplemented expression cultures of *E. coli* BL21 (DE3) transformants yielded small amount of soluble proteins.

Previous studies have demonstrated that copper enhances the activity and proper folding of laccases obtained from bacterial sources (Durao et al., 2008, Malhotra et al., 2004). Copper ions are needed by laccases for enzymatic activity and addition of copper ions to expression cultures may produce soluble active proteins (Ihssen et al., 2015). However copper chloride supplemented induced *E. coli* expression culture cultivated at 15^{o}C yielded low level expression of proteins. Solubility test of proteins from copper supplemented cultures yielded low amount of soluble proteins.

Expression of psychrophilic or cold adapted genes in mesophilic host such as *E. coli* is often difficult, due to the optimum growth temperature of *E. coli* at 37^{o}C. psychrophilic or cold adapted enzymes are heat labile, therefore expression of the genes encoding psychrophilic enzymes in a mesophilic host optimum temperature may affect the proper folding of the expressed proteins or denaturation of the proteins (Feller et al., 1997, Margesin et al., 2008). Previous reports suggest that low temperature cultivation of *E. coli* expression strain can improve soluble protein production (Sørensen and Mortesen, 2005). However, this was not the case in this study, as low temperature cultivation of *E. coli* BL21 (DE3) harbouring the

recombinant plasmid at suboptimal temperatures yielded insoluble proteins. Even expression of the laccase genes in psychrophilic *Pseudomonas* Psm26D7 yielded insoluble proteins.

Solubility of recombinant protein production in *E. coli* can be improved by several ways which include co-expression of molecular chaperones which improves the proper folding of the proteins. Cultivation of *E. coli* at low temperature by co-expression of chaperones Cpn60 and Cpn10 from a psychrophilic bacterium have been shown to improve activity and proper folding of recombinant proteins by allowing growth of *E. coli* at suboptimal temperature (Ferrer et al., 2004). The use of fusion proteins such as maltose binding proteins, thioredoxin can also enhance solubility of recombinant proteins (Sørensen and Mortesen, 2005).

Static cultivation of expression cultures supplemented with copper after induction have also been shown to improve solubility of laccases from bacterial sources (Durao et al., 2008). However, copper was supplemented to expression cultures in this study, but the static cultivation condition was not utilised due to limited time.

Further work needs to be done to improve the solubility of the recombinant laccase proteins to obtain more soluble and active proteins. This may include as stated above, by co-expression of molecular chaperones and use of fusion proteins.

The copper supplementation of the expression cultures can be tested further (static cultivation) by using concentrations of copper chloride, lower than 0.5mM utilised in this study to improve solubility of the proteins, as higher concentrations can be toxic to cells. This can be done in conjunction with, utilising low concentrations of both inducers (IPTG and m-Toluic acid) in the different expression hosts.

The use of a different culture media may also be an alternative to improve yield and solubility of the recombinant proteins, however Terrific broth, an enriched medium was used in this study (data not shown) to cultivate *E. coli* expression host and the outcome was consistent with the use of LB medium.

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Appendices

Appendix A: Primers

Table 9: shows all primers used for PCR reactions in this thesis

Primers	Sequence	Use
P11f6-F	GAAGGAGATATACATATGATTCATCAGCTTCCTATGACATTG	Amplification of P11f6 gene
P11f6-R	GTGATGGTGGTGATGATGCGCAGCGCCACGTACAATGAC	
P11G3-F	GAAGGAGATATACATATGACGAACACGTTCCCGATGACAATG	Amplification of P11G3 gene
P11G3-R	GTGATGGTGGTGATGATGATGGCGACGGACGATGACCAGTGCCATC	
P2G3-F	GAAGGAGATATACATATGCTGGCACAGCTTGATGATGTGAC	Amplification of P2G3 & P11G5 genes
P2G3-R	GTGATGGTGGTGATGATGAATGGCTAAACTGCGCACGACAATC	
pETite-F	TAATACGACTCACTATAGGG	Sequencing & colony PCR
pETite-R	CTCAAGACCCGTTTAGAGGC	
pHH100-F	TAAACGGGTCTTGAGGGGTTGGATCCTCTAGCTAGAGTCA	Amplification of plasmid pHH100
pHH00-R	TAATTGCGTTGCGCTCACTGGACGAAAGGGCCTCGTGATA	
pETite-F	TATCACGAGGCCCTTTCGTCCAGTGAGCGCAACGCAATTA	Amplification of four recombinant laccase DNA
pETite-R	TGACTCTAGCTAGAGGATCCAACCCCTCAAGACCCGTTTA	

Appendix B: Sequencing result

The sequencing results were analysed by Multiple sequence alignment with hierarchical clustering (MultAlin.com). The reference DNA was designated WT and laccase gene sequences from sequencing results were designated P11f6, P11G3, P11G5 and P2G3 respectively. The red areas indicate a match between the reference DNA and sequencing results of the sequenced laccase genes, which signifies the coding region. The dark areas flanking the coding regions are flanking sequences from the pETite plasmid. The images of multiple sequence alignments are presented as follows:

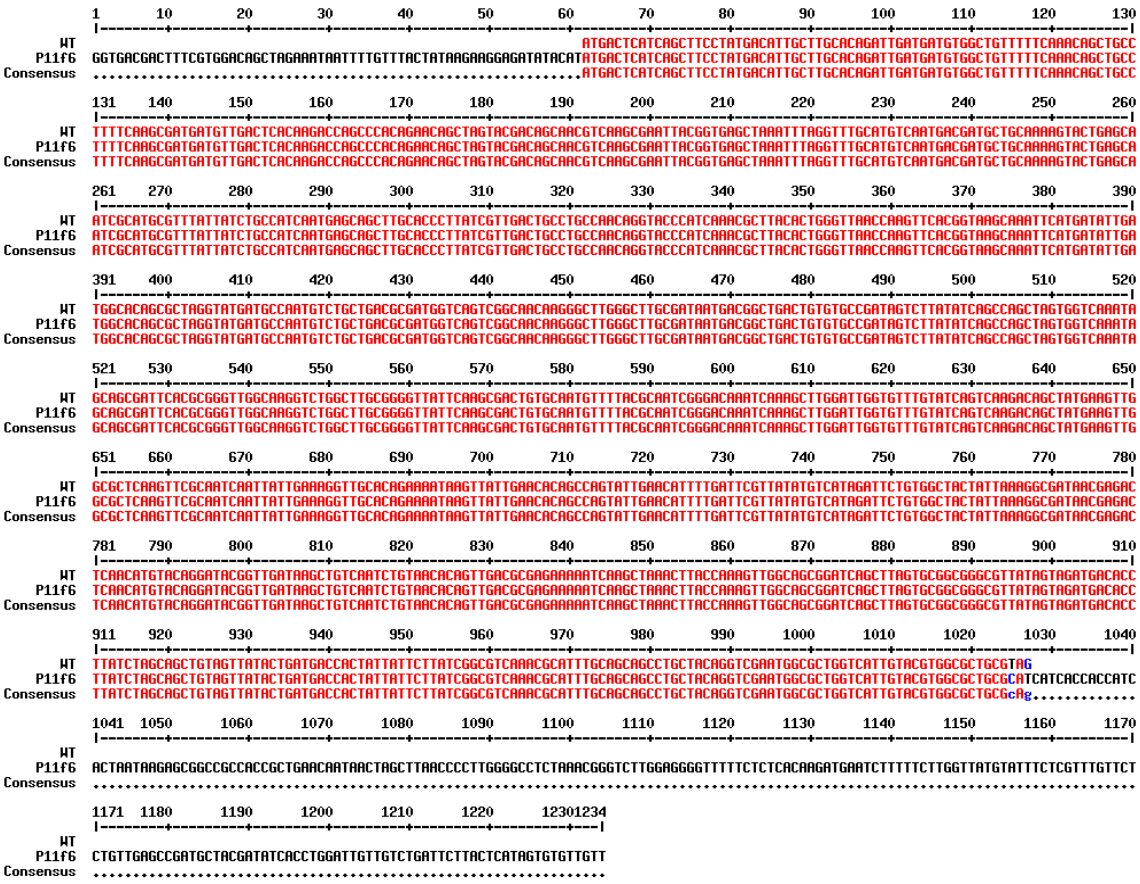

Fig. B-1: P11f6 sequences analysed with multiple sequence alignment (MultAlin)

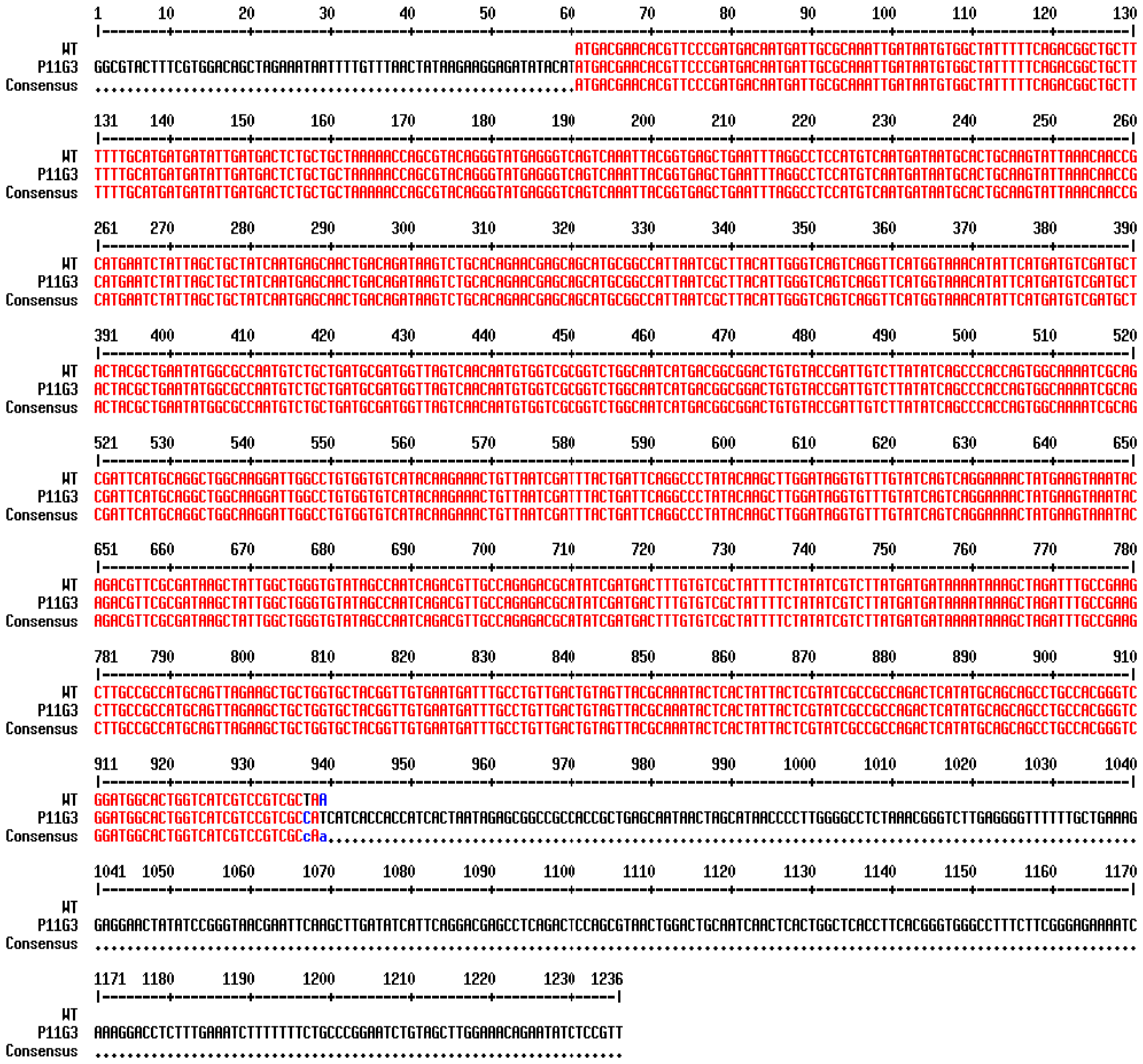

Fig.B-2: P11G3 sequences analysed with multiple sequence alignment (MultAlin)

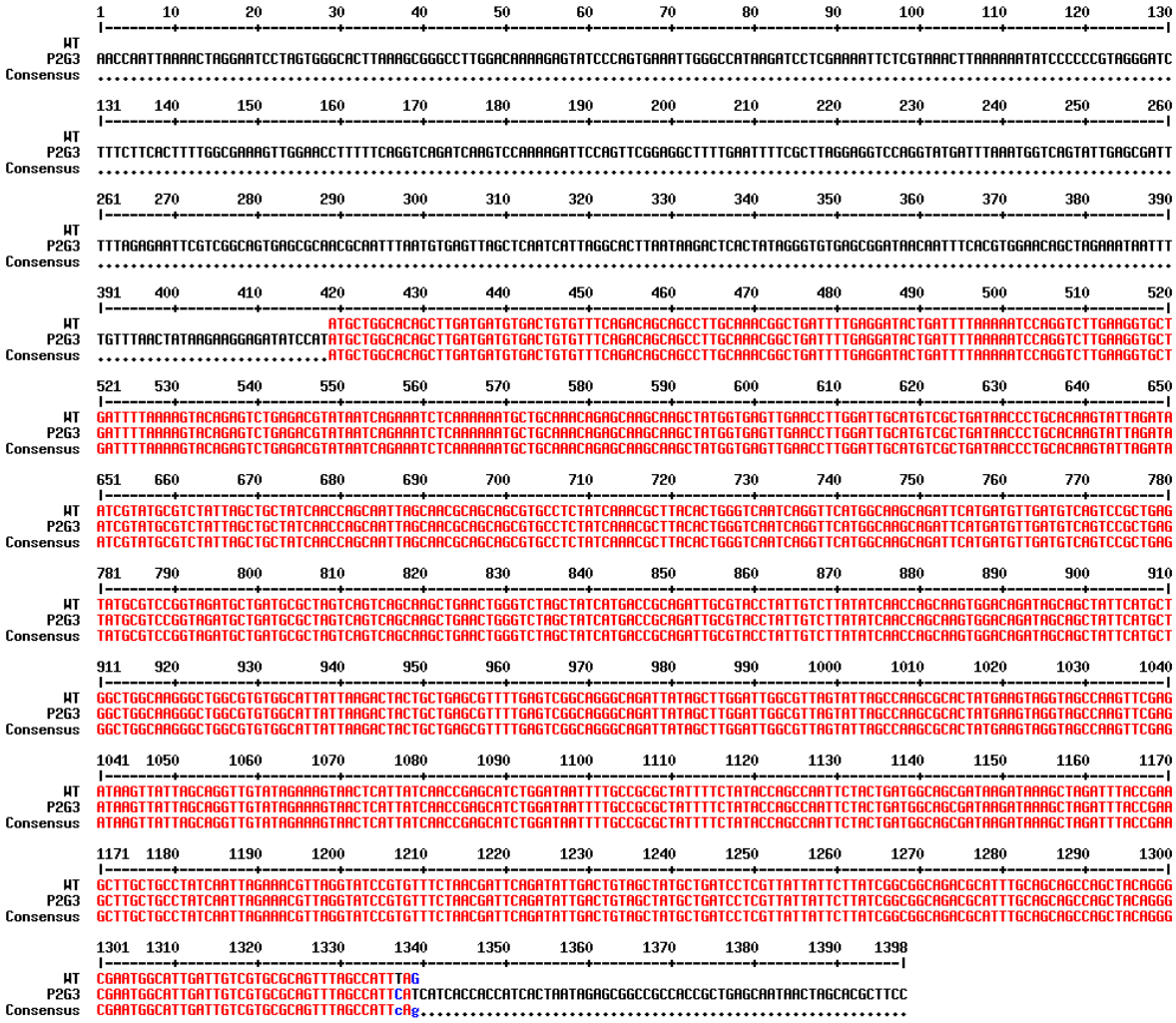

Fig.B-3: P2G3 sequences analysed with multiple sequence alignment (MultAlin)

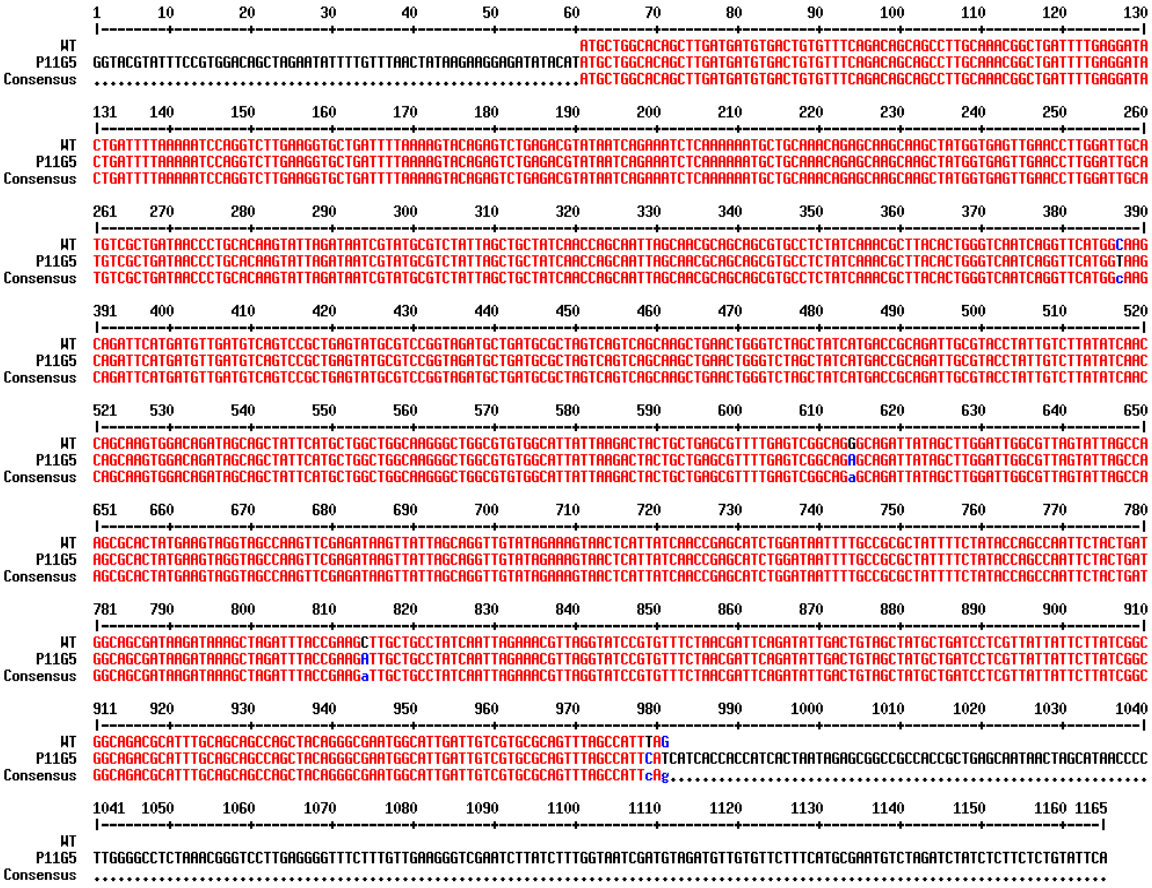

Fig.B-4: P11G5 laccase gene sequences analysed with multiple sequence alignment (MultAlin) showing the differences in 3 sequences between P2G3 and P11G5. The 3 sequences are found in the sequences between row 380-390, 610-620 & 810-820 respectively.

Appendix C: Protein ladder

Protein ladder were used to estimate the expected molecular weights of the recombinant proteins expressed in this study by SDS-PAGE analysis. Unstained protein ladder (New England Bilolabs) and Precision Plus Protein Prestained standards in Dual colour (Biorad) were used in this study.

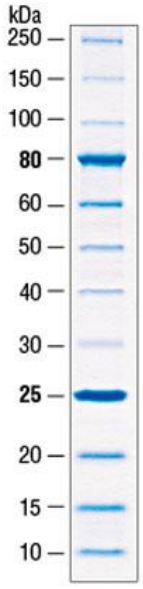

Fig.C-1: Unstained protein ladder (NEB), Broad range (10-250 kDa)

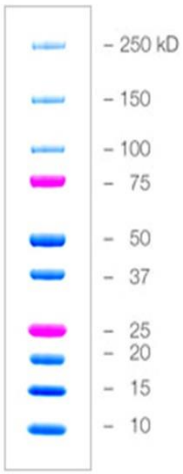

Fig.C-2: Prestained Precision Plus Protein standards (Biorad), Broad range (10-250 kDa)

Appendix D: Agarose gel electrophoresis

1kb GeneRuler DNA ladder was used as a reference during verification of the PCR products by agarose gel electrophoresis.

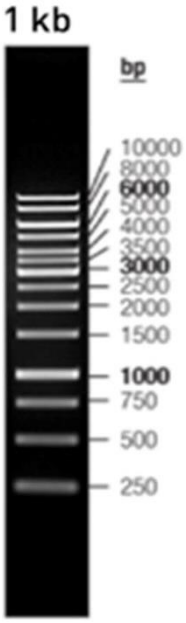

Fig.D-1: 1kb GeneRuler DNA ladder