Cell surface display of palladium binding peptide on *Saccharomyces cerevisiae* EBY100 cells using the a-agglutinin anchor system developed for the biosorption of Pd (II)

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ABSTRACT

Platinum group metals (PGMs) are concentrated in the earth's core and mantle and have low natural abundances. Due to the limited availability of PGMs; their recovery and reuse from waste solutions is economically attractive. Conventional methods used for the removal of PGMs from effluents include precipitation, ion exchange, and reverse osmosis; these methods have many shortfalls and this had led to a search for new technologies for the removal of metals from effluents, one such technology is biosorption. The primary objective of this research project was to develop a genetically engineered *Saccharomyces cerevisiae* EBY100 strain for the biosorption of Pd(II) in aqueous solutions. The palladium binding peptide was successfully expressed on *Saccharomyces cerevisiae* EBY100 cells by means of cell surface display using the a-agglutinin anchor system; this was confirmed by means of DNA sequencing, flow cytometry and microscopy. The maximum adsorption capacity of genetically transformed *Saccharomyces cerevisiae* EBY100 cells for Pd(II) was 125mg/g.

Keywords: Cell surface display; Saccharomyces cerevisiae EBY100; pYD5 plasmid vector; Biosorption; Palladium; Flow cytometry; Fluorescence microscopy

INTRODUCTION

Precious metals are widely used in various industries due to their physical and chemical properties (Ramesh *et al*., 2008; Won *et al*., 2010); this group of metals include gold, silver, platinum and palladium. However, their demand and supply is not balanced due to their limited availability (Wolowicz and Hubicki, 2009; Marinho *et al*., 2011). Their limited availability makes the recovery of precious metals from waste solutions economically attractive (Won *et al*., 2010).

Industrial recycling techniques have been widely used in the recovery of precious metals from wastewaters (Jacobsen 2005; Won *et al*., 2010). These techniques include adsorption by ion exchange resin, solvent extraction as well as reduction of the precipitate of metals by reagents (Won *et al*., 2010). These techniques have the following disadvantages: they are very costly, require extensive labour and time and they generate large quantities of secondary waste (Won *et al*., 2010). The above mentioned shortfalls of these methods have led to pursuit for new technologies for the recovery of precious metals from waste solutions which are of low cost, eco-friendly and generate little secondary waste (Won *et al*., 2010). One such technology is biosorption.

Biosorption is a process that employs living and dead biomass, including microorganisms. The process of biosorption is dependent on the components of the cell, especially the cell surface and the spatial structure of the cell wall (Wang and Chen, 2009). The cell surface and cell wall consist of a complex network of various binging sites with diverse affinities for metal ions (Wang and Chen, 2009; Malejko *et al*., 2012). However; there is still a need to further research and develop biosorption systems which are more improved. Improvements can be through the use of genetic engineering; by the display of GEPI in the cell wall of biomass which bind to specific metals.

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GEPI can be defined as a sequence of amino acids that specifically and selectively bind to inorganic surfaces (Sarikaya *et al*., 2003). The most common screening method for identifying and isolating GEPI is by means of biopanning. Biopanning is the most common screening

method for identifying and isolating GEPI. GEPI are commonly biopanned using phage display (PD) (Sarikaya *et al*., 2003).

In this method, a large number of random peptides are screened for their inorganic binding properties using the combinatorial approach. Thereafter these peptides get displayed onto surfaces of bacteriophages (Brown, 1997). These are then exposed to the target compound and those that recognize and adhere to the target compound are recovered and analyzed (Brown, 1997). This process gets repeated several times to enrich specificity and high affinity binders (Evans *et al*., 2008). At the end of the process, the amino acid sequences of these GEPI become deduced by DNA sequencing and thereafter get catalogued (Evans *et al*., 2008).

The display of GEPI on phages or cells has a wide range of applications; this includes their application in the selectivity to bind to a wide variety of metals. Stanley Brown is one of the pioneers on the studies of metal-binding peptides. He performed one of the earliest experiments of isolating metal-binding peptides; by isolating gold (Au) binding peptides from a bacterial combinatorial peptide library (Brown, 1997; Kriplani and Kay, 2005). Since then, many more peptide sequences specific to other metals have been identified; these include peptide sequences specific to silver (Ag) (Naik *et al*., 2002; Naik *et al*., 2004; Kriplani and Kay, 2005), platinum (Pt) and palladium (Pd) (Sarikaya *et al*., 2003; Kriplani and Kay, 2005) amongst others. To date, little or no studies have been reported on GEPI which binds to platinum expressed on the surface of Saccharomyces cerevisiae. In light of this, this study was focused on the display of a platinum-binding peptide on the surface of *Saccharomyces cerevisiae*.

The palladium binding peptide (PdBP) represented by the peptide sequence SVTQNKY used in this study was obtained from literature (Sarakiya *et al*. 2003). Sarakiya *et al*. (2003) expressed the PdBP onto phages, in contrast to their study; in this study the PdBP were displayed onto the cell surfaces of *Saccharomyces cerevisiae* EBY100 cells using the a-agglutinin protein display system.

The a-agglutinin protein display system is the most common yeast display system which was pioneered by Boder and Wittrup (Shibasaki *et al*., 2009). The a-agglutinin is present on the cell wall of a-type mating yeast cells and is used as a mating adhesion receptor protein; this protein has two subunits, Aga1 and Aga2 which are encoded by the *aga1* and *aga2* genes respectively (Zhao *et al*., 2001). The a-agglutinin display system utilizes *Saccharomyces cerevisiae* EBY100 cells which contain the Aga1 gene under the regulation of the GAL 1 promoter and a pYD plasmid vector encoding the Aga2 gene which is fused to the gene of interest and an epitope (Li *et al*., 2014). After the *Saccharomyces cerevisiae* EBY100 cells have been transformed with the pDY1 plasmid vector which contains the protein of interest, the cells become induced with galactose. Induction by galactose activates the galactose inducible GAL 1 promoter which in turn leads to the expression of the protein of interest fused to the epitope and the Aga2 protein which is linked by two disulphide bonds to the Aga1 protein anchored covalently to the cell wall of *Saccharomyces cerevisiae* EBY100 cells by a glycosylphosphatidylinositol (GPI) anchor (Li *et al*., 2014); this is illustrated in figure 1.

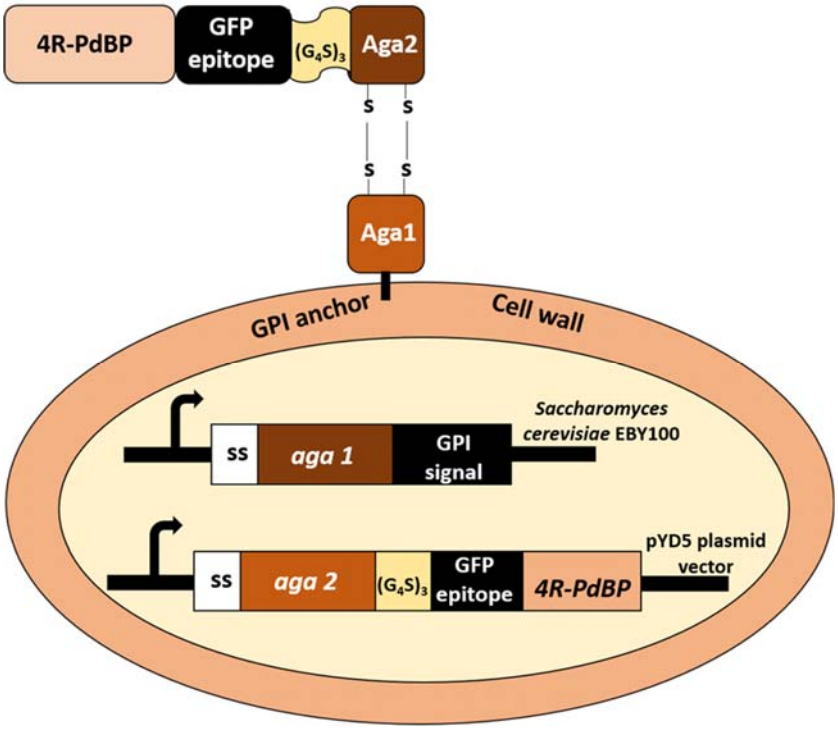

Figure 1: The principle of surface display based on the a-agglutinin system, utilizing Saccharomyces cerevisiae EBY100 and pYD5 plasmid vector. The metal binding peptide, 4R-PdBP was cloned in-frame with aga2 gene in the pYD5 plasmid vector and introduced into Saccharomyces cerevisiae EBY100 that overexpressed the aga1 gene. The resulting proteins, Aga1 and Aga2-4R-PdBP, get connected by disulfide bonds in the secretion process and are exported together on the surface of the cell where Aga1 remains attached to the cell wall by the glycosylphosphatidylinositol (GPI) anchor. Adopted from Borodina *et al*. 2010.

Two molecular tailoring strategies were incorporated into modifying PdBP for this study; this was for purposes of stabilizing the peptide. According to the classical chain entropy model a disulfide bond is said to stabilize the native protein by reducing entropy of its unfolded state (Pace *et al*., 1988; Hagihara *et al*., 2002). Inserting a pair of cysteine residues to a protein results into formation of disulphide bonds; (Darby and Creighton, 1995); it is for this reason that a pair of cysteine residues were added to the PdBP in this study. Another manner in which the stability of peptides can be enhanced is by means of oligomerization. According to Sinha and Surolia (2005), oligomerization enhances the conformational stability of a protein; this was indicated in results obtained in their study whereby tetramerization endowed a protein far greater conformational stability compared to its monomer counterpart. The PdBP in this study was tetramerized to form 4R-PdBP for purposes on enhancing the conformational stability of the peptide.

In this study; 4R-PtPB was expressed on the surface of *Saccharomyces cerevisiae* EBY100 using the yeast display vector, pYD5 which was developed from pYD1 by Wang *et al*. (2005). According to Wang *et al*. (2005); the affinity in the pYD5 vector is increased by 3-fold as compared to pYD1; this was achieved by reconstruction of the pYD1 vector to increase affinity to protein of interest by leaving the N-terminus of the heterologous protein free, rather than having the heterologous protein being sterically hindered.

MATERIALS AND METHODS

Peptides, Yeast, Bacterial and Plasmids used in this study

The palladium binding peptide (PdBP) sequence (SVTQNKY) used in this study was obtained from literature (Sarakiya *et al*. 2003). The modified 4R-PdBP which was tetramerized and had cysteines added to it was procured from Blue Heron Biotech (USA). *Saccharomyces cerevisiae* EBY100 cells (*MATa AGA1::GAL1-AGA1::URA3 ura3-52 trp1 leu2Δ1 his3Δ200 pep4::HIS3 prb1Δ1.6R can1 GAL*) were procured from American Type Culture Collection, USA (ATCC). NEB® 5-alpha competent *Escherichia coli* cells (*fhuA2* (*argF-lacZ)U169 phoA glnV44 80 (lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17*) were procured from New England Biolabs. The pYD5 is a yeast display vector that was modified by Wang *et al*., (2005) from the commercial pYD1 yeast display vector and it was obtained from Prof Oliver Kerscher

(College of William and Mary Williamsburg, VA, USA). The pYD5 was further modified to replace the V5 epitope fluorescent protein with the green fluorescent protein (GFP); the pYD5 plasmid vector was sent to Blue Heron Gene Synthesis Company (USA) to replace the V5 epitope with Blue Heron Biotech's generic GFP epitope as a reporter gene.

Plasmid construction

Restriction enzymes *EcoRI* and *NheI* procured from Thermo Fisher Scientific were used to cut the 4R-PdBP to its proper sequence. A mixture containing the following was made: 2 µL of NEB Buffer 1 (10x), 0.5 µL of BSA (100x), 1 µL of *NheI* (10U/µL), 1 µL of *EcoRI* (10U/µL), and 16 µL of 4R-PdBP. The mixture was placed in a 37 °C water bath for 2 hours. The 4R-PdBP was then ligated into pYD5 yeast display vectors in accordance to the protocol DNA ligation with T4 DNA Ligase (1U/µL) from Thermo Fisher Scientific. The resulting successfully ligated product was termed pYD5/4R-PdBP.

The ligation products were transformed to DH5α *Escherichia coli* cells using the High Efficiency Transformation protocol from New England Biolabs. Recombinant DH5α *Escherichia coli* cells were then spread onto LB plates (1.0% Tryptone, 0.5% Yeast Extract, 1% NaCl, and 100µg/ml of ampicillin). The plates were placed in a 37°C incubator overnight. After overnight incubation at 37°C, single colonies were isolated and mixed with 10 µL of nuclease-free water. 1 µL of the solution was then used as a template for bacterial colony PCR and sequencing. For 50µL reactions: 10 µL of 5x Phusion HF buffer, 1µL of 10 mM dNTPS, 2.5 µL of 10 µM forward and reverse primers, 1 µL of template DNA, 0.5 µL of Phusion DNA polymerase, and 32.5 µl of nuclease free water were added. Cycling conditions were as follows: 1 cycle of initial denaturation at 98 °C for 30 seconds, 35 cycles of 98 °C for 10 seconds, 45 °C for 30 seconds, and 72 °C for 1 minute, and 1 cycle of 72 °C for 10 minutes. Two pairs of forward (PdBPF1, PdBPF2) and reverse (PdBPR1, PdBPR2) primers used in this study are indicated in table 1. The PCR products were analysed by means of gel electrophoresis (100 volts for 40 minutes) using 1x TAE buffer, 0.8% agarose, and ethidium bromide solution. PCR products were loaded alongside a DNA ladder mix (Fermentas, St. Leon-Rot, Germany). Samples were resolved by exposure to a potential difference of 95 V for 45 minutes and were visualised on a UV Transilluminator. Pureyield Plasmid Miniprep (Promega)was utilized to isolate the plasmid DNA from

recombinant DH5α *Escherichia coli* cells. The isolated plasmids were then digested enzymatically using *EcoRI* and *NheI* (Thermo Fisher Scientific) to linearize the plasmid DNA. The samples were sent to the Inqaba Biotec (South Africa) for sequencing to confirm positive clones.

Table 1: Primers used in this study

Primer ID	Sequence
PdBPF1	5'CCAGCATCCTGCAGAAC3'
PdBPF2	5'GATGCCGGTGAAGAAAGAG3'
PdBPR1	5'GATGCCGGTGAAGAAAGAG3'
PdBPR2	5'CTAAAAGTACAGTGGGAACAAAGTC 3'

Transformation of *Saccharomyces cerevisiae* EBY100 cells with pYD5/4R-PdBP

pYD5/4R-PdBP DNA was electroporated into *Saccharomyces cerevisiae* EBY100, in accordance with the S.c. EasyComp™ Kit (Thermo Fisher Scientific) protocol; however with a few variations. Four different transformation conditions were conducted to determine the optimal transformation conditions for *Saccharomyces cerevisiae* EBY100 cells with pYD5/4R-PdBP as well as the optimal conditions for the expression of the 4R-PdBP in transformed *Saccharomyces cerevisiae* EBY100 cells; this was achieved by varying plasmid DNA concentration as well as excluding or including the utilization of carrier DNA. UltraPure™ Salmon Sperm DNA (Thermo Fisher Scientific) was utilized as the carrier DNA. The four different transformation conditions which were conducted for the *Saccharomyces cerevisiae* EBY100 cells with pYD5/4R-PdBP (P4) were as follows: (1) 50 ng plasmid DNA with 50 µg carrier DNA (P4.1), (2) 50 ng plasmid DNA no carrier DNA (P4.2), (3) 100 ng plasmid DNA with 50 µg carrier DNA (P4.1C) and (4) 100 ng plasmid DNA with no carrier DNA (P4.2C). The electroporated *Saccharomyces cerevisiae* EBY100 cells were spread onto Minimal Dextrose (MD) plates that contained 0.01% leucine. The plates were incubated at 30°C for three days to allow for single colonies to grow. After the third day of incubation, two colonies were picked and were inoculated into 10ml YNB-CAA growth medium containing 2% glucose as a carbon source. Flasks were placed in a shaking incubator at 30 °C at 150rpm overnight until cell cultures reached OD600 readings of between 2 and 5.

Induction of the expression of 4R-PdBP in transformed *Saccharomyces cerevisiae* EBY100 cells and growth characterization of the transformed cells

The successfully transformed *Saccharomyces cerevisiae* EBY100 cells which were initially grown in YNB-CAA growth medium containing 2% glucose as a carbon source were centrifuged at 3000 x g for 10 minutes at room temperature. The supernatant was discarded and the cell pellets were resuspended in YNB-CAA medium containing 2% galactose to induce the expression of the 4R-PdBP. The cells were incubated in a shaking incubator at 20°C at 200rpm. Cell density readings were taken using a UV/VIS spectrophotometer at OD600. OD600 readings should be between 0.5 and 1, to ensure that the cells continue to grow in log-phase. Cell cultures were assayed over a 48-hour time period; at 0, 12, 24, 36 and 48 hours to determine the optimal induction time required for maximum display of the 4R-PdBP in *Saccharomyces cerevisiae* EBY100 cells. For each time point, OD600 readings were taken and a volume of cells that is equivalent to 2 OD600 units was taken. The samples were stored at 4°C until all time points are collected.

Flow cytometric and fluorescence microscopic analysis of GFP-tagged 4R-PdBP expressed in surface engineered *Saccharomyces cerevisiae* EBY100 cells

The gene encoding the green fluorescent protein (GFP) was fused to 4R-PdB and assembled into the GAL expression cassette; this was to allow for the expression of 4R-PdB in *Saccharomyces cerevisiae* EBY100 to be detected by flow cytometry and visualized by fluorescence microscopy**.** Cell cultures were assayed over a 48-hours at the following intervals: 0, 12, 24, 36 and 48 hours to determine the optimal induction time required for maximum display of 4R-PdB in *Saccharomyces cerevisiae* EBY100. Cell samples were centrifuged at 3000 x g for 10 minutes at 4°C. The supernatants were discarded and the pellets were resuspended in 250 µL of 1X PBS. The cells were centrifuged again at 3000 x g for 10 minutes at 4°C, the supernatants were discarded and resuspended again in 250 µL of 1X PBS. For flow cytometry cells were transferred into a small culture tube and analyzed using the BD FACSAria (Becton Dickenson) cell sorter using the FITC channel. Wild-type *Saccharomyces cerevisiae* EBY100 cells were included as negative controls to provide the baseline from which the values for percentage of expression of 4R-PdBP in *Saccharomyces cerevisiae* EBY100 cells were calculated. The obtained flow cytometry data was analysed using FlowJo V10.
Fluorescence Microscopic analysis was performed for the cell population that had the highest expression levels of 4R-PdBP based on cytometry data. For fluorescence microscopy a small

aliquot of cells were spread onto a glass slide, covered with a coverslip and observed under the 40x magnification objective lens using the AxioVert A1 inverted fluorescence microscope (Carl Zeiss) equipped with an AxioCam Cm1 camera (Carl Zeiss). The excitation and emission wavelengths were set at 450–490 nm and 515–565 nm respectively. Images were enhanced by adjusting contrast and brightness and zooming in.

Preparation of dead biomass and modification of biomass by means of freeze drying

After determining the induction time required for maximum display of the metal binding peptides; cell cultures were induced again in accordance with the induction protocol stated above. The induced cells were centrifuged at 3000 x g for 10 minutes at 4°C. The supernatants were discarded and the pellets were resuspended in ultrapure water. The cells were centrifuged again at 3000 x g for 10 minutes at 4°C. The cells were autoclaved at 121°C for 5 minutes to obtain dead biomass and thereafter were immediately frozen at -70ºC overnight. The cells were then transferred to a freeze dryer (Martin Christ Alpha 3-4 LSCbasic) for 48hrs; in order to obtain fine powdered biomass. The freeze dried cells were then stored at freezer at -70ºC.

Preparation of stock solutions for biosorption studies

The stock solutions of Pd (II) (1000ppm) were prepared by dissolving measured quantities of PdCl2 (Merk) in ultrapure water. The stock solutions were diluted with ultrapure water to obtain the desired initial metal concentrations of 10mg/L, 20 mg/L, 30 mg/L and 50 mg/L. The pH levels of the solutions were adjusted using dilute HCl or NaOH.

Batch adsorption studies of Pd(II) by 4R-PdBP surface displaying *Saccharomyces cerevisiae* EBY100 cells

Batch experiments were conducted to determine the effects of pH, contact time, initial metal ions concentration and competing ions on the biosorption of Pd (II) by 4R-PdBP surface displaying *Saccharomyces cerevisiae* EBY100 cells. For the experiments, 0.1g of biomass was added to 500ml of metal solution in 1000mL Erlenmeyer flasks. The flasks were agitated on a rotary shaker (150rpm) at room temperature. 1mL samples were collected for analysis and stored at 4°C until all required time points were. The collected samples were analysed for metal concentration using Inductively Coupled Plasma mass spectrometry (ICP, Spectro Arcos

FHS12, Boschstroisse, Germany). The amount of metal ions adsorbed per unit biomass and the percentage of metal removal were obtained using equations (1) and (2) respectively:

$$q_e = \frac{(C_0 - C_e)\, x\, V}{m} \tag{1}$$

$$\%\, Adsorbed = \frac{(C_0 - C_e) x\, 100}{C_0} \tag{2}$$

where q_e is the amount of metal ion adsorbed onto the unit mass of the adsorbent (mg/g), C_0 and C_e are the initial and final equilibrium metal concentrations (mg/L), V is the volume (L) and m is the mass of the biosorbent (g).

Effect of contact time on the biosorption of Pd(II)

The effects of contact time on the biosorption were carried out at pH 3 with 0.1g of adsorbent dosage at room temperature using initial metal concentration of 10 mg/L. The studies were conducted over 48 hours and samples were collected at the following pre-determined time intervals: the first sample was collected after 5 min, thereafter samples were collected every 15 minutes for the first 3 hours, followed by a collection of samples every 30 minutes for 6 hours, 3 hours sample intervals followed for 12 hours and lastly samples were collected every 6 hours for the remaining 24 hours.

Effect of pH on the biosorption of Pd(II)

The effect of pH on the sorption was studied at pH 0.5 1, 2 and 3 with 0.1g of adsorbent dosage at room temperature, for 12hrs using initial metal concentration of 10mg/1. The first sample was collected after 5 min, thereafter samples were collected every 15 minutes for the first 3 hours, followed by a collection of samples every 30 minutes for 6 hours and the last samples were collected every hour for 3 hours.

Effect of initial ion concentration on the biosorption of Pd(II)

The effect of initial metal concentrations on the sorption was investigated by changing the initial metal ion concentrations to 10 mg/L, 20 mg/L, 30 mg/L and 50 mg/L with an adsorbent dosage of 0.1g, at pH 3 at room temperature for 8 hours.

RESULTS AND DISCUSSION

Plasmid construction

4R-PdBP was successfully ligated into the pYD5 vector to form 4R-PdBP/pYD5 and this was confirmed by colony PCR amplification and gel electrophoresis; data not shown. Sequence results obtained in this study representative of colony PCR containing 4R-PdBP/pYD5 (P4) are indicated in figure 2. These sequence results indicate that 4R-PdBP was successfully ligated into pYD5 vectors to form 4R-PdBP/pYD5 and the ligated plasmids were successfully transformed into NEB® 5-alpha (DH5α) *Escherichia coli* cells.

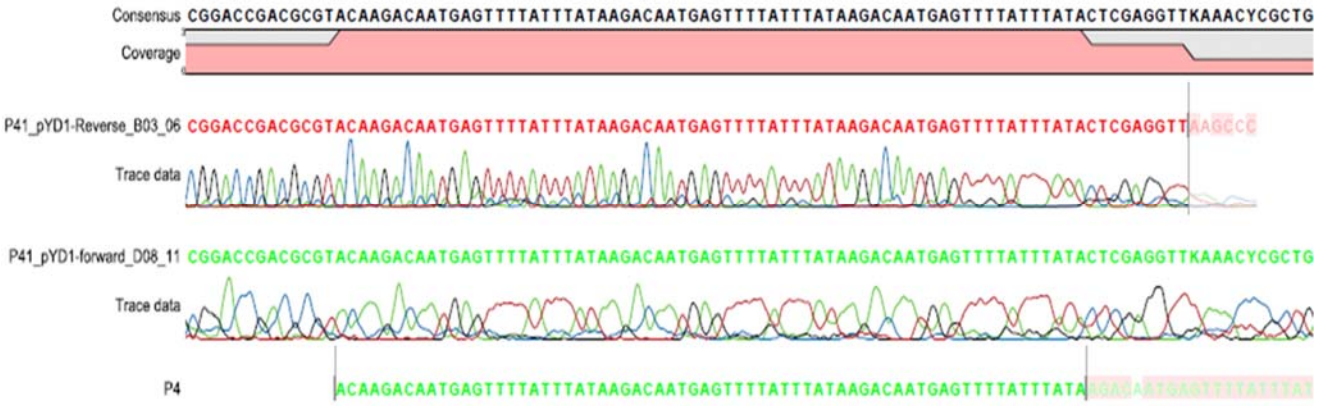

Figure 2: Sequence data obtained from colony PCR of NEB® 5-alpha (DH5α) *Escherichia coli* cells transformed with the 4R-PdBP/pYD5 plasmid.

Flow cytometric and fluorescence microscopic analysis of GFP-tagged 4R-PdBP in induced *Saccharomyces cerevisiae* EBY100 cells

It was evident from the results obtained from flow cytometry analysis confirmed that *Saccharomyces cerevisiae* EBY100 cells were efficiently transformed with pYD5/4R-PdBP

and successfully expressed 4R-PdBP in induced cells represented by transformants P4.1, P4.1C, P4.2 and P4.2C; as indicated from the histograms obtained from the FITC channel as indicated in figure 3. It was also evident from the histograms obtained from flow cytometry data that the expression of 4R-PdBP occurred immediately in transformants P4.1, P4.1C, P4.2 and P4.2C after being induced with galactose as indicated by the MFI data of GFP. Maximum GFP expression occurred within 12 hours of induction. Subsequent to reaching maximum GFP expression levels, the GFP expression levels declined throughout the remaining 60 hours; reasons behind this will be investigated further in future studies. Furthermore data obtained indicated that the P41C transformant produced a sub-population of cells with the highest transformation efficiency amongst P4 transformation variants; 10% of P41C sub-population cells were positive for GFP expression and the population had the highest copy number of the 4R-PdBP. The transformation mixture of P41C contained 50 ng plasmid DNA as well as 50 µg carrier DNA; this is an indication that carrier DNA did play a role in enhancing the transformation efficiency of P4 parental cells in this instance. These results are similar to those of studies conducted by Pham *et al*. (2011); their studies indicated that carrier DNA does increase transformation efficiency. It is evident and worth noting from the results obtained in this study that although the expression of 4R-PdBP by transformant P41C represented by the percentage of cells expressing GFP and the copy number of GFP in cells was successful and rapid, they were both not at optimal levels; therefore there is a need for optimizing the expression system to yield higher productivity in future studies. There are several strategies which can be utilized in future studies for purposes of optimizing the transformation efficiencies of the genes of interest as well as to increase the expression levels of the genes of interest; these include (1) engineering the host cell, (2) engineering recombinant DNA sequences and (3) optimizing the environmental / external parameters (Liu *et al*., 2013). These strategies may then result into an increase in the surface area available for biosorption and may thus lead to an increase in the biosorption efficiency, rate and capacity of the transformant P41C for Pd(II).

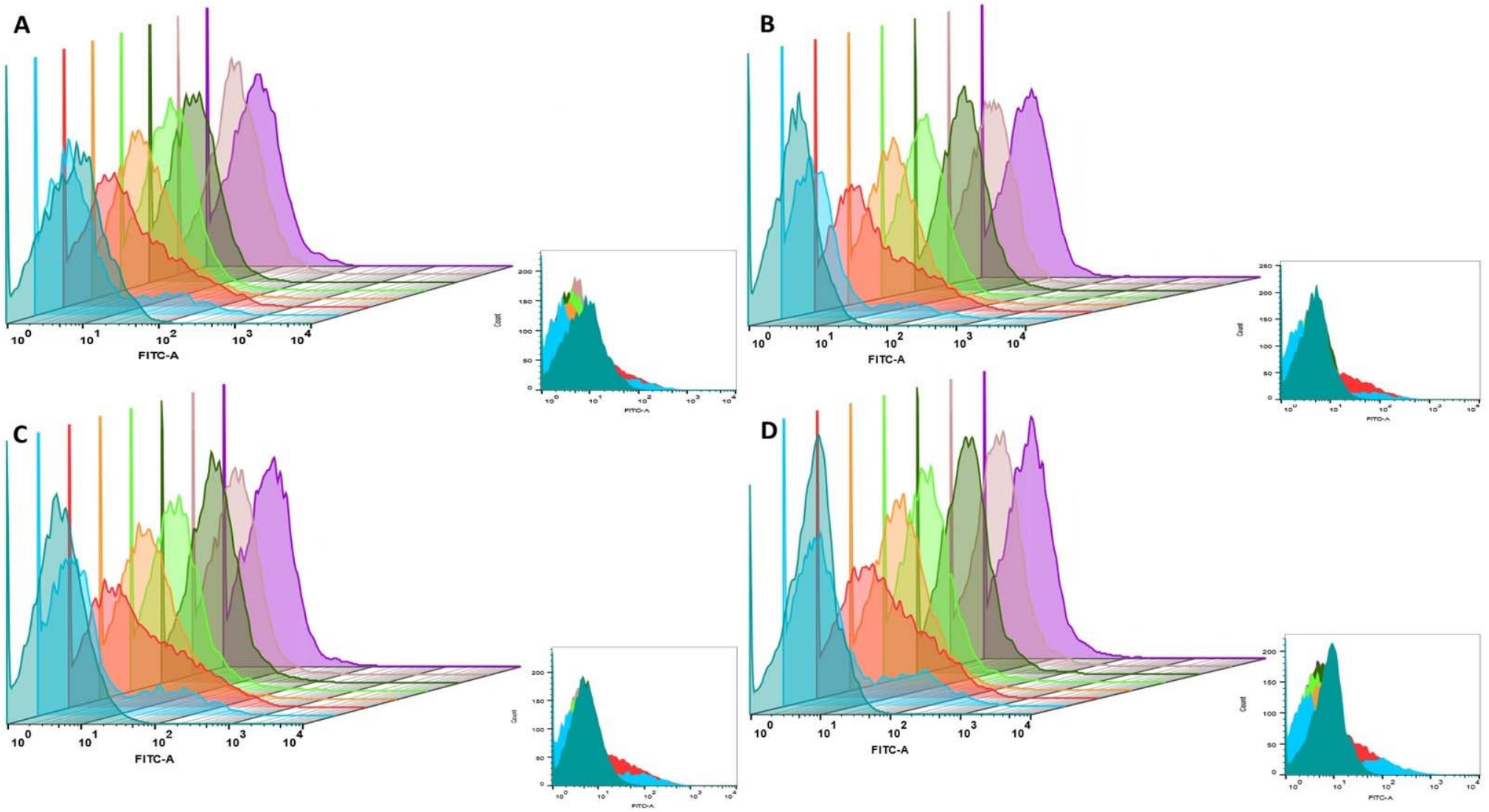

Figure 3: Flow cytometric histograms of galactose induced Saccharomyces cerevisiae EBY100 cells which were transduced with 4R-Pd-BP/pYD5 under the following four conditions (1) 50 ng plasmid DNA no carrier DNA (P4.1), (2) 100 ng plasmid DNA with no carrier DNA (P4.2), (3) 50 ng plasmid DNA with 50 µg carrier DNA (P4.1C) and (4) 100 ng plasmid DNA with 50 µg carrier DNA (P4.2C); these four different transformation conditions are represented by diagrams A, B, C and D respectively. These histogram represent the fluorescence profiles obtained from

induced P41, P42, P41C and P42C transformants over a period of 72 hours as well as that of their respective controls P4.1 GLU, P4.2 GLU, P4.1C GLU and P4.2C GLU. The horizontal axis represents the signal intensity of FITC and the vertical axis represents the number of events (cells) per FITC channel.

The advantage of using fluorescence microscopy lies in the ability to visualize the presence of the expression of fluorescent fusion proteins (FFPs) in cells (Ali *et al*. 2018); as was the case in this study. Fluorescence microscopy allowed for the visualization of GFP fused with 4R-PdBP; therefore the results obtained in this study confirmed that *Saccharomyces cerevisiae* EBY100 cells were efficiently transformed with pYD5/4R-PdBP and successfully expressed 4R-PdBP on the surface of induced cells represented by transformants P4.1, P4.1C, P4.2 and P4.2C. The fluorescence microscope images in figure 4 are representative of transformants P4.1, P4.1C, P4.2 and P4.2C where maximum expression levels of 4R-PdBP were detected as indicated by flow cytometry data. Maximum expression levels of 4R-PdBP were detected after 12 hours of cells being induced in galactose.

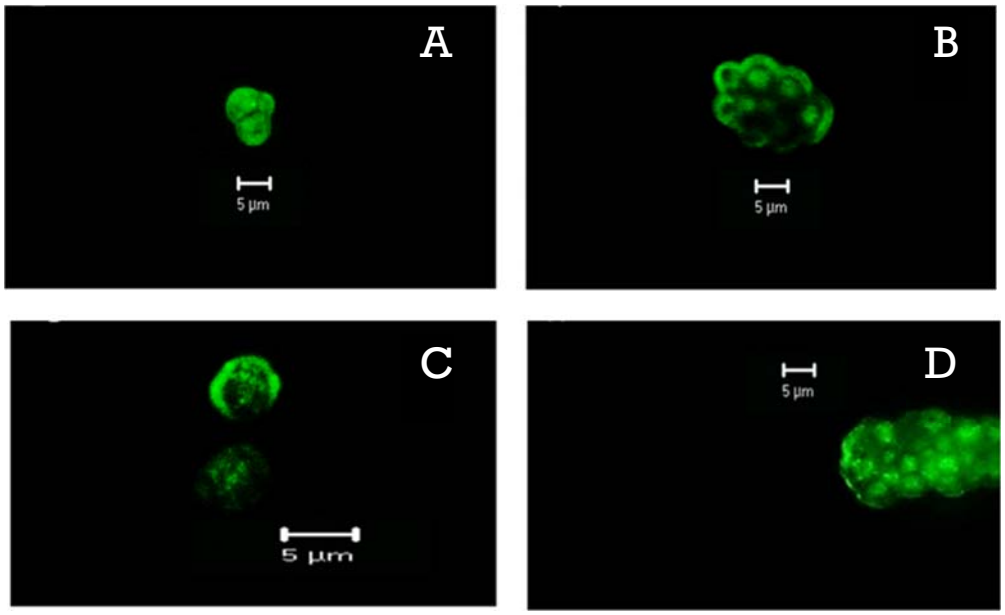

Figure 4: The fluorescence microscope images are indicative of maximum expression levels of 4R-PdBP in Saccharomyces cerevisiae EBY100 cells after 12 hours of induction in galactose. Fluorescence micrographs A, B, C, D, represent P42, P41C, P42C and P41 respectively showing autofluorescence with filters for green fluorescent protein (GFP); this is indicative of successful cell surface display of 4R-PdBP by *Saccharomyces cerevisiae* EBY100 cells.

Batch biosorption studies

As previously stated; four different transformation experiments were conducted for P4 for purposes of evaluating the effects with which the different conditions of transformation have will have on the expression of 4R-PdBP in transformed *Saccharomyces cerevisiae* EBY100 cells; the resulting transformant which had the highest copy numbers of the metal binding peptides were chosen to conduct biosorption studies. The transformant P41C had the highest copy number of 4R-PdBP and was thus chosen for conducting biosorption studies.

Effect of contact time on the biosorption of Pd(II)

Figure 5 illustrates the results of the effect of contact time on the biosorption of Pd(II) by transformant P41C over 48 hours. It was observed that the adsorptive capacity increased with increase in contact time. The biosorption of Pd(II) by P41C was very rapid; results indicated that 73% of Pd(II) was adsorbed by the P41C transformant within the first 5 minutes of the experiment; thereafter equilibrium was reached and a total of 90% of the Pd(II) was adsorbed within 4.5 hours of the experiment. This value of 90% remained relatively constant ± 1.3% throughout the remaining ± 43.5 hours of the study; data not shown on the plot. This exhibited biosorption pattern from this study can be characterized as being biphasic; whereby the initial biosorption phase was rapid, followed by the second phase where biosorption was gradual, slow and progressing towards equilibrium (Gadd, 2009). The lower reaction rates in second phase of the may be due to the existence of sites with low reactivity or surface nucleation-precipitation (Borda and Sparks, 2008; Gadd, 2009); this will be explored further in future studies.

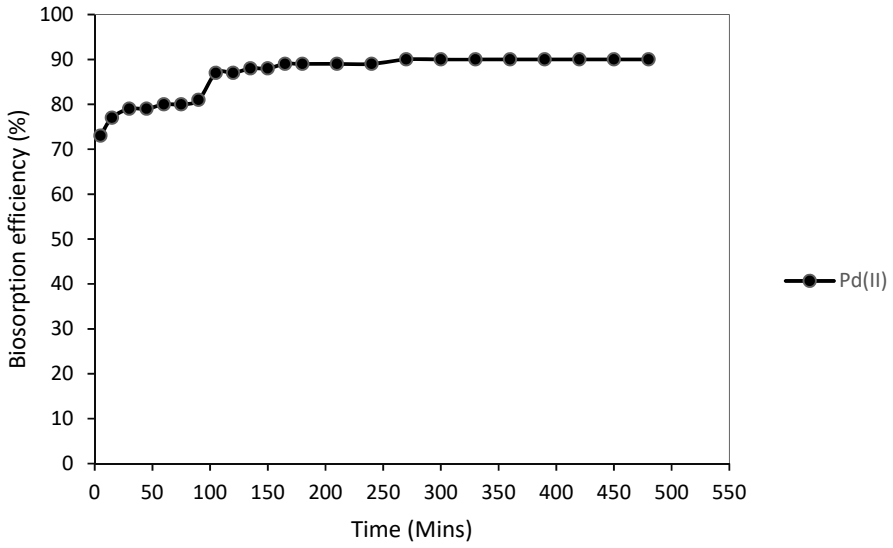

Figure 5: The biosorption efficiency of Pd (II) by transformant P41C as a function of contact time over 48 hours. The following parameters were kept constant throughout the experiments as follows: pH 3, 0.1g of adsorbent dosage, room temperature and initial metal concentration of 10 mg/L

The effect of pH on the biosorption of Pd(II)

The effect of the initial pH on the biosorption of Pd(II) by transformant P41C was studied at the pH range from 0.5 to 3.0 over 8 hours (Figure 6). The lowest and highest biosorption efficiencies of Pd(II) by P41C for were obtained at pH 0.5 and pH 3.0 respectively as shown

in figure 6; thus indication that the biosorption efficiency of Pd(II) by transformant P41C increased with the increase in pH. This may be explained by the effect that pH has on the chemical mechanism of the adsorption process.

The concentration of chloride ions has an effect on the chemical form at which platinum group metals will exist in a solution at equilibrium state; when a solution is strongly acidic, i.e. low pH, then anionic Pd(II) as ($PdCl_4^{2-}$) will predominate (Godlewska-Zyłkiewicz *et al*., 2019). According to literature, the low biosorption Pd(II) observed in strong acidic medium is an effect of chloride ions competing with anionic chlorocomplexes of palladium present in the solution for the protonated functional groups of the biosorbent (Godlewska-Zyłkiewicz *et al*., 2019). Furthermore it is said that at low pH the biosorbent ligands can become saturated with hydronium ions thus leading to repulsive forces which may prevent biosorption (Alpat *et al*., 2010). As mentioned above, the biosorption capacities of Pd(II) by P41C were low when the aqueous solution was strong acidic; therefore these statements elucidate the cause of the results obtained in this study.

According to Espinosa-Ortiz *et al*. (2016), increasing the pH of an aqueous solution results into an increase in the dissociation of protons as well as the presence of negatively charged groups in a biosorbent, thus resulting in an increase in the number of binding sites available the biosorption of metal ions; therefore this can be what attributes to the increases in the biosorption efficiency Pd(II) by P41C as the pH of the solution was increased.

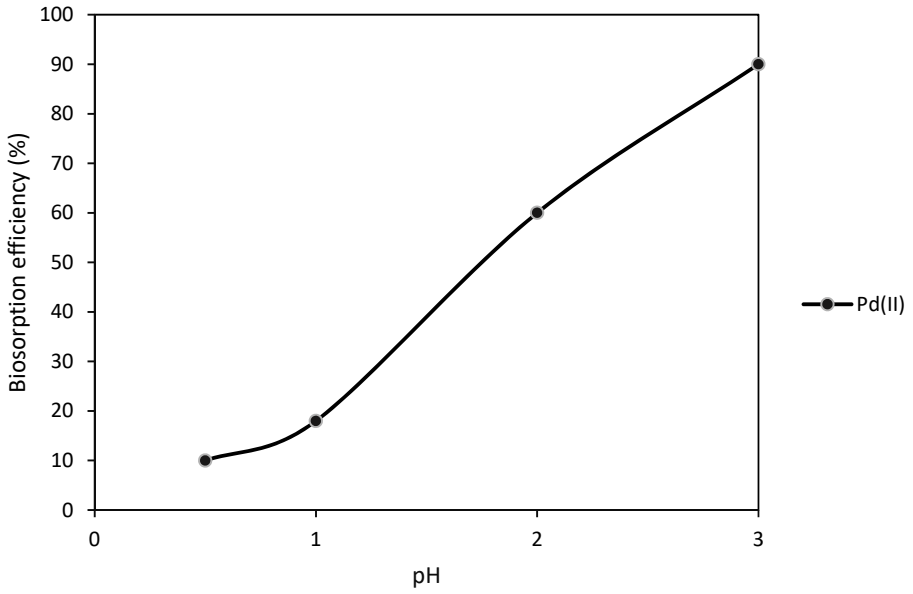

Figure 6: The biosorption efficiencies of Pd(II) by transformant P41 as a function of pH. The following parameters were kept constant throughout the experiments as follows: 0.1g of adsorbent dosage, room temperature, 8 hours contact time and initial metal concentration of 10 mg/L

The effect of initial metal concentration on the biosorption of Pd(II)

The correlation between the initial concentration of Pd(II) on the biosorption capacity of P41C is illustrated in figure 7. Metal concentrations of 10, 20, 30 and 50 mg/l were utilized to analyze the effect of initial metal concentration on the biosorption capacities of P41C for Pd(II) in a single metal solution; these experiments were carried out over 8 hours, at pH 3 with 0.1g of adsorbent dosage at room temperature. The results indicated that the biosorption capacity i.e. q_e (mg/g) of P41C for Pd(II) increased with the increase in metal concentration. 125 mg/g was the highest biosorption capacity obtained by P41C for the biosorption of Pd(II). This is a clear indication that biosorption is dependent on the initial metal concentration of the metals. According to Sudha *et al*. (2007), at lower metal concentration the ratio of the initial number of metal ions available to the adsorption sites is low; thus the low biosorption capacity of the adsorbent; whereas at higher metal concentration, the available adsorption sites become fewer; thus the dependence of biosorption capacity of P41C on the initial concentration of Pd(II). The Pd(II) adsorption capacity of the genetically modified *Saccharomyces cerevisiae* EBY100 strain expressing 4R-PdBP at pH 3 was comparable to that of the wild type *Saccharomyces cerevisiae* at pH 1 (23.0mg/g – 23.3mg/g) (Saitoh *et al*., 2020) and at pH 2.5 (0.042 mg/g)

(Godlewska-Zyłkiewicz *et al*., 2019).

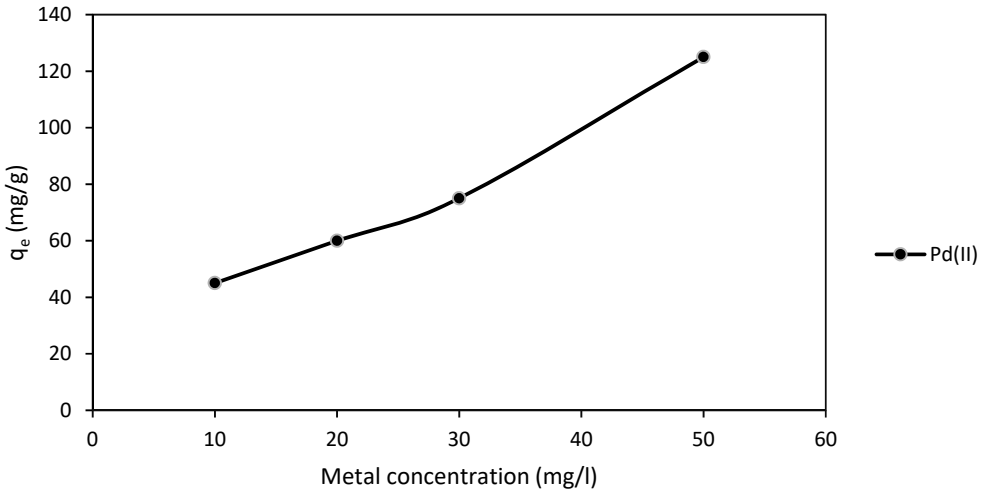

Figure 7: The biosorption capacity of P41 for Pd(II) as a function of initial metal concentration. The following parameters were kept constant throughout the experiments as follows: pH 3, 0.1g of adsorbent dosage, 8 hour contact time and room temperature. Initial metal concentrations of 10 mg/l, 20 mg/l, 30 mg/l and 50 mg/l were utilized.

CONCLUSIONS

This study described the successful development of a novel *Saccharomyces cerevisiae* EBY100 strain which was a result of genetic transformation using a pYD5 plasmid vector. The data presented here has indicated that by using the *Saccharomyces cerevisiae* a-agglutinin as an anchor region and secretion signal peptide, the palladium binding peptide (4R-PdBP) was successfully transported to the cell surface. Transformation conditions were varied to determine the optimal transformation conditions for *Saccharomyces cerevisiae* EBY100 cells with pYD5/4R-PdBP as well as the optimal conditions for the cell surface display of 4R-PdBP; this was achieved by varying plasmid DNA concentration as well as excluding or including the utilization of carrier DNA and results obtained indicated that carrier DNA did play a role in enhancing the transformation efficiency. The P41C transformant produced a sub-population of cells with the highest transformation efficiency amongst P4 transformation variants; 10% of P41C sub-population cells were positive for GFP expression and the population had the highest copy number of the 4R-PdBP. It is however evident from the results obtained in this study that although the expression of 4R-PdBP by transformant P41C was successful and rapid, it was

not optimal; therefore there is scope for improvement with regards to optimization in future studies. Optimizing the expression of 4R-PdBP by transformant P41C may result into an increase in the surface area available for biosorption and may thus lead to an increase in the biosorption efficiency, rate and capacity of the transformant P41C for Pd(II).

The P41C cells which are representative of 4R-PdBP surface displaying *Saccharomyces cerevisiae* EBY100 cells exhibited Pd(II) biosorption abilities from an aqueous solution. Biosorption experiments were conducted using 0.1g of dead biomass which was prepared using a freeze dryer. The biosorption studies conducted in this study indicated that the factors pH, contact time and initial metal concentration do have an effect on the biosorption of Pd(II) by the P41C cells. The biosorption efficiency of Pd(II) by the P41C cells increased with increasing pH, contact time and initial metal concentration. 90% uptake of Pd(II) was achieved by P41 cells at pH 3 within 4.5 hours at room temperature. The P41C cells achieved maximum biosorption capacity of 125mg/g for Pd(II). These results are an indication of the potential use of 4R-PdBP surface displaying *Saccharomyces cerevisiae* EBY100 cells for the removal of palladium from industrial wastes. A better understanding of the biosorption mechanism of Pd(II) onto 4R-PdBP surface displaying *Saccharomyces cerevisiae* EBY100 cells could contribute to the improvement of results obtained in this study; this will be explored in future studies.

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