# Characterization of Hepatitis B surface Antigen gene containing recombinant *Pichia pastoris*

By

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A thesis submitted to the Department of Mathematics and Natural Sciences in partial fulfillment of the requirements for the degree of Bachelor of Science in Biotechnology

Mathematics and Natural Sciences Department

BRAC University

October 2019

# Declaration

It is hereby declared that

1. The thesis submitted is my own original work while completing a degree at Brac University.
2. The thesis does not contain material previously published or written by a third party, except where this is appropriately cited through full and accurate referencing.
3. The thesis does not contain material which has been accepted, or submitted, for any other degree or diploma at a university or other institution.
4. I have acknowledged all the main sources of help.

**Student's Full Name & Signature:**

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# Approval

The thesis titled "Characterization of Hepatitis B surface antigen gene containing recombinant *P. pastoris*" submitted by Afra Abreshmi (15136009) Of Spring, 2015 has been accepted as satisfactory in partial fulfillment of the requirement for the degree of Bachelors of Science in Biotechnology on 3rd October 2019.

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3) The paper properly credits the meaningful contributions of co-authors and co-researchers.

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# Abstract

Hepatitis B is a potentially life-threatening liver infection caused by the Hepatitis B virus. It is a major health problem worldwide. So, the development of safe and effective vaccines against this disease can be a huge triumph for mankind. *Pichia. pastoris* is a suitable host for inducing the production of the Hepatitis B surface antigen (HBsAg) inside it's body as well as it can be used for commercially large scale production. This can be done by inserting the HBsAg gene into the plasmid vector of *P. pastoris* yeast under the alcohol oxidase (AOX) promoter and let them grow in suitable condition. Our study was conducted to ensure the insertion of the HBsAg gene under the AOX promoter into the yeast *P. pastoris* after allowing them to be stored as Master Cell Bank (MCB) and Working Cell Bank (WCB). Thus, the first aim of this study was to characterize the Master Cell Bank and Working Cell Bank by Phenotypic tests; like microbial purity, identity and viability. Secondly, HBsAg gene in *P. pastoris* was analyzed by polymerase chain reaction (PCR) using two sets of primers named F1R1 and F2R2, followed by performing gel electrophoresis and Sanger sequencing. Characterization of the cell banks as well as ensuring the recombinant HBsAg gene indicates that visually all the *P. pastoris* contain the HBsAg gene. Finally, the main objective of this study was to ensure the expression, purification, and characterization of HBsAg in yeast *P. pastoris*. The study was carried out successfully.



**Keywords:** *P. pastoris*; HBsAg; Vaccine; AOX promoter; F1R1 primers; F2R2 primers

# Dedication

Dedicated to my parents and my sisters; Major S.M. Shahadat Hossain, Mrs. Ferdousi Hossain, Rubaiyat Binte Hossain, and Risalat Binte Hossain

# Acknowledgment

First and foremost, I would like to convey my gratitude to the Almighty for keeping me healthy and giving me the patience throughout my thesis work.

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(Afra Abreshmi)

October 2019

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# List of Acronyms

| | |
|---|---|
| SCD | Soybean Casein Digest Medium |
| MMH | Minimal Methanol Medium |
| YPD | Yeast Extract Peptone Dextrose Medium |
| BMMY | Buffered Methanol Complex Medium |
| BMGY | Buffered Glycerol Complex Medium |
| WCB | Working Cell Bank |
| MCB | Master Cell Bank |
| PCR | Polymerase Chain Reaction |
| CFU | Colony Forming Unit |
| AOX | Alcohol Oxidase |
| HBsAg | Hepatitis B Surface Antigen |

# CHAPTER-1

# INTRODUCTION

*Pichia pastoris* is a methylotrophic yeast. It utilizes reduced one-carbon compounds; like methane and methanol and produces enzymes involved in methane metabolism at a high level. *Pichia pastoris* has become a significant expression system and has been used frequently. It has two alcohol oxidase genes; AOX1 and AOX2 under strong inducible promoters. The promoters get induced in the presence of methanol. This is why the desired gene (gene of interest) is inserted under these promoters. The Hepatitis B surface antigen (HBsAg) gene can be inserted under the AOX1 promoter. In the presence of methanol, the AOX1 promoter gets induced and starts synthesizing the HBsAg. There are some factors which make *Pichia pastoris* widely acceptable. *Pichia pastoris* is a single-celled microorganism that can be easily manipulated. It also has similarity with *Saccharomyces cerevisiae* in terms of genetic manipulation techniques. The AOX1 promoter is uniquely suitable for the controlled expression of foreign genes. Furthermore, the expression system of *Pichia pastoris* is fast and inexpensive in terms of using the growth media. However, being a eukaryote this yeast is capable of post-translational modifications; like proteolytic processing, folding, disulfide bond formation, and glycosylation.

## 1.1 Hepatitis B surface antigen expression in yeast

Hepatitis B is a liver infection caused by the Hepatitis B virus. Amongst different types of hepatitis, B and C can become chronic. So, the number of efforts have been put together to find out the structural and behavioral characteristics of this virus. However, it has been very difficult to get a positive result from these efforts as this virus only replicates in human and chimpanzee liver. So, steps had been taken to produce the vaccine against this virus using the expression system of *E. coli* which did not end successfully. This was because, in *E. coli* cells, the HBsAg gene product seems to be either unstable or to cause effects deleterious to the host, or both. (MIYANOHARA et al., 1983)

## 1.2 *Pichia pastoris* expression system

Being a methylotrophic yeast *Pichia pastoris* has been used widely as an expression host for different pharmaceutical proteins. It is mainly due to the ease of use, relatively rapid expression

times and low cost (Byrne, 2015). *P. pastoris* is a methylotrophic yeast which is able to metabolize methanol. There are a number of components in the metabolic pathways of this yeast. These include:

1. Alcohol oxidase
2. Catalase
3. Formaldehyde dehydrogenase
4. Formate dehydrogenase
5. Dihydroxyacetone synthase
6. Dihydroxyacetone kinase
7. Fructose-1,6-bisphosphate aldolase
8. Fructose-1,6-bisphosphate
9. Formaldehyde reductase

The expression strategy of *P. pastoris* can be demonstrated as follows:

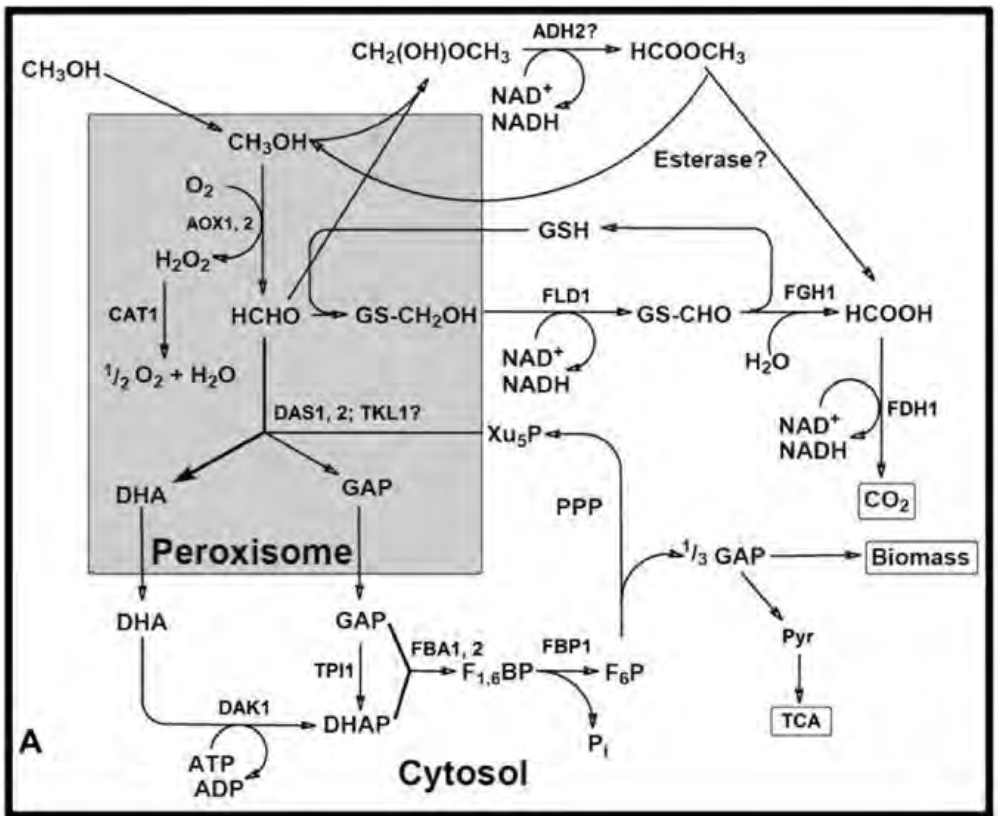



Figure 1: *P. pastoris* expression system

## 1.3 *P. pastoris* as a methylotrophic yeast

*P. pastoris* is one of the four genera that is capable of metabolizing methanol. The other three genera are *Candida, Hansenula, and Torulopsis.* Here only the metabolic pathway of *P. pastoris* is focused. The first step of this metabolic pathway is the oxidation of methanol to formaldehyde that generates hydrogen peroxide in the process by the enzyme Alcohol Oxidase (AOX). The toxicity of hydrogen peroxide is avoided due to the occurrence of the first step inside a specialized organelle named the peroxisome. It sequesters away the toxic hydrogen peroxide from the rest of the cell.AOX is a homo-octamer with each subunit containing one non-covalently bonded FAD (Flavin Adenine di-nucleotide) co-factor. AOX has a poor affinity for oxygen and methylotrophic yeast compensate for this large amount of deficiency by synthesizing a large amount of the enzyme. There are two genes that encode for the AOX. These are AOX1 and AOX2. However, AOX is responsible for the vast majority of alcohol oxidase activity in the cell. Expression of the AOX1 gene is tightly regulated and induced by methanol to high levels. The expression of the AOX1 gene is controlled at the level of transcription. So, the presence of methanol appears to be essential to induce high levels of transcription. The presence of another gene named the His4 gene has a significant role in the pathway as well. It appears to encode a trifunctional enzyme catalyzing the second (phosphoribosyl-ATP pyrophosphohydrolase), third (phosphoribosyl-AMP cyclohydrolase), and tenth (histidinol dehydrogenase) steps in histidine biosynthesis. (Crane and Gould, 2015).

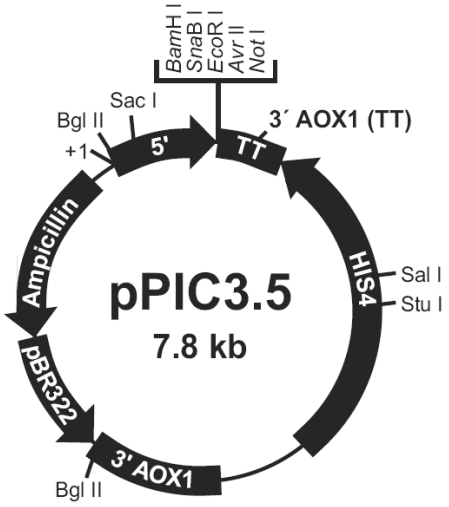



Figure 2: *P. pastoris* plasmid vector

## 1.4 Expression of HBsAg in *P. pastoris*

To develop the strain for expressing the desired hepatitis B surface antigen, the HBsAg was inserted into a region under the AOX1 promoter. In the presence of methanol, the promoter can start transcribing the region under it. The GS115 is the wild type strain of *P. pastoris* that has been used in the project. After the insertion of the desired gene, the vector obtained is called the Hepatitis B –pHIL-D2. In this transformed yeast strain the mutation is supposed to occur in one or more auxotrophic genes to allow for selection. The insertion of another gene of 2.4 kb called His4 gene or the Histidine dehydrogenase gene complements the defective His 4 gene of the host cell line. Thus the *P. pastoris* transformants are selected for the His4+ phenotype. They can grow on minimal Agar plates in the absence of added Histidine.

## 1.5 Starting strain ( MCB and WCB)

The overall procedure of the cloning of the plasmid vector and the preparation of the Master cell bank and the Working cell bank was carried out by a renowned pharmaceutical of our country. So, the rest of the part has been done through this project and that will be the focal point for this thesis report.

## 1.6 Advantages of *P. pastoris* over *Saccharomyces cerevisiae*

Although there are a number of similarities between these two yeasts *P. pastoris* and *Saccharomyces cerevisiae*, there are also a few reasons that make *P. pastoris* a better option. These can be as follows:

1. Expression in *P. pastoris* can be controlled under tightly regulated and highly expressed AOX1 promoter which is induced by methanol; a cheap inducer.
2. The expression cassette can be inserted into the host genome, thereby avoiding potential plasmid instability problems.
3. The culture medium consists of carbon source such as glycerol and simple salts; which are as well as inexpensive.
4. The optimum pH for growth is around 5 a pH at which most microorganisms do not grow well. So, it reduces the chances of contamination.

5. Studies show that *P. pastoris* is non-pathogenic and devoid of toxins, allergens, and pyrogens.

## 1.7 Risk factors of handling *P. pastoris*

In general, this yeast *P. pastoris* is non-pathogenic and do not cause any serious disease amongst people. However, just like other microorganism *P.* spp also has some negative effects reportedly to some articles. For instance, *Pichia anomala* is emerging yeast causing serious nosocomial infections in newborn and immune-compromised children. It was reported that nosocomial port catheter infection due to *P. anomala* in three children who were receiving cancer chemotherapy, bloodstream infection in a preterm infant and in an infant with severe combined immunodeficiency was diagnosed in Turkey. However, all patients were treated with amphotericin B and fluconazole (Bakir et al., 2004). Another case of this yeast had been reported in Brazil from October 2002 to January 2004 where 1,046 children were admitted to the pediatric ICU, 17 of whom developed *P. anomala* fungemia (attack rate, 1.6%). The overall mortality rate was shockingly 41.2%. Two patients received no antifungal treatment; others were treated with amphotericin B( Pasqualotto et al., 2016). However, it has not significantly reported that handling *P.* spp in the laboratory can cause a serious disease or infection. Still, it would be wise to be careful while handling any yeast, be it *P. pastoris* or any other yeast.

## 1.8 Objective

*P. pastoris* has two AOX genes under strong inducible promoters. The promoters get induced in the presence of methanol. So, the gene of interest can be inserted under the AOX1 and AOX2 promoters. For the research purpose, the Hepatitis B surface antigen gene was inserted under the AOX1 promoter. So, in the presence of methanol, the recombined *P. pastoris* can be grown and characterized.

This research was carried out to know whether the *P. pastoris* strain has been recombined successfully or not through the genotype testing. Also, testing the viability and purity of the strain (provided by reputed Pharmaceuticals) was also carried out after the storage in -80° C.

# CHAPTER-2

# MATERIALS AND METHOD

For the research, the following materials and methods have been used.

## 2.1 Materials:

For the research the following materials and reagents have been used:

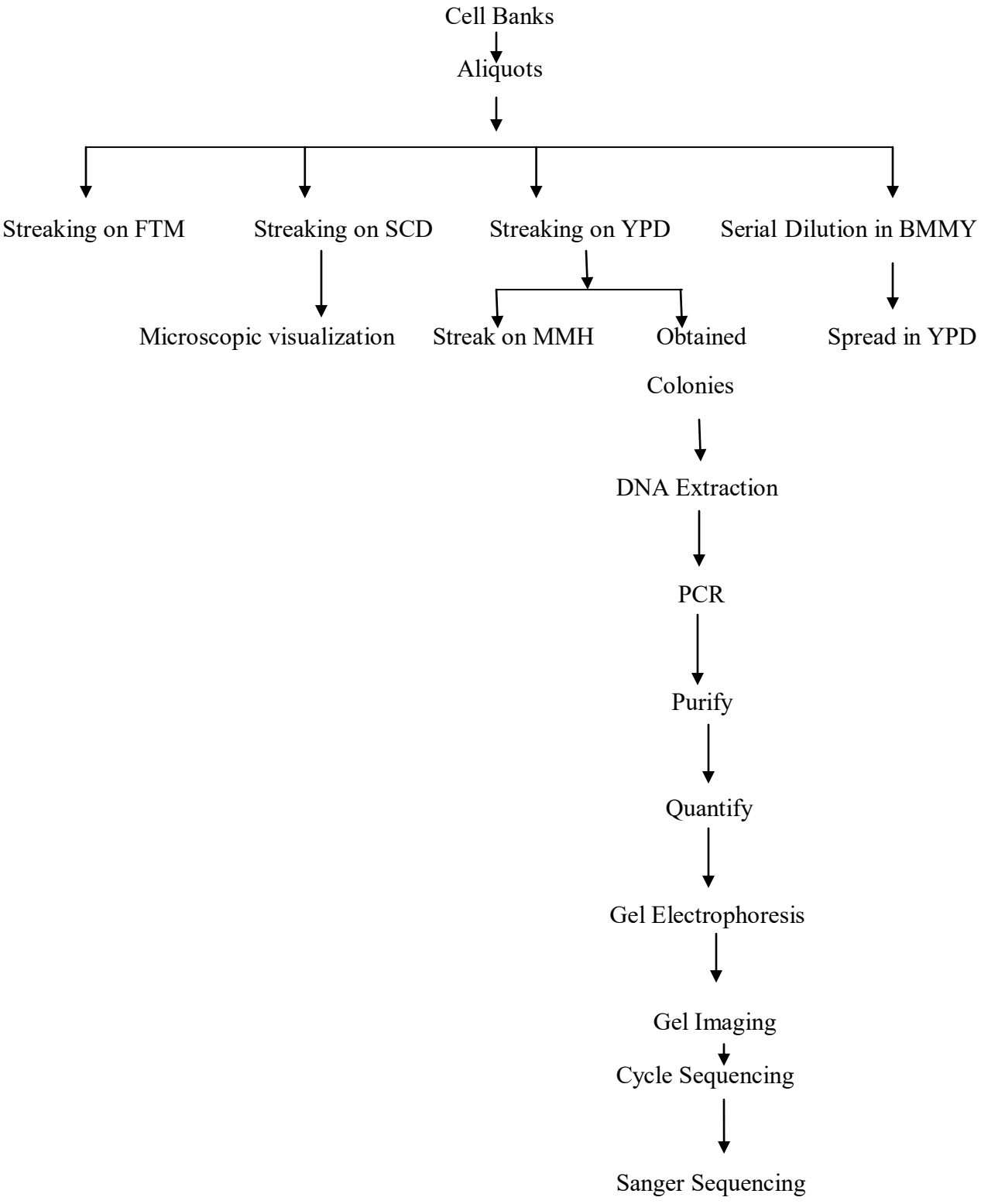
- FTM media plates
- SCD media plates
- YPD agar media plates
- MMH agar media plates
- BMMY broth
- BMGY broth
- Nuclease free water
- Deionized water
- Gel red
- Loading dye

For the research study the following equipment and machinery have been used:

- Laminar hood
- Microbalance machine
- Conical flask
- Falcon tubes
- Streaking loops
- Plastic Spreaders
- PCR hood
- Micropipettes and tips
- PCR tubes and Eppendorf tubes
- Centrifuge machine
- Short spinner
- PCR machine
- Gel electrophoresis machine

## 2.2 Brief outline of the methodology

The following outline would help to give an idea about the overall research work.



## 2.3.1Descriptions of the media and broth used

For the research four types of agar media and two broths have been used. These are described as follows:

1. YPD media: it stands for the yeast extract peptone dextrose media. It is a complete media for the growth of yeast *P. pastoris.* It contains yeast extract, double distilled water, peptone, and dextrose. This media cannot be used as a selection media for auxotroph mutants rather it is used for the growth of the yeast only. It was also used for the viability testing of the yeast.
2. SCD media: it stands for the soybean casein digest media. This media is mainly used to test the purity or the sterility of the yeast. The combination of pancreatic digest of casein and papaic digest of soybean meal makes this medium nutritious by providing amino acids and long-chain peptides for the growth of microorganisms. Natural sugars in soybean promote the growth of fastidious organisms. Dextrose monohydrate is the fermentable source of carbon and dipotassium hydrogen phosphate serves as the buffer in the medium. Sodium chloride maintains the osmotic balance of the medium. This media also has a pH of 7.10-7.50 and at 25°C for 3-4 days promote the growth of only *P.pastoris.* ( Technical Data, soybean casein digest media; Himedia.2011 )
3. FTM media: it stands for Fluid Thioglycollate media. It is also a media that is used for the purity or sterility testing of yeast. This media also has a pH of 7.10 (± 0.2) and at 25°C for 3-4 days the *P. pastoris* growth is observed.
4. MMH media: it stands for Minimal Methanol media. The methanol in this media induces the AOX1 promoter for the growth of *P. pastoris.* So, it also acts as the media for identity testing of the yeast. After growing on YPD media, the yeasts were grown on MMH media for reassuring the growth of the yeast. However, the media did not contain any added histidine. So, the growth of the yeast on this media indicates that the yeast could utilize its own histidine and also utilize the methanol in the media as a sole carbon source.
5. BMMY and BMGY broth: these broths stand for buffered methanol complex media and Buffered glycerol complex media respectively. These were used to serial dilute the yeast colonies for their viability testing. However, there had been no significant difference in using these two broths as they both promote the growth of yeast. However, glycerol might

act as an inhibitor for the growth of yeast. So, for the research BMMY, broth media was used mainly.

### 2.3.2 YPD and SCD media preparation

For preparing YPD agar the following steps were followed:

- For 12 plates 16.25 g of YPD media powder was suspended into a conical flask of 250 ml of distilled water based on 65g of powder in per liter water (as per as the instructions written on the container).
- On a heat stirring machine at 90°C at 200 rpm, the conical flask was placed for about an hour.
- It was then autoclaved for 15 minutes at 121°C.

For preparing SCD agar the following steps were followed:

- For 6 plates 4.8 g of SCD media powder was suspended into a conical flask of 120 ml of distilled water based on 40 g of powder per liter water (as per as the instructions written on the container).
- On a heat stirring machine at 90°C at 200 rpm, the conical flask was placed for about half an hour.
- It was then autoclaved for 15 minutes at 121°C.

The rest of the media including MMH, FTM, BMMY were provided by reputed Pharmaceuticals, in Bangladesh.

## 2.4 Preparations of aliquots from MCB and WCB

After taking out the MCB and WCB (provided by reputed Pharmaceutical.) from -80°C, 4 aliquots (containing 100 µl of the sample in each container) for both MCB and WCB were prepared.

## 2.5 Identity testing

The identity testing of *P. pastoris* was carried out by streaking the sample from both of the aliquots of the MCB and WCB on YPD media. The yeast was then allowed to grow on the media for 48 hours at 25°C. After the growth was observed eight colonies from one YPD (of WCB) media plate

was streaked on eight quadrants of MMH media. Same was done for the MCB. After 48 hours of incubation at 25, °C growth was observed on MMH for both MCB and WCB.

## 2.6 Purity testing

For purity testing the sample from both of the aliquots of MCB and WCB were streaked on FTM media and SCD media. After four days of incubation at 25, °C growth was observed on both FTM and SCD media for MCB and WCB. The morphological characteristics of the yeast were observed under a microscope for SCD media.

## 2.7 Viability testing

For viability testing, the aliquots of MCB and WCB were serially diluted in BMMY broth media. In 900 µl of BMMY broth 100 µl of sample was added for serial dilution. The aliquots were diluted 10 times using the following formula:

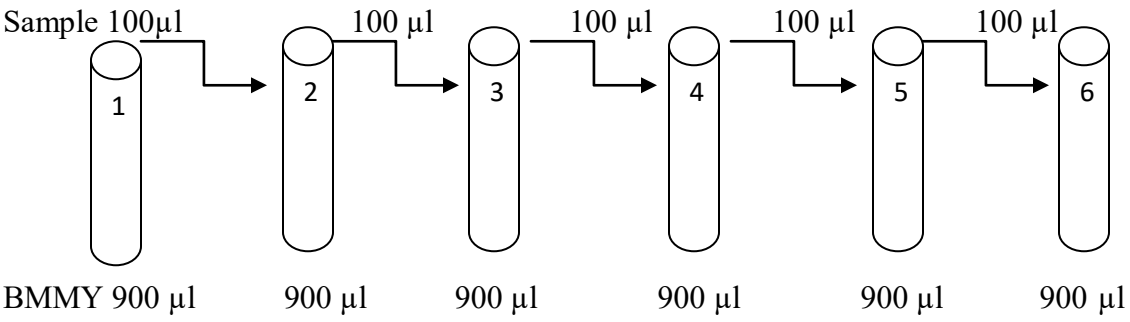



Figure 3: Serial dilution of samples in BMMY broth for viability testing

After serial dilution, 30 µl of the solution from dilution 4, 5 and 6 each were spread on the YPD agar media for both MCB and WCB using spreaders. After 48 hours of incubation at 25, °C growth were observed on every media plate, colonies were counted visually and the CFU was calculated for each of the plate using the following formula:

$$\text{CFU/ml} = \frac{number\ of\ colonies \times dilution}{volume\ of\ sample\ spreading\ (ml)}$$

## 2.8 Genotyping

For the genotyping test, the colonies obtained from the YPD media after 48 hours of incubation at 25°C (during the identity testing) were used.

### 2.8.1 DNA extraction

The DNA extraction method was quite a short and convenient method. The method is called the colony PCR method. The protocol is as follows:

- The single colony from each of the plate was mixed in 50 µl of BMMY broth.
- The solution was vortexed for 15 seconds and spun down for a few seconds.
- The whole solution was then transferred into 4 PCR tubes for both MCB and WCB. (so total of 8 tubes).
- The tubes were then placed inside the PCR machine at 95°C for 10 minutes. During this time the yeast cells went through a heat shock period.
- After 10 minutes the tubes were placed on ice immediately for the yeast cells to undergo cold shock.
- After keeping on ice for 5 minutes the solution was centrifuged at 13,000 rpm for 5 minutes at 4°C.
- The supernatant was then transferred to a fresh PCR tube. This supernatant contains the extracted DNA.

### 2.8.2 PCR

After the DNA was extracted it was amplified using PCR. For the PCR process, two sets of primers F1R1 and F2R2 were used to amplify two specific regions. These regions contained the GOI; which is the HBsAg. Four different combinations of PCR solutions were prepared. These are:

1. MCB template with master mix containing F1R1 primer
2. MCB template with master mix containing F2R2 primer
3. WCB template with master mix containing F1R1 primer

4. WCB template with master mix containing F2R2 primer

The HBsAg genes were amplified using a pre-designed set of primers specific for the Hepatitis B surface antigen gene. The sequences of the primers along with the obtained product size are as follows:

Table 1: primers used for the experiment:

| Name of the primers | Primers sequences | Product size |
|---|---|---|
| HBV-F1 | ACCATGGAGAATATCACATCAGG | 637 bp |
| HBV-R1 | AGGGACTCAAGATGTTGTACAGAC | 637bp |
| HBV-F2 | GACAAGAATCCTCACAATACCG | 486 bp |
| HBV-R2 | ACGAACCACTGAACAAATGGCAC | 486 bp |

The Master Mix was prepared using the following components:

- 10X colony PCR buffer = 5 µl
- 25 mM MgCl = 3 µl
- 10 mM dNTPs = 1 µl
- F1/R1 primer = 2.5 µl
- R1/R2 primer = 2.5 µl
- Taq polymerase = 0.3 µl
- Nuclease free water = 34.7 µl
- Template = 1 µl

Total 50 µl of solution.

The PCR followed the following protocol:

- 95°C for 1 minute
- 95°C for 30 seconds
- 62°C 1 minute
- 72°C 1 minute
- 72°C 6 minutes
- 4°C for ∞

### 2.8.3 PCR product purification and quantification

After the PCR amplification, the products were purified using the Qiagen Minelute kit and following the protocol. The protocol is as follows:

- Five volumes of Buffer PB were added to 1 volume of the PCR reaction and were mixed.
- MinElute columns were placed in a provided 2 ml collection tube in a suitable rack.
- Sodium acetate was added to see the color change and was then centrifuged for 1 min at 13,000rpm.
- The disposed solution at the bottom was discarded. The MinElute column was placed back into the same tube.
- 700 µl Buffer PE was added to the MinElute column and centrifuged for 1 min at 13,000rpm.
- Flow-through was discarded and the MinElute column was placed back in the same tube.
- The column was centrifuged for an additional 1 min at 13,000rpm.
- The MinElute column was then placed in a clean 1.5 ml microcentrifuge tube.
- To elute DNA 10 µl of nuclease-free $H_2O$ was added to the center of the membrane,
- The column was allowed to stand for 1 min and then centrifuged for 1 min.
- Finally, the flow-through was collected.

After purifying the PCR product it was quantified using the nanodrop machine using 2 µl of samples.

### 2.8.4 Gel Electrophoresis and Gel imaging

Before purifying the PCR product, it was gel electrophorized. For this,6 samples for MCB with F1R1 primer (named sample 16-21) and 6 samples for MCB with F2R2 primer were used for gel run. Same was done for WCB. Five µl of sample mixed with 2 µl of loading dye were loaded in 50 ml of 1% Gel of electrophoresis machine. Five µl of DNA ladder of 100 bp was also added and the sample ran at 100 Voltage. After the run was finished the gel was placed in a container containing 200 ml of distilled water and 3µl of GelRed (staining dye) for post staining. The gel was kept there for about 20 minutes. After that, the gel was washed with distilled water in a container thoroughly. The gel was then placed in a gel imaging machine and the IMAGE LAB software was used to get the gel image.

### 2.8.5 Cycle sequencing

After getting the proper gel image the samples were cycle sequenced using the following steps to prepare the Master Mix:

- Big dye = 0.2 µl
- Sequence buffer = 2 µl
- Nuclease free water = 6.5 µl
- Primer = 0.3 µl
- Template = 1 µl

### 2.8.6 Sanger sequencing

After a successful cycle sequencing, the further Sanger sequencing was carried out in the laboratory of Dhaka University. Through this, the sequences of the inserted genes were obtained.

# Chapter -3

# Results

After the methods were carried out the following results were obtained:

## 3.1 Identity testing

After the incubation of *P. pastoris* on the YPD media for 48 hours, and from YPD streaking on MMH and incubation for another 48 hours the following results were obtained:

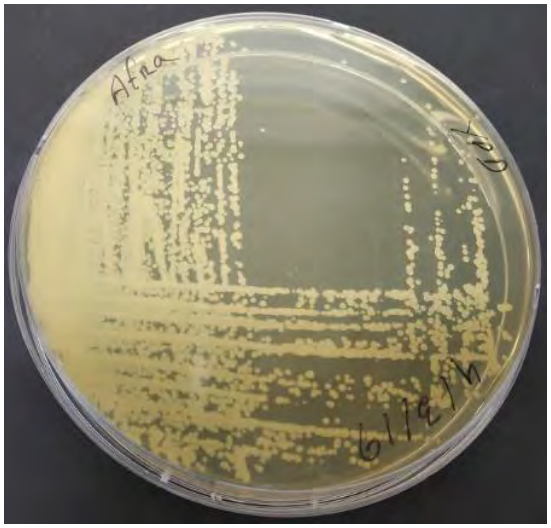


Figure 4: The growth of WCB sample on YPD media after 48 hours of incubation at 25°C

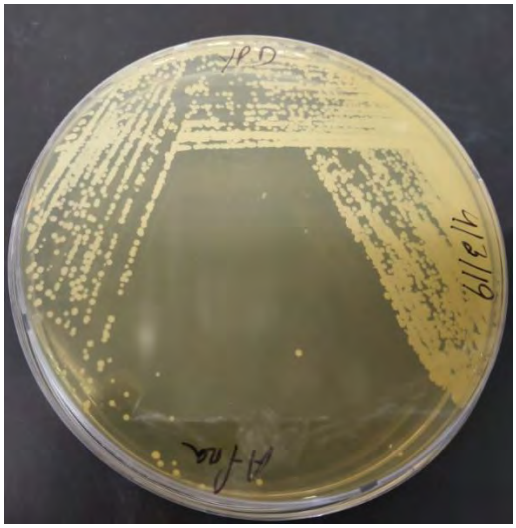


Figure 5: The growth of MCB sample on YPD media after 48 hours of incubation at 25°C

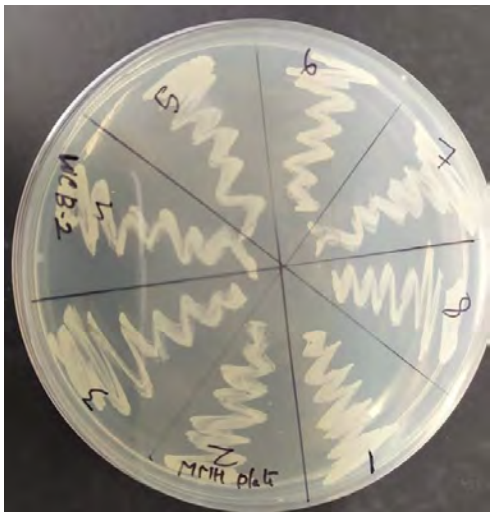



Figure 6: The growth of WCB sample on MMH after 48 hours of incubation at 25°C

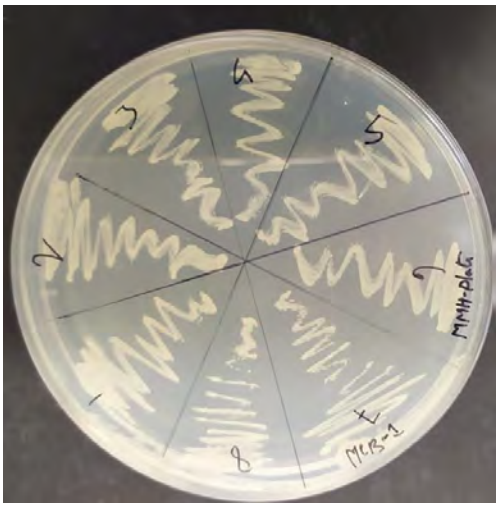



Figure 7: The growth of MCB sample on MMH after 48 hours of incubation at 25°C

## 3.2 Purity testing

After the incubation of the MCB and WCB both on FTM and SCD media for four hours at 25°C, the following results were obtained:

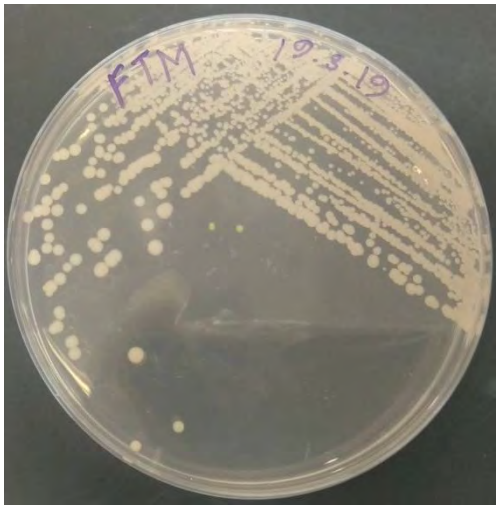



Figure 8: The growth of WCB sample on FTM after 4 days of incubation at 25°C

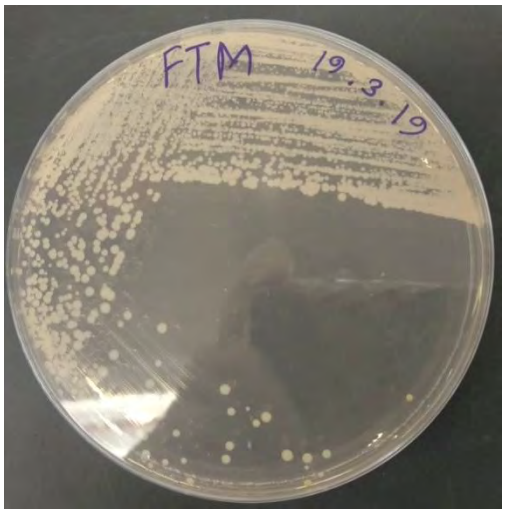



Figure 9: The growth of MCB sample on FTM after 4 days of incubation at 25°C

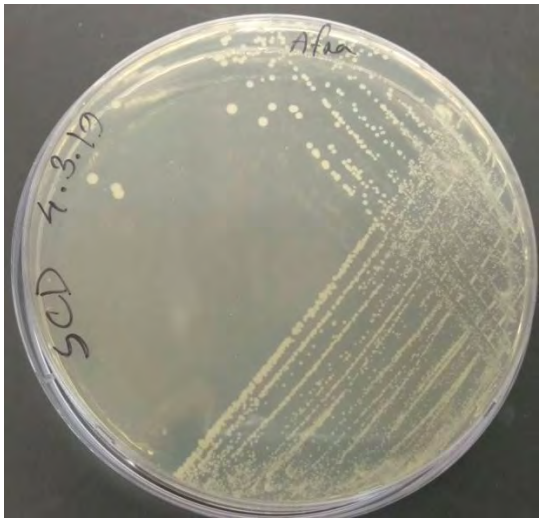



Figure 10: The growth of WCB sample on SCD after 4 days of incubation at 25°C

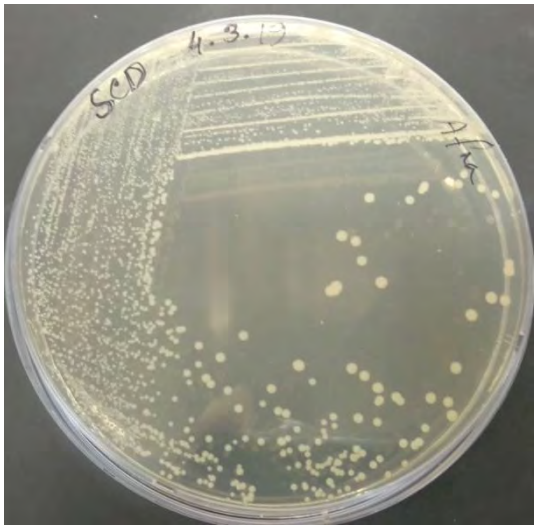



Figure 11: The growth of MCB sample on SCD after 4 days of incubation at 25°C

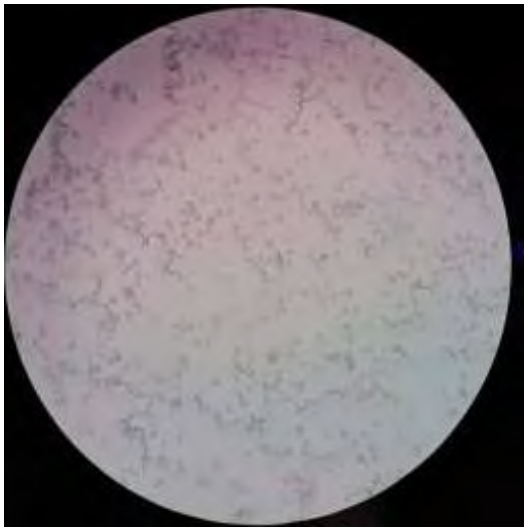


Figure 12: The visualization of SCD colonies under the microscope

## 3.3 Viability testing

After the serial dilution, followed by spreading of dilution 4,5 and 6on YPD media the following results were obtained:

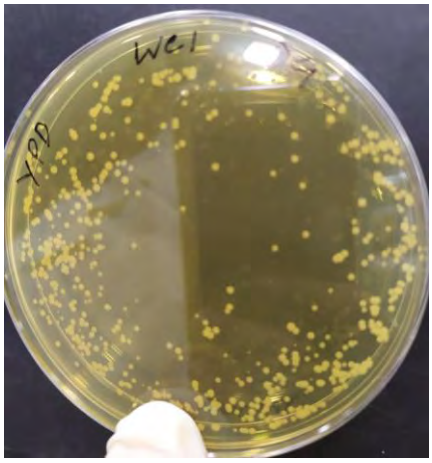



Figure13: Colonies of WCB serial dilution 4

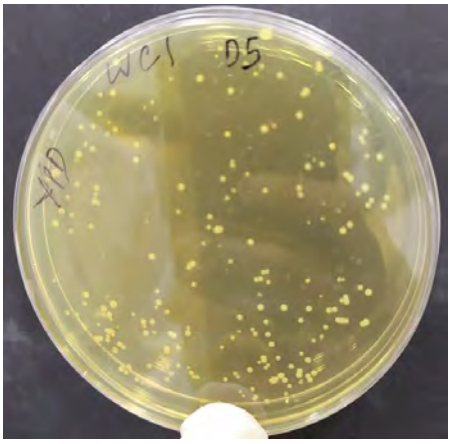



Figure 14: Colonies of WCB serial dilution 5

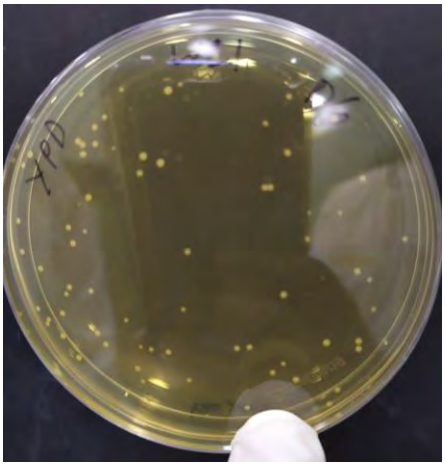



Figure 15: Colonies of WCB serial dilution 6

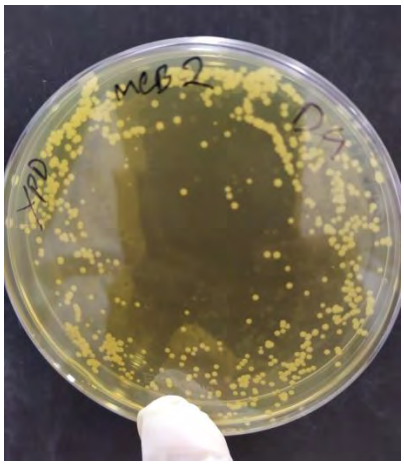



Figure 16: Colonies of MCB serial dilution 4

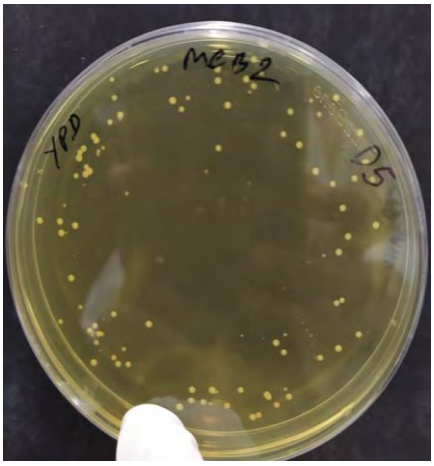



Figure 17: Colonies of MCB serial dilution 5

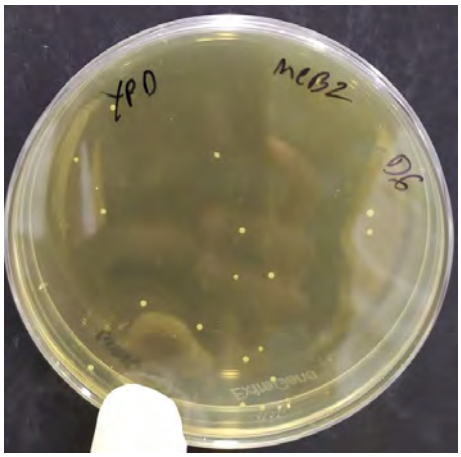



Figure 18: Colonies of MCB serial dilution 6

Using the formula the CFU for each of the dilution are as follows: (here the volume of the sample was 20 µl)

$$\text{CFU/ml} = \frac{number\ of\ colonies \times dilution}{volume\ of\ sample\ spreading\ (ml)}$$

CFU for WCB dilution 4 = $369 \times 10\text{^}4 \times 1000/20 = 1.84\times10^8$

CFU for WCB dilution 5 = $302 \times 10\text{^}5 \times 1000/20 = 1.5\times10^9$

CFU for WCB dilution 6 = $74 \times 10\text{^}6 \times 1000/20 = 3.7\times10^9$

CFU for MCB dilution 4 = $338 \times 10\text{^}4 \times 1000/20 = 1.69\times10^8$

CFU for MCB dilution 5 = $85 \times 10\text{^}5 \times 1000/20 = 4.25\times10^8$

CFU for MCB dilution 6 = $21 \times 10\text{^}6 \times 1000/20 = 1.05\times10^9$

## 3.4 Genotyping

Throughout the genotyping process, the following results were obtained successfully.

### 3.4.1 DNA extraction

The DNA from the colonies were extracted successfully which was proven through the rest of the results.

### 3.4.2 PCR

The PCR amplification was done successfully which was proven by the rest of the gel imaging.

### 3.4.3 Quantification

After the quantification of the samples, the results were successful because the quantity and the purity of the samples were generous enough. It can be proven through the table given below:

Table 2: Quantification of PCR products

| CELL BANK | PRIMER | PURITY(260/280 nm) | QUANTITY (nanogram) |
|---|---|---|---|
| WCB | F1R1 | 1.8 | 25.5 |
| WCB | F2R2 | 1.61 | 30.75 |
| MCB | F1R1 | 1.68 | 26.67 |
| MCB | F2R2 | 1.78 | 41.08 |

### 3.4.4 Gel image

After the Gel Electrophoresis was carried out, the following gel images were obtained using the IMAGE LAB SOFTWARE:

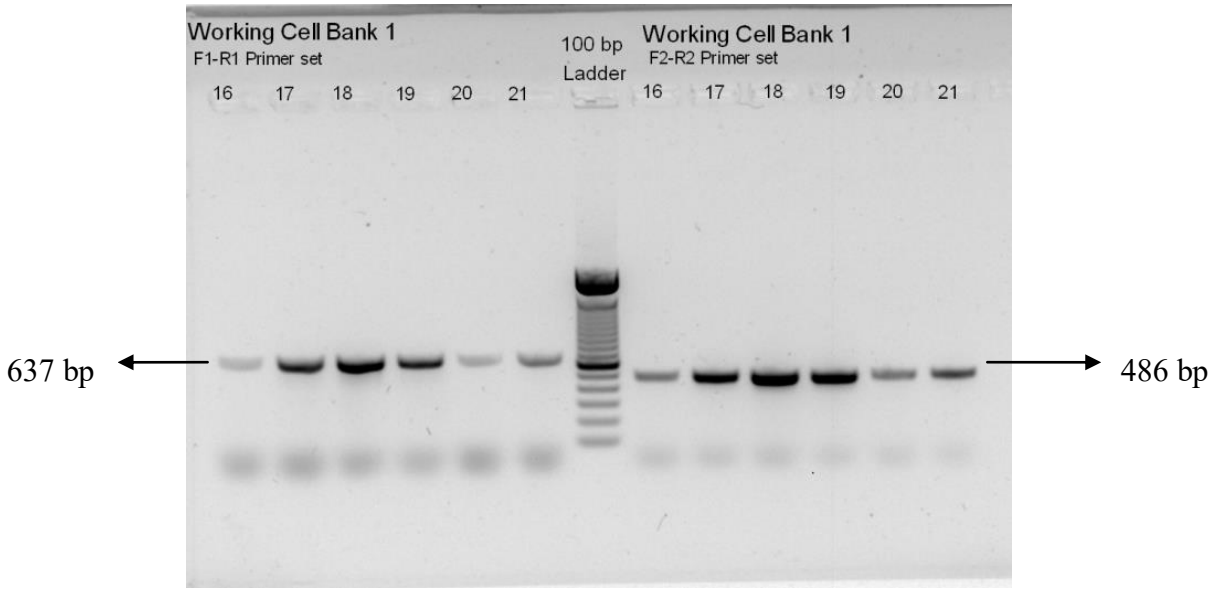



Figure 19: Gel image of the working cell bank using both F1R1 and F2R2 primers; where 16-21 are sample numbers

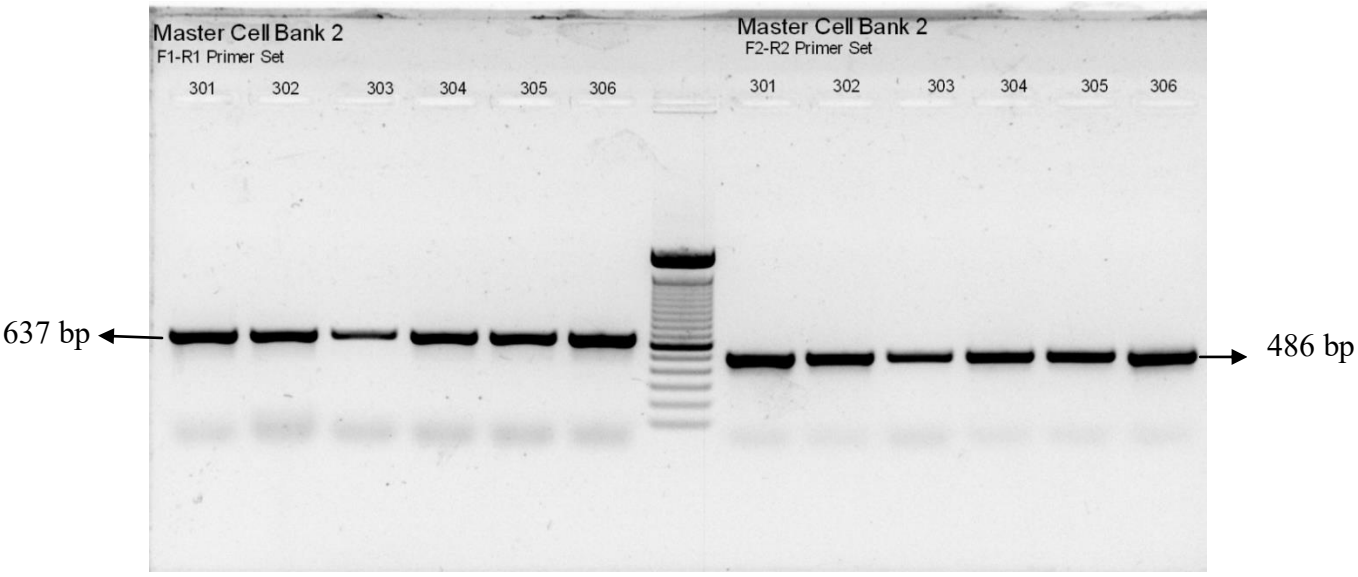



Figure 20: Gel image of the master cell bank using both F1R1 and F2R2 primers; where 301-306 are sample numbers


Figure 23: these are the sequences of the Working cell Bank for the HBV-F1primer; obtained using the Chromas software

Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

| Description | Max Score | Total Score | Query Cover | E value | Per. Ident | Accession |
|---|---|---|---|---|---|---|
| Hepatitis B virus isolate 246HBV2013 polymerase (pol) gene, partial cds | 219 | 219 | 100% | 3e-53 | 93.38% | MH272597.1 |
| Hepatitis B virus isolate 2589_NL_2002 S protein (S) gene, partial cds | 213 | 213 | 100% | 1e-51 | 92.72% | MH521492.1 |
| Hepatitis B virus isolate 2467_NL_2002 S protein (S) gene, partial cds | 213 | 213 | 100% | 1e-51 | 92.72% | MH521415.1 |
| Hepatitis B virus isolate 4357_NL_2004 S protein (S) gene, partial cds | 213 | 213 | 100% | 1e-51 | 92.72% | MH521350.1 |
| Hepatitis B virus isolate 2335_NL_2002 S protein (S) gene, partial cds | 213 | 213 | 100% | 1e-51 | 92.72% | MH521329.1 |
| Hepatitis B virus isolate FL10, complete genome | 213 | 213 | 100% | 1e-51 | 92.72% | KT749832.1 |
| Hepatitis B virus isolate 666, complete genome | 213 | 213 | 100% | 1e-51 | 92.72% | MF772360.1 |

Figure 25: Hit Lists for MCB sequence for HBV-F1 primer

Sequence of Master Cell Bank for HBV-F2 primer:

>ACAAATCTAAACTCGTGGTGGACTTCTCTCATTTTCTAGGGGGATCTCCCGTGTGTCTT
GGCCAAAATTCGCAGTCCCCAACCTCCAATCACTCACCAACCTCCTGTCCTCCAATTTG
TCCTGGTTATCGCTGGATGTGTCTGCGGCGTTTTATCATATTCCTCTTCATCCTGCTGCT
ATGCCTCATCTTCTTATTGGTTCTTCTGGATTATCAAGGTATGTTGCCCGTTTGTCCTCT
AATTCCAGGATCAACAACAACCAGTACGGGACCATGCAAAACCTGCACGACTCCTGCT
CAAGGCAACTCTATGTTTCCCTCATGTTGCTGTACAAAACCTACGGATGGAAATTGCAC
CTGTATTCCCATCCCATCGTCCTGGGCTTTCGCAAAATACCTATGGGAGTGGGCCTCAG
TCCGTTTCTCTTGGCTCAGTTTACTAGTGCCATTTG

This sequence was used to find out the significant match for it using the BLAST tool. The top hit lists are as follows:

Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

| Description | Max Score | Total Score | Query Cover | E value | Per. Ident | Accession |
|---|---|---|---|---|---|---|
| Hepatitis B virus isolate 92491_NL_2004 S protein (S) gene, partial cds | 811 | 811 | 98% | 0.0 | 99.55% | KX659966.1 |
| Hepatitis B virus isolate Cuba45 polymerase (P) and large S protein (S) genes, partial cds | 811 | 811 | 98% | 0.0 | 99.55% | KM606881.1 |
| Hepatitis B virus isolate Cuba100a polymerase (P) and large S protein (S) genes, partial cds | 811 | 811 | 98% | 0.0 | 99.55% | KM606843.1 |
| Hepatitis B virus isolate C133 from Belgium, complete genome | 811 | 811 | 98% | 0.0 | 99.55% | EU859938.1 |
| Hepatitis B virus isolate MSM25-04 HBsAg gene, partial cds | 811 | 811 | 98% | 0.0 | 99.55% | DQ988438.1 |
| Hepatitis B virus isolate MSM13-95 pre-S2 protein gene, partial cds | 811 | 811 | 100% | 0.0 | 99.12% | DQ631602.1 |
| Hepatitis B virus clone pEco63, complete genome | 811 | 811 | 98% | 0.0 | 99.55% | AY128092.1 |
| Synthetic construct Hepatitis B virus 1.28-mer overlength sequence | 811 | 811 | 98% | 0.0 | 99.55% | AF305422.1 |

Figure 26: Hit Lists for MCB sequence for HBV-F2 primer

Sequence of Working Cell Bank for HBV-F1 primer::

>TGCGGGGTTTTTCTTCGTTGACAGGAATCCTCTAATACCGCATAGTCTATACTCGTGGT
GGACTTCTCTCAATTTTCTAGGGGGATCTCCCGTGTGTCTTGTCCTTTGTCGCAGTCCCC
AACCTCCGATCACTCATAACCTCCTGTCCTCCAATTTGTCCTGGTTATCGCTGGATGTGT
CTGCCGCGTTTTATCCTATTCCTCTTCTCCCGGCTGCTATGCCTGATCTTCTTATGGGTT
CTTCTGAATTAGCAATGTAAGTAGCCCGTTTGTCCTCTAATTCCAGGATCAAAAAAAAC
CATTACAGGACCATGCATAACCTGCACAACTCCTGCAATAGGCAACTCTATGTTTCCCT
CATGTTGCTGTACAAAACCTACTTATGGAAATTGACCTGTATTCCCATCCCAACGTCCT
GGACTTTCACTAAATACCTATAGGAGTGGGCCTCAGTCCGTTTCTCTTGGATCAGTTTA
CTACTGCCATTTGTTCAGTGGTTCGGAGAGCTTTCCCGCACTGTTTAGCTTTCAGCTATA
TAGATGATGCTGGTATTGCGGGGCAACCTGATACATCTTCTCGAATGCCC

This sequence was used to find out the significant match for it using the BLAST tool. The top hit lists are as follows:

Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

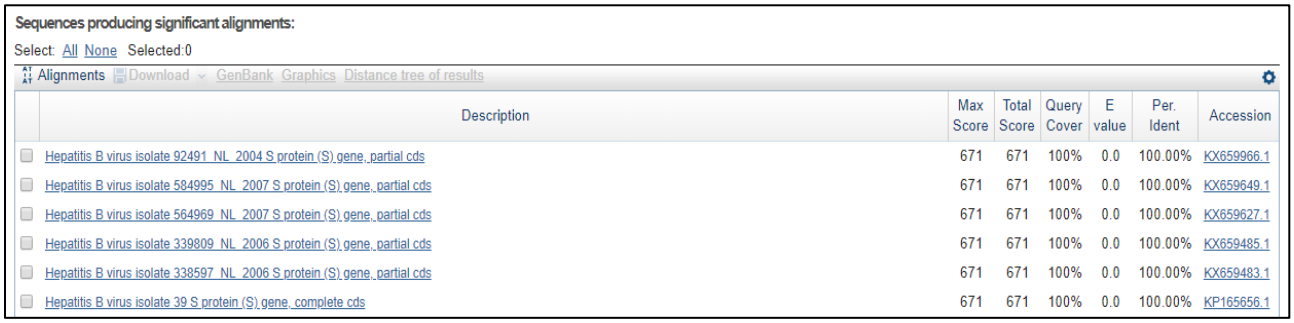
| Description | Max Score | Total Score | Query Cover | E value | Per. Ident | Accession |
|---|---|---|---|---|---|---|
| Hepatitis B virus isolate 92409_NL_2004 S protein (S) gene, partial cds | 712 | 712 | 95% | 0.0 | 90.44% | KX659965.1 |
| Hepatitis B virus isolate 117873_NL_2004 S protein (S) gene, partial cds | 712 | 712 | 95% | 0.0 | 90.44% | KX659188.1 |
| Hepatitis B virus isolate Cuba45 polymerase (P) and large S protein (S) genes, partial cds | 712 | 712 | 95% | 0.0 | 90.44% | KM606881.1 |
| Hepatitis B virus isolate Cuba100a polymerase (P) and large S protein (S) genes, partial cds | 712 | 712 | 95% | 0.0 | 90.44% | KM606843.1 |
| Hepatitis B virus S gene, partial cds, isolate: M8 | 712 | 712 | 95% | 0.0 | 90.44% | AB809490.1 |
| Hepatitis B virus isolate PM1-C10 polymerase gene, complete cds | 712 | 712 | 95% | 0.0 | 90.44% | JQ707532.1 |
| Hepatitis B virus isolate MSM32-04 HBsAg gene, partial cds | 712 | 712 | 95% | 0.0 | 90.44% | DQ988393.1 |
| Hepatitis B virus isolate MSM24-04 HBsAg gene, partial cds | 712 | 712 | 95% | 0.0 | 90.44% | DQ988369.1 |
| Hepatitis B virus HBsAg gene for surface antigen, partial cds, isolate: patient 1 | 712 | 712 | 95% | 0.0 | 90.44% | AB289692.1 |
| Hepatitis B virus clone pEco63, complete genome | 712 | 712 | 95% | 0.0 | 90.44% | AY128092.1 |
| Synthetic construct Hepatitis B virus 1.28-mer overlength sequence | 712 | 712 | 95% | 0.0 | 90.44% | AF305422.1 |

Figure 27: Hit Lists for WCB sequence for HBV-F1 primer

Sequence of Working Cell Bank for HBV-2 primer::

TCAAAATCTAGACTCGTGGTGGACTTCTCTCAATTTTCTAGGGGGATCTCCCGTGTGTC
TTGGCCAAAATTCGCAGTCCCCAACCTCCAATCACTCACCAACCTCCTGTCCTCCAATT
TGTCCTGGTTATCGCTGGATGTGTCTGCGGCGTTTTATCATATTCCTCTTCATCCTGCTG
CTATGCCTCATCTTCTTATTGGTTCTTCTGGATTATCAAGGTATGTTGCCCGTTTGTCCT
CTAATTCCAGGATCAACAACAACCAGTACGGGACCATGCAAAACCTGCACGACTCCTG
CTCAAGGCAACTCTATGTTTCCCTCATGTTGCTGTACAAAACCTACGGATGGAAATTGC
ACCTGTATTCCCATCCCATCGTCCTGGGCTTTCGCAAAATACCTATGGGAGTGGGCCTC
AATCCGTTTCTCTTGGCTCAATTTACTAGTGCCATTTGTTCATTGGTTCATAAAATAACT
CTAACCTG

This sequence was used to find out the significant match for it using the BLAST tool. The top hit lists are as follows



**Sequences producing significant alignments:**

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

| Description | Max Score | Total Score | Query Cover | E value | Per. Ident | Accession |
|---|---|---|---|---|---|---|
| Hepatitis B virus isolate 92491_NL_2004 S protein (S) gene, partial cds | 671 | 671 | 100% | 0.0 | 100.00% | KX659966.1 |
| Hepatitis B virus isolate 584995_NL_2007 S protein (S) gene, partial cds | 671 | 671 | 100% | 0.0 | 100.00% | KX659649.1 |
| Hepatitis B virus isolate 564969_NL_2007 S protein (S) gene, partial cds | 671 | 671 | 100% | 0.0 | 100.00% | KX659627.1 |
| Hepatitis B virus isolate 339809_NL_2006 S protein (S) gene, partial cds | 671 | 671 | 100% | 0.0 | 100.00% | KX659485.1 |
| Hepatitis B virus isolate 338597_NL_2006 S protein (S) gene, partial cds | 671 | 671 | 100% | 0.0 | 100.00% | KX659483.1 |
| Hepatitis B virus isolate 39 S protein (S) gene, complete cds | 671 | 671 | 100% | 0.0 | 100.00% | KP165656.1 |

Figure 28: Hit Lists for WCB sequence for HBV-F2 primer

# CHAPTER -4

# DISCUSSION

The thesis project was basically done in two different parts. One is the characterization of the cell banks using the phenotype testing like the microbiological purity testing, identity testing, and viability testing. The other part was the genotype testing which included the DNA extraction, PCR amplification and obtaining a specific size of band through the Gel Electrophoresis technique and getting the sequence using the Sanger sequencing method. The whole project was done to ensure the recombination of the HBsAg into the *P. pastoris* plasmid vector and also, to analyze the stability and the purity of the strain at the same time. All of these analyses were done successfully through the thesis project in the following manner.

While growing the yeast on the YPD media and on MMH media for their identity testing, no sign of contamination was seen. Only the desired yeast *P. pastoris* was grown in both of the media. White-colored small round colonies were observed. Any other type of colonies was not present. This indicates that in spite of being stored at -80°C, the yeast strain was able to utilize the Methanol in the media as their sole carbon source and survived without the presence of added Histidine in the media. The yeast *P. pastoris* is even capable of utilizing methanol as its sole carbon source and secrete desired product in the media. The increased methanol feed rate resulted in a proportionally increased specific rate of product secretion to the medium (Jahic et al., 2002). It indicates that in the presence of high amount of methanol, *P. pastoris* is able to utilize it more and can grow more. This is because the majority of *P. pastoris* processes described so far utilize methanol as a substrate and inducer for heterologous protein production (Mattanovich et al., 2009).

At the same time, no sign of contamination was seen in the FTM and SCD media after the growth of the yeast. This was justified by visualizing the colonies under a microscope. It was observed that only very small, spherical-shaped cells were grown. Some of the cells were also in the state of budding as well. So, the morphological characteristics indicate the yeast strain was in a pure form. However, several secreted *P. pastoris* proteins can be observed as contaminants in culture supernatants and required elaborate product purification and analytical effort. A detailed characterization of the secretome would significantly improve production and quality control of

biopharmaceuticals produced with this expression system (Mattanovich et al., 2009). Luckily, this troubleshooting was not observed during our experiment.

Viability testing, showed no significant decrease in viability. From the results, it was seen that for the MCB the number of single colonies decreased in approximately four times after each dilution. For WCB the number of single colonies decreased in approximately three times after each dilution. So, the cells were able to retain their viability despite being stored at -80°C. The Phenotyping analysis was a success.

For the genotype testing part, the main aim was to find out the desired band size of the inserted genes and the sequences of those gene segments. The AOX1 promoter is a strong promoter but it is repressed by unlimited growth on glycerol (Jahic et al., 2002). So, the DNA extraction procedure was done using the BMMY broth media rather than the BMGY broth media, as the BMGY broth media contains glycerol. After the PCR amplification of the specific genes (the HBsAg gene) using two different sets of primer; F1R1 and F2R2 the products were purified followed by a quantification process. These purified PCR products went through the Gel Electrophoresis and the bands were analyzed using the IMAGE LAB software. From the gel images (figure 19 and 20) it was clearly seen that the band size of the gene region of WCB amplified by F1R1 was approximately 637bp. The band size of the gene region of MCB amplified by F1R1 was also approximately 637 bp. The Pharmaceutical Company estimated that the desired band size would be 680 bp. As the band size obtained was somewhat near to 680 bp, the bands were of HBsAg gene's. However, the band size of the gene region of WCB amplified by F2R2 was approximately 486 bp. It was the same for the band size of the gene region of MCB amplified by F2R2. The estimated size of the bands was 480 bp for the regions amplified by the F2R2 primers. So, the genes were the HBsAg genes.

So, to prove and ensure that the genes were of the HBsAg the sequences of those regions were obtained and compared with other sequences using the BLAST tool. After the sequences went through BLAST, it was observed that these sequences are similar to that of the sequences of the Hepatitis B surface antigen genes. This was proven by comparing the obtained sequences with the Hit lists of the sequences (figure 25, 26, 27 and 28). So, the genotype testing was also a success because the HBsAg gene was successfully inserted.

# CHAPTER-5

# CONCLUSION

Our thesis project could prove and recommend that the HBsAg gene was successfully inserted and recombined into the plasmid vector of the given strain of *P. pastoris.* Also, it could prove the viability, identity, and purity of the strain were retained after being stored at -80°C. So, the characterization of the strain including the microbiological analysis and the genotype testing was done prevalently.

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