Addressing lipid impurities in *Pichia pastoris* cells producing virus like particles: genetic and bioprocess studies

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I, Sushobhan Kalyankumar Bandyopadhyay, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Signed:.....

Date:.....26th August 2019.....

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Abstract

Production of hepatitis B surface antigen (HBsAg) virus like particles (VLPs) occurs intracellularly in *Pichia pastoris* (*P. pastoris*). During extraction by cell disruption, host cell DNA and lipid impurities are released into the feed stream fouling the downstream process membranes and matrices. Effect of commercial triacylglycerol *Candida rugosa* (*C. rugosa*) lipase (CRL) on *P. pastoris* homogenate is investigated. *P. pastoris* homogenate was treated with CRL (0.0001 to 0.1 mg/mL) resulting in removal of triacylglycerol and increase in throughput by 60%, during constant flux filtration using 0.45µm polyethylene sulfone single layer disc membrane at 800 litres per square meter per hour. A strategy was employed where *C. rugosa* lipase 3 (CRL lip3) gene was localized in three different cellular locations (cytosol, endoplasmic reticulum, and cell surface) and encoded them under the control of P_{ENO1} promoter alongside of the HBsAg gene under P_{AOX1}. Developed strains were tested for expression of HBsAg along with optimisation of potentially toxic intracellular CRL lip3 expression. The CRL lip3 production did not influence HBsAg expression. A cell growth and gene induction regimes were identified with CRL lip3 localised to cell cytosol which enabled maximal HBsAg expression with independent control and CRL lip3 expression to peak only at the end of cultivation.

Strains from the strategies which produced both, HBsAg as well as CRL lip3, were selected and further scaled-up to 0.25L bioreactor. The maximal sequential expression of HBsAg protein up to 20mg/L and CRL lip3 up to 150mg/L was confirmed. The formation of HBsAg VLP produced from co-expression culture was also confirmed using transmission electron microscopy. Downstream benefits of introducing lipases to VLP-containing *P. pastoris* homogenate and feasibility of cell engineering to produce CRL lip3 in a manner that does not otherwise compromise host cell performance was confirmed.

Impact statement

The work contained in this thesis is an attempt to address critical questions towards the process improvement in manufacturing of virus like particles (VLP) produced intracellularly in *P. pastoris*. The manufacturing of the VLP is complex which demands time and cost that occur during process development. There is often loss of performance during manufacturing due to fouling of microporous membranes and matrices that are used in solid-liquid separation and chromatography. The fouling issue is more evident during VLP manufacturing from yeast homogenate which increases the burden on manufacturing process and contributes to the high overall cost. This increase in cost ranges from 40% to 70% of the complete production cost which can ranges from £40M to £520M as per published report in 2017 (Plotkin *et al.*, 2017). Such high production costs are necessary for manufacturing of highly pure vaccines and proteins (impurities < 0.001%) in order to meet regulatory standards and specifications. The material obtained from the genetically engineered organism producing these vaccines has intrinsic variabilities which requires a high degree of process understanding to design processes sufficiently robust to most specifications from one batch to another. These leads to the overall development and validation of such manufacturing processes being a major cost and time component in the development of these products critical to global health and can lead to overall time to market up to 10 years.

Currently there are various techniques and materials used to tackle the fouling of process membrane and matrices which increases the overall throughput obtained from single process runs. Even though such materials such as diatomaceous earth filters exist these special materials contribute to the overall cost and are difficult to use in large scale process. This study will help researchers understand and devise strategies towards better routes for manufacturing of vaccines. The tools and techniques described here will enable the biotechnology industry to design efficient manufacturing platforms that can be used with already present manufacturing process settings.

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List of abbreviations and symbols

σ	Sigma
α-MF	Alpha mating factor secretion signal
ϵ	Epsilon
(v/v)	Volume per volume
(w/v)	Weight per volume
°C	Degree centigrade
4nP	Para-nitrophenol
A	Area
ADH1	Alcohol dehydrogenase (promoter)
AEBSF	4-benzenesulfonyl fluoride hydrochloride
AEX	Anion exchanger
AOX1	Alcohol oxidase (promoter)
B	Biotin
BCA	Bicinchoninic acid assay
BD	Below detection
bp	Base pair
BSA	Bovine serum albumin
BSM	Basal salt medium
CAD	Charged Aerosol Detector
CAD software	Computer-aided drafting software
CALB	*Candida antarctica* lipase B
CER	Carbon dioxide emission rate
CEX	cation exchanger
CHAPS	3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate hydrate
CIP	Clean in Place
CNC	Computer Numerical Control
CRL	*Candida rugosa* lipase
CRL lip3	*Candida rugosa* lipase 3
CV	Column Volume
D	Dextrose
Da	Dalton
DAG	Diacylglycerol
DAQ	Data acquisition
DCW	Dry cell weight
DMF	Dimethylformamide
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DO	Dissolved oxygen
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ELSD	Evaporating Light Scattering Detector
ENO1	Enolase (promoter)

ER	Endoplasmic reticulum
ESI MS	Electrospray Ionization Mass Spectrometry
EGFP	Enhanced Green Fluorescence Protein
FF	Fast Flow
FLO9	Flocculin protein
FT	Flow through
g	Gravity
GAP	Glyceraldehyde 3-phosphate dehydrogenase (promoter)
Glc	Glucose
GY	Glycerol
GPI	Glycosylphosphatidylinositol
GFP	Green Fluorescent Protein
H_2O	Water
HBsAg	Hepatitis B surface antigen
HCP	Host cell protein
HEPES	(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIC	Hydrophobic interaction chromatography
HPLC	High performance liquid chromatography
HPLC-MS	High performance liquid chromatography – mass spectrometry
HRP	Horseradish Peroxidase
HAS	Human serum albumin
ICL1	Isocitrate lyase (promoter)
IgG	Immunoglobulin G
IPA	Isopropyl alcohol
kDa	Kilodalton
Lab	Laboratory
LB	Lysogeny broth
LDS	Lithium dodecyl sulphate
LMH	Liters/m^2/hour
M	Molar
MAG	Monoglycerides
MCB	Master Cell Bank
MES	2-(N-morpholino) ethane sulfonic acid
Mins	Minutes
MOPS	3-(N-morpholino) propane sulfonic acid
MS	Mass Spectrometry
MTX	Methotrexate
MUB	4-Methylumbelliferyl substrate
$Mut^{(+,s,-)}$	Methanol utilization phenotype positive, slow or negative
MW	Molecular Weight
MWCO	Molecular Weight Cut Off
OD_{600nm}	Optical density at 600nm
ORF	Open Reading Frame
P_{AOX1}	Alcohol oxidase (promoter)
P_{ENO1}	Enolase (promoter)

P_{THI11}	Thiamine biosynthesis (promoter)
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline (buffer)
PBS-T	Phosphate Buffered Saline Tween (buffer)
PCR	Polymerase chain reaction
PDB	Protein Data Bank
PDMS	Polydimethylsiloxane
PEEK	Polyether ether ketone
PES	Polyethersulfone
pH	potential of hydrogen
PHO1	*P. pastoris* acid phosphatase (secretion signal)
PHO89	Phosphatase-responsive gene (promoter)
PIR	Protein internal repeats
PMSF	Phenyl methane sulphonyl fluoride
PSI	Pound-force per square inch
PTM_1	*P. pastoris* trace metals
RCF	Relative centrifugal force
RE	Restriction Enzyme
RIU	Relative intensity units
RO	Reverse-osmosis (water)
RP-HPLC	Reversed-Phase High-Performance Liquid Chromatography
RPM	Rotations per minute
S	Sorbitol
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SLM	Standard liter per minute
SPV	Sulfo-phospho vanillin
TAG	Triglyceride
TBE	Tris borate EDTA (buffer)
TBS	Tris-buffer saline (buffer)
TBST	Tris-buffer saline - tween (buffer)
TE	Tris & EDTA (buffer)
TEM	Transmission electron microscopy
TFA	Trifluoroacetic acid
THI11	Thiamine biosynthesis (promoter)
Tris	Tris aminomethane
USD	Ultra-scale down
V	Volume
V5 CRL lip3	Simian virus 5 peptide tagged *Candida rugosa* lipase 3
VLP	Virus Like Particle
vvm	Volume per volume per minute
WCB	Working cell bank
WCW	Wet cell weight
WT	wild type
YNB	Yeast nitrogen base
YPD	Yeast extract Peptone Dextrose

1 Introduction

1.1 Background and production of biologicals in *Pichia pastoris*

The modern biopharmaceutical industry largely comprises of biological molecules derived from recombinant DNA technology. Bacterial and fungal species have made a major contribution to the success of these biopharmaceuticals. Fungal species like methylotrophic yeast *Pichia pastoris* (*P. pastoris*) was first developed by Philips Petroleum Company in the 1960s. It was later contracted to Salk Institute Biotechnology Industrial Associates Inc. (SIBIA, La Jolla, CA, USA) to develop *P. pastoris* as a heterologous protein expression system. Since then *P. pastoris* has been widely used for production of biologicals (Serrano-Cinca *et al.* , 2005). These biologicals range from small peptides to large self-assembling virus like particles (VLPs) including high value vaccines like HBsAg VLPs, ENGERIX® B (Glaxo-SmithKline) (Meehl and Stadheim, 2014; Wang *et al.* , 2017) and also include human papillomavirus (HPV) which causes cervical cancer. Two such well known vaccines produced in yeast are Gardasil™ (Merck) and Cervarix™ (Glaxo-SmithKline). *P. pastoris* is used to produce vaccines as it capable of synthesising proteins in its native conformations and required immunogenicity. Due to such capability of synthesising proteins, *P. pastoris* and other yeasts are widely used to manufacture vaccine candidates (Wang *et al.*, 2016).

The recombinantly produced biologicals are processed and purified before they can be fit for human use. Certain difficulties involving impurities are faced during processing of these biologicals. Such impurities are cell debris, host cell DNA and host cell lipids which adhere to process membranes and matrices resulting into fouling of these membranes and matrices. For instance, it is seen that most of the products in case of VLP purification gets lost during clarification along with cell debris. The overall recovery in VLP production is found to be only 10%, with loss due to co-precipitation and unassembled capsid proteins. To improve purification, ammonium sulphate and polyethylene glycol (PEG) precipitation are carried out as described by Josefsberg and Buckland, 2012. Nevertheless, purification through PEG

precipitation gives rise to problems such as aggregation of nucleic acids during membrane filtration steps leading to inconsistent product recovery and purity.

The second difficulty is seen when the cells are disrupted and host cell protein, DNA and lipids along with carbohydrates and proteins in form of impurities, effects the downstream processing of required product which is released along with these impurities. To remove nucleic acid impurities, nucleases like benzonase have been in used in earlier studies (Balasundaram *et al.* , 2009). DNA impurities have been addressed by use of engineered cells which can release nuclease enzyme during cell lysis and hence reduce the viscosity of the overall cell lysate. The change in viscosity gives better recovery with higher purity and clarification performance during the process. Addition of chemicals like nucleases adds cost to the overall process and their removal poses new challenges during scale-up. The problem of having high levels of lipids and DNA contamination in feed stock or cell lysate still needs to be addressed to improve downstream yields and purity of the VLPs (Pattenden *et al.* , 2005).

Yeast lipids majorly consist of non-polar lipids which are stored in compartment referred as lipid particles, lipid droplet or oily bodies. They form the building blocks for membrane lipid synthesis and are stored as biologically inert form of fatty acids and sterols (Grillitsch *et al.* , 2011). It has also been found that yeast culture conditions change the percentage of accumulation of triacylglycerol's in the cell (Grillitsch *et al.* , 2014; Koch *et al.* , 2014). The degradation of lipids in yeast cells occurs by hydrolysis of triacylglycerol and sterol esters by lipases and hydrolases respectively, playing an important role in lipid metabolism. The importance of lipid fouling on process membranes and matrices needs to be studied. To reduce the lipid fouling, the triacylglycerol lipases can be used, which in turn will help in process improvement.

1.2 Fouling of process membranes and matrices during bioprocessing of intracellular *P. pastoris* products

After cell disruption the host cell DNA makes the supernatant very viscous and in turn contributes to reduced yield by blocking the membranes. Reducing viscosity gives better recovery of the product. This is normally achieved using enzymes to degrade the DNA. But the use of enzymes necessitates the use of methods for their subsequent removal, and furthermore it becomes expensive to use large quantities of purified enzymes, increasing the overall cost of production.

A study by Balasundaram *et al.,* 2009 shows that it may be possible to combine both enzymatic degradation of native lipids and VLP expression to reduce intrinsic DNA levels. By use of a specific cell targeting mechanism and synthetic biology techniques, the enzyme of interest can be targeted to specific regions of the cell. Such cell targeting keeps the enzyme active and at the same time does not allow it to interfere with the formation of the VLPs. The authors described that by utilising this approach, the viscosity of the feed material changed considerably, which contributed to better purity and clarity during the VLPs purification process (Balasundaram *et al.* , 2009). This gives rise to a scenario where enzymes such as lipase can be co-expressed with VLPs. Although it also raises new questions including what would be the effect of such co-expression on the cell and in turn, how this might affect the yield of the product of interest. Also, it should be noted that the lipase might inhibit neutral lipid formation.

These neutral lipids may be needed for VLP formation owing to the lipid membrane coating on the capsid. Additionally, commercially available lipases can be secreted by the host cells and are purified from the supernatant of the harvested cell broth. It is to be noted that maintaining the enzymes inside the host cells during production becomes critical in such an approach. This is particularly advantageous when the cells are washed and then homogenized

in the preferred medium. By doing so, the lipid degrading enzymes will not be lost and can so act under the desired condition.

Further study is required to show if this approach is not only cost effective but also sufficiently reduces lipid-associated fouling during vaccine manufacture. It has been reported by Bracewell *et. al.* 2008, that fouling during the primary recovery step in the manufacturing process has a significant effect on intracellular product purification. It was found that due to fouling during the clarification step, the resulting feed used for chromatography separation posed difficulties. To take into consideration and attempt to overcome such fouling the size of the guard filters as well as the chromatography column are increased. Fouling was also found to affect the binding capacities of the purification column, thus indicating that a more clarified supernatant would require a smaller column and be more efficient. Further investigation confirmed that lipid aggregation was the main cause of the fouling, associated with less clarification during the primary recovery step (Bracewell *et al.* , 2008).

Though these studies sought to understand fouling during bio-manufacturing, similar problems are faced during the recovery and purification of VLPs in their manufacturing processes. VLPs produced in yeast, generally are recovered using disruption techniques along with use of detergent. Such methods, however, generate large quantities of fouling contaminants. These contaminants not only foul the membranes during primary recovery and reduce the yield of overall VLP recovery during this step but also foul the subsequent columns used in the chromatography steps.

Other than host cell DNA, lipid impurities pose a considerable amount of fouling to membranes and purification resins. Lipid fouling occurring during downstream processing, can not only give rise to process related issues such as back pressure, decreased flux rates and increase the total process time, but also effects the dynamic binding capacities in resins. Being hydrophobic in nature lipids can even alter the ligand properties of the resin itself. Hence these give rise to inconsistent elution profiles and can cause a major issue in reproducibility of the purification process. It has been shown that a higher proportion of lipid reduces the binding

of VLPs to a hydrophobic interaction column. Here one of the most common chromatography columns, hydrophobic interaction chromatography, was used for purification of VLPs. It was found that performance such as binding capacity of the chromatography column was severely affected by the lipid impurities. Even after repeated clean-in-place cycles to remove the lipid impurities, the recovery of the VLPs was found to be reduced by 70% over 40 cycles. This study showed that the host cell lipids were the major cause of the fouling problem in VLP purification (Jin *et al.* , 2010). The fouling effect can be visualised using confocal microscopy where the lipids were dyed using a BODIPY 493/503 dye having a specificity to lipids. The fouling of Sepharose beads after loading the column with lipid rich feed and dying the beads with BODIPY dye is shown in Figure 1.1.

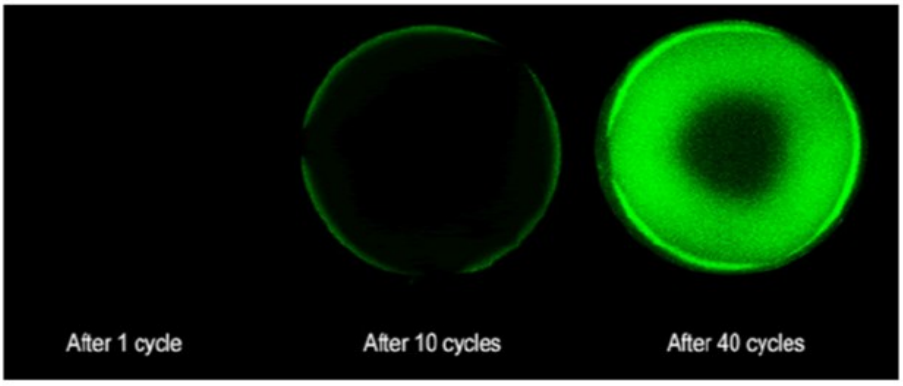


Figure 1.1: Confocal image of Butyl-S 6 FF Sepharose beads shown after repeated loading cycles using a BODIPY 493/503 dye (Jin *et al.* , 2010).

The advantages of removal of fouling agents like lipids before purification was also studied using monolith purification columns. Monolith columns were used to observe and test the purification efficiency under various binding conditions. The impact of the crude material was visualised using confocal microscopy which revealed that the lipid layers formed on the top sections of the monolith column. The lipids were also found to travel through the column hence fouling the entire column. The lipid was removed using amberlite beads which would absorb the lipid from the feed stream. But this also resulted in loss of 20% VLP. The application of lipid removal before the purification did improve the recovery and binding of the VLPs to the columns (Burden *et al.* , 2012). It was shown that removal of lipids before the

column purification greatly improved the performance of the step and reduced the amount and rate of the fouling process. The study suggested that the method of lipid removal should be studied in further detail.

1.2.1 Current understanding of HBsAg production in *P. pastoris*

A recent study shows how the commonly known Hepatitis B surface antigen (HBsAg) are intracellularly produced in yeast. It is not well understood how the VLPs are assembled. However as shown in transmission electron microscopy studies, the VLPs forms tubular structures along with monomer units of the particles in the endoplasmic reticulum. These tubular structures ultimately dissociate to form complete VLPs during further downstream processing (Lünsdorf *et al.* , 2011). This process of HBsAg forming VLPs and then getting released when cell is disrupted after the production is shown in Figure 1.2. Considering such hypothesis, the HBsAg gene was cloned under P_{AOX1} and transfected in *P. pastoris* cells.

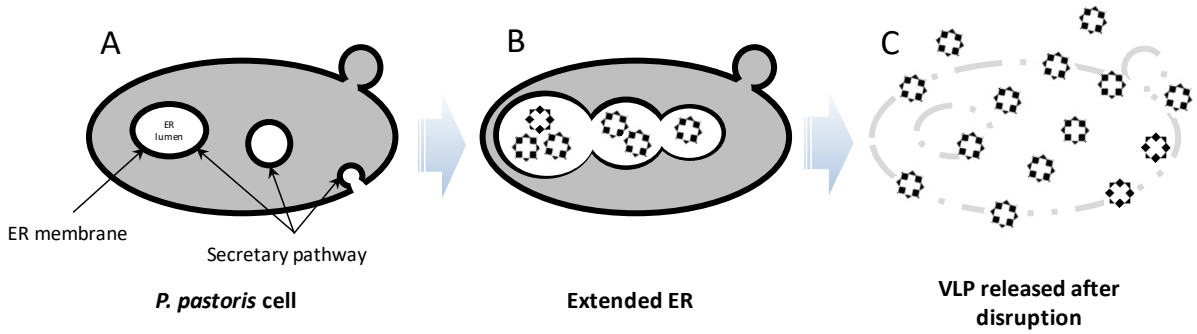


Figure 1.2: HBsAg expression leading to post homogenization VLP formation in *P. pastoris*

The expression of HBsAg in P. pastoris (A) shows the normal yeast cell with circles showing the endoplasmic reticulum (ER) and a representation of the secretory pathway. (B) The distended ER HBsAg is produced and forms subunits in the ER region as shown in a previous study by Lünsdorf et al., 2011 on the production of HBsAg and its localization in the cell. (C) The disruption of yeast cell and release of HBsAg VLP subunits into the yeast disrupted media.

1.2.1.1 Production and purification of HBsAg from *P. pastoris*

Virus like particle vaccines are divided into two major classes, namely enveloped and non-enveloped VLPs. These VLPs are produced in various expression systems including bacterial,

yeast, insect cells mammalian cells, plant cells and cell free systems (Kushnir *et al.* , 2012). HBsAg VLPs fall under the non-enveloped category of vaccines. They are majorly produced in yeast (*P. pastoris* and *Hansenula polymorpha*) and marketed by companies like Merck and Glaxo-SmithKline (GSK).

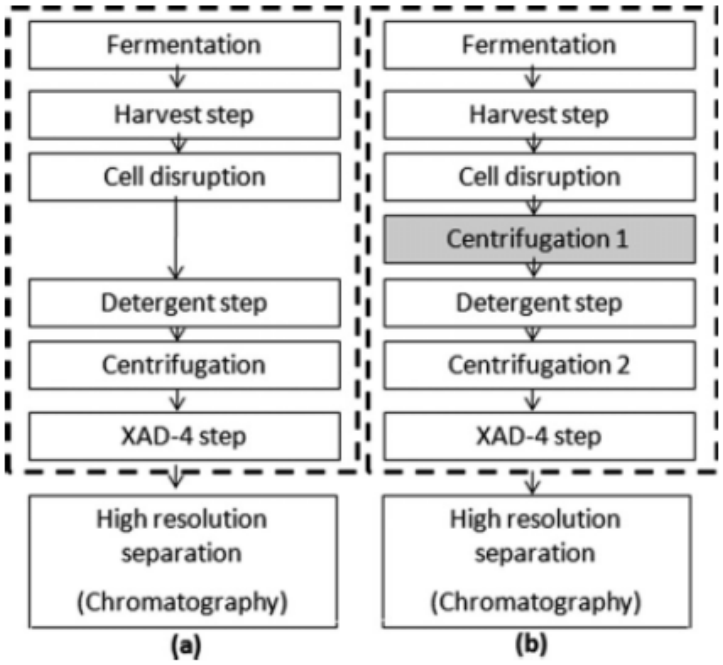


Figure 1.3: Conventional process steps undertaken for production of HBsAg VLPs (Kee *et al.* , 2010).

The production of HBsAg conventionally under taken includes cell disruption by homogenization followed by detergent steps and clarification as highlighted by the dotted line in the above Figure1.3. HBsAg VLPs are then extracted from the clarified product with the help of detergent wash using Triton-X 100 and then by using anion ion beads (XAD-4) before purifying the product with the used of traditional chromatography matrices (Kee *et al.* , 2010).

It has been reported that HBsAg antigens are formed and accumulated in the ER of the host cell. Although it has been suggested that the VLP assembly takes place after the disruption and extraction of the HBsAg subunits from the host cells (Gurramkonda *et al.* , 2013).

Gurramkonda *et al.,* 2013 showed in their study the high-density batch production of HBsAg with *P. pastoris* under Alcohol oxidase I promoter (P_{AOX1}). P_{AOX1} is the most popular

but strictly-regulated methanol inducible promoter for heterologous protein expression in *P. pastoris*. Methanol was continuous feed under fed batch conditions with a maximum concentration of ~7 grams of HBsAg per litre of culture was obtained. Out of which only 2.3 grams per litre of soluble HBsAg was recovered (Gurramkonda *et al.* , 2009). This study showed the optimal conditions for the VLP production and extraction from yeast cells which has been used in the present work.

It is evident from the previous mentioned study that for the extraction of soluble HBsAg subunits after cell disruption, the use of triton X-100 is required. The cell disruption at 600bar using a high-pressure homogenizer along with presence of EDTA/detergent is shown to be optimal for the extraction of HBsAg VLPs from the yeast cells as shown by Zahid *et al.* , 2015.

1.3 Methods for lipid quantification from *P. pastoris*

With the recent interest in lipids as contaminants and fouling agents to the filter membranes, there are various methods used to help understand the process of fouling and to develop possible solution to address such issues. To do so one needs to quantify the lipids of interest, methods of which, are discussed below.

1.3.1 Colorimetric methods for lipid quantification

The main colorimetric methods used for quantification of total lipids from bacterial, fungal as well as eukaryotic cells are the Nile red dye binding assay and the sulfo-phospho vanillin (SPV) method. Nile red assay has the advantage of being used to stain live cells which can then be studied under the microscope for observing the localization of lipid molecules inside the cells. This is particularly advantageous when investigating metabolic pathways of cells. An example of Nile red assay methodology is given by Poli *et al.* 2014 Isopropanol and Nile Red in concentrations of 5% and 500 µg/L was used to stain yeast cells in PBS-KCl buffer.

When the Nile red quantification technique was compared with more traditional techniques like the gravimetric method, similar results were obtained. The authors also suggested that this method not only uses less organic solvent but can also be used as a fast screening method. Another study used a different type of solvent system for the fluorescent lipophilic dye Nile Red for the detection and quantification of intracellular lipid molecules. In this experimental setup a rapid screen is possible by using dimethyl sulfoxide (DMSO). This solvent improves the cell permeability and eliminates the need for subsequent washing steps. This method was also used on a broad variety of ascomycete and basidiomycete yeast species and was validated against classical gravimetric methods (Sitepu *et al.* , 2012).

An alternative method for the quantification of the total lipid content colorimetrically is Sulfo-phospho-vanillin method. This method has been used in various instances for the quantitative measurement of lipids within algal cultures. A study by Mishra *et al.*, 2014 showed that the lipids from algal culture reacted with sulphuric acid to form an adduct which then binds with the vanillin to give rise to a distinctive pink coloration. This was then quantified using a spectrophotometer at 530nm wavelength. In the same study the method was used on various fungal species namely *Chlorella vulgaris*, Monoraphidium *sp.*, *Ettlia sp.* and *Nannochloropsis oceanica.* where a large amount of lipid accumulation in the cell was observed. The results were validated and tested using gas chromatography as an orthogonal technique (Mishra *et al.* , 2014).

In another study the high throughput analysis of total lipids was achieved by using 96-well plates which showed that the method does not need large amount of sample and multiple numbers of samples can be analysed at a time. It also helps in correction of background absorbance with consistent colour formation hence giving better accuracy in the results. This approach was also tested on a variety of samples and it was seen that there was no difference between the results obtained from the macro-gravitation methods and the developed colorimetric method (Cheng *et al.* , 2011). In summary, yeast neutral lipids are quantified using various colorimetric methods like Nile red and SPV method. But these methods have their own drawbacks along with the advantages. Even though they are easy and can be used on live

cells in case of Nile red they cannot be used for identification and quantification of the lipid classes from yeast cells. For the proper elucidations of more sophisticated techniques like HPLC with Evaporating Light Scattering Detector (ELSD) or Charged Aerosol Detector (CAD) along with mass spectrometry has been used.

1.3.2 HPLC methods for lipid quantification

The quantification of a large range of lipid classes has been done using high performance liquid chromatography with evaporative light-scattering detection (ELSD). Multiple studies have investigated different solvents to obtain optimal separation between lipid classes. The chromatography performance obtained from these combinations of solvents was tested on samples derived from rat liver, heart, kidney and brain. This method is found to be suitable for separation of various lipid classes with relatively simple analytical technique like HPLC-ELSD (Homan and Anderson, 1998). It was found that the major classes of lipids from animal tissues vary in their polarity from cholesterols to phosphatidylcholines. Such differences were difficult to quantify using HPLC methods. Better quantification was done using HPLC-MS method (Christie, 1985).

Seeking to identify triacylglycerols and phospholipids using mass spectrometry Grillitsch *et al.* described that in *Saccharomyces cerevisiae* there are multiple combinations of acyl chains in the triacylglycerol and phospholipids which are based on major fatty acids found in yeast (Grillitsch *et al.* , 2011; Ivashov *et al.* , 2013).

The complexity in analysing the samples increases when more classes of lipids are identified from yeast cell lines. A shotgun approach has been used to identify and quantify as many as 250 species of lipids which covered about 21 major lipid classes in *S. cerevisiae* (Ejsing *et al.* , 2009). The method of HPLC with an Evaporating Light Scattering Detector has been compared with electrospray ionization tandem mass spectrometry. Others have identified and developed methods which involve HPLC with ELSD and HPLC with ESI MS/MS methods. Using these techniques, they were able to identify the monoacetylated and diacetylated fatty acids and sophorolipids produced by yeast species (Ribeiro *et al.* , 2012).

The use of HPLC along with mass spectrometry has not only been used to study lipids classes from animal tissue but it has also been used for studying standard commercial oils that are used in the food industry. Lin *et al.* elucidated that ten-molecular species of diacylglycerols and 74 triacylglycerols species could be identified using mass spectrometry along with liquid chromatography. In this analysis new hydroxyl fatty acids were identified. The classes of lipids where compared between two commercially available oil and differences in hydroxyl fatty acids were found. Such differences can only be identified by using sensitive techniques like mass spectrometry along with HPLC (Lin and Chen, 2014).

The quantification of lipids that are responsible for fouling is one of the approaches to study membrane fouling. This can also be investigated using confocal microscopy, as demonstrated by a study into the impact of yeast and *Escherichia coli* fouling on cross flow filtration. A difference in fouling was seen between yeast suspensions and fouling caused by bacterial culture mixed with fungal cells. The resistance varied significantly with the concentration and characteristics of the fouling material. It is also suggests that such device can be useful in studying the cake formation or characteristics of the fouling cake (Hassan *et al.* , 2013).

1.3.3 Use of enzymes for removal of impurities from process feed

Lipases, being the major enzyme responsible for hydrolysis of lipids are of great importance in cellular metabolism. As these enzymes hydrolyse lipids, they can be used to remove lipid contaminants which are found intrinsically in yeast cells. Microbial lipases act on various substrates, are highly active and stable in organic solvents. Due to these properties, they are used in various processes which include the production of detergents, paper, biodiesel, biopolymers, pharmaceutical derivatives, and food processing. But these industries are still limited by the high cost of lipase production and there is a need for highly active and stable lipases which can be used in pharmaceutical processes. A review by Anobom *et al.,* 2014 describes the present scenario in biotechnological applications of lipases and various methods

that are used to create a more active and selective bio catalytic enzyme, improving the biotechnology applications based on lipases (Anobom *et al.* , 2014).

1.3.3.1 Bacterial lipase and the structural importance for its activity

The importance and function of a lipase is very much related to the structure and mechanism of action. An example of a lipase from a yeast species is *Candida antarctica* lipase B (CALB) which hydrolyses the carboxyl ester bonds of the triacylglycerol at low temperatures. The information on the function of these lipases has been studied using computational techniques by Ganjalikhany *et al.,* 2012 where it is demonstrated that temperature affects the flexibility of the lid region of CALB. The open-closed conformation was studied with simulations ranging up to 60ns at various temperature conditions (Ganjalikhany *et al.* , 2012). It was found that the conformation is open at lower temperatures and closed at higher temperatures between 35 °C and 50 °C. Furthermore, it was found that these structural changes were due to the movement of a helical structure and its side chains. This suggested that the function of the lipases might be due to the structural movement in the domain near the active site of the molecule.

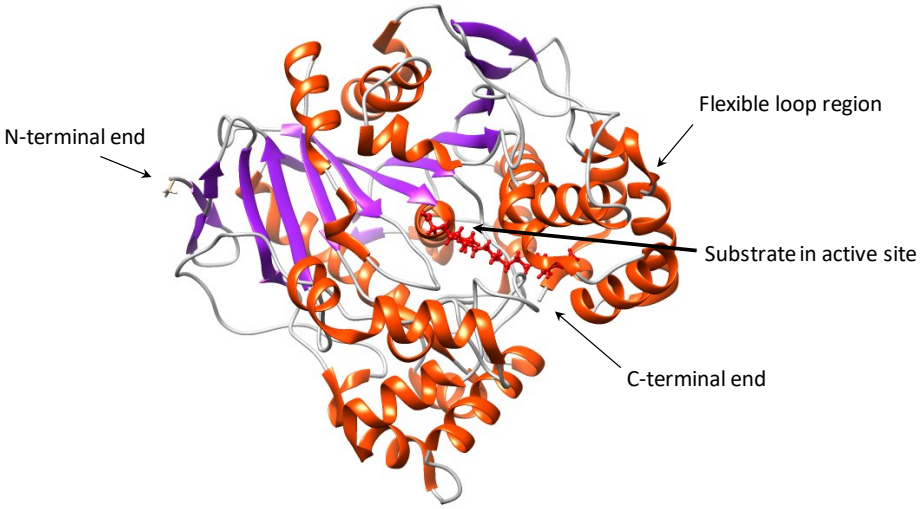


Figure 1.4: Crystal structure of *C. rugosa* lipase showing the active site near to the C-terminal end of the protein

The crystal structure of C. rugosa lipase obtained from protein structure database (PDB-1LLF). As active site of C. rugosa lipase enzyme is found near to the C-terminal region of the protein, this suggests that the C-terminal might interact with the activity of the enzyme.

The crystal structure of lipases showed that at the catalytic site there is a well-defined lid as well as a hydrolase domain which take part in the catalytic reaction. This region is defined by a set of amino acid residues including Ser184, Asp334 and His366. When this structure is compared with other known structures the similarities in the domains was seen. It was also found that the fatty acid molecule interacts near to the catalytic site. This suggested that there is a conformational change required for interfacial activation (Ericsson *et al.* , 2008).

The overall study stresses that lipase action is dependent on the structural changes that occur during hydrolysis of triacylglycerols. Also, it should be noted that lipase action needs the enzyme to be in solvent liquid interface. In case of lipid hydrolysis, the lipase enzyme acts best when it has access to the hydrophobic fatty acid chain which is associated near the catalytic site during enzyme reaction (Uppenberg *et al.* , 1995). Here lipase acts on the bonds between oxygen and carbon of triacylglycerol, in the presence of water molecules, generating glycerol and fatty acid as shown in Figure 1.5.

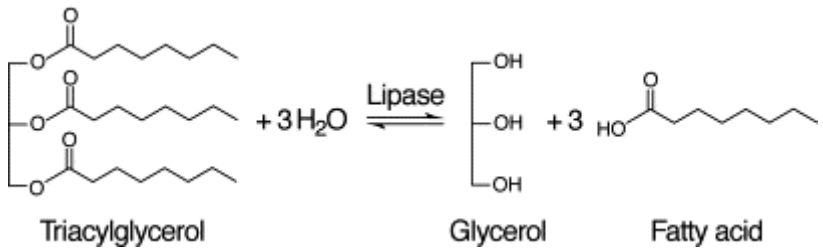


Figure 1.5: A schematic diagram of reaction mechanism of triglyceride lipase giving rise to glycerol and fatty acid after hydrolysis(Nirit U. and Kiryat-Tivon, 2011)
Lipase acts on triacylglycerol in presence of water (H_2O) producing one glycerol molecule and three fatty acid molecules. The above Figure is a representation of the chemical reaction occurring, where the lipase acts on the carboxyl linkages of the triacylglycerols.

Various crystal structure has been solved to describe the active site of lipases from different species. A detailed structural view showing the active site from *Pseudomonas aeruginosa* in a model built using the X-ray coordinates has been determined by Schrag *et al.* (Schrag *et al.* , 1997). In another study on *C. antarctica* lipase, the enzyme was crystallized to explain the mechanism of action and their ability to hydrolyse the triacylglycerol's in the aqueous - oil

interface. It is known that that there were no sequence similarities between lipases from *C. antarctica* and other lipases belonging to the sample class of proteins. The active site sequence was found to be conserved between all the lipases. The study by Uppenberg *et al.* , 1994 concluded that the catalytic domain had an open and closed confirmation, and this restricts the access of the active site to the substrate molecules. It is believed that due to this restriction there is a high degree of substrate specificity and high stereo-specificity (Uppenberg *et al.* , 1994). Even though the *C. antarctica* lipase sequences has no sequence similarity, the conserved active site and confirmations of the catalytic domain helps in its activity. In case of *Candida rugosa*, whose structure and function has also been studied shows conserved active site sequence. It is evident from the crystal structure shown in Figure 1.6 (B) that the catalytic domain of the protein is near the C-terminal region of the protein (Pletnev *et al.* , 2003).

Recently, studies have been conducted for selection of C-terminal and N-terminal secretion tags for strategies like surface display outlined in the minireview by Tanaka and Kondo, 2015. The surface display technique has been used for whole cell bio-catalysis as reviewed by Kuroda *et al.* (Kuroda and Ueda, 2013). These secretion tags are a topic of interest and they are synthesised along with the protein sequence considering the proximity of the active site of the protein to the terminal sequences. A schematic diagram for different options of surface display strategy used for different surface display applications are shown in the following Figure 1.6.

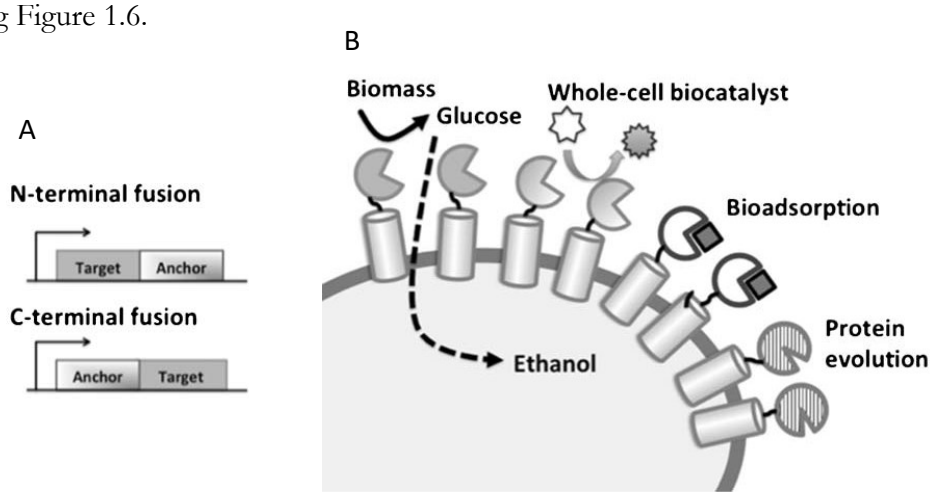


Figure 1.6: Yeast surface display strategies and applications (Tanaka *et al.*, 2012)

The schematic diagram (A) shows the two major strategies for the placement of attachment for surface display, namely N-terminal and C-terminal. The arrow shows the position of the promoter sequence used for transcription of the proteins. (B) shows the different surface display strategies where the protein is anchored to the cell surface and can be used as whole cell biocatalyst or bio-adsorption or for protein evolution. For construction of a strain consisting of an active surface display, the anchor tag sequence is kept away from the active site of the protein of interest.

In the present study, for the ease of detection, V5 epitope tag from simian virus had been used. The position of the tag is very important in obtaining an active enzyme. Various studies suggested that the tag should be used in the N-terminal region of the protein so that it does not interact with the C-terminal end having the active site residues. The following Figure 1.7 describes in detail the active site residues and possible position for the V5 epitope tag.

An example of production of lipase in yeast can be found from the study done by Rotticci-Mulder *et al.,* 2001 where the genes coding for the enzyme where cloned under the control of alcohol oxidase promoter. The gene was expressed such that the proteins were secreted and purified for further study (Rotticci-Mulder *et al.*, 2001). These are examples of lipase production in *P. pastoris* where the enzyme is secreted in culture broth. In the context of the present problem, the enzyme of interest needs to be localized inside the cell, for example in the periplasmic space. For this, protein expression needs to be controlled and hence it would require to be under the control of promoter, which is itself highly regulated and subjected to induction to activate it.

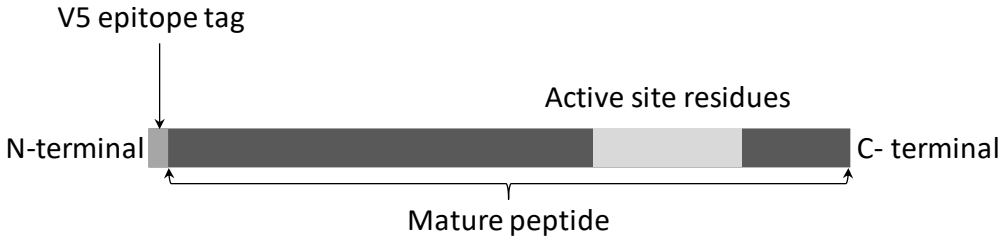


Figure 1.7: Insertion of V5 epitope tag away from active site of *C. rugosa* lipase
The above schematic diagram shows the active site residues of C. rugosa lipase protein near the C-terminal end of the protein and possible site for V5 tag integration towards the N-terminal ends of the protein. To reduce the possibility of interaction of V5 tag sequence

with the active site of the protein, the protein is cloned with the V5 tag in the N-terminal region.

Other types of lipases like lipase A from yeast species of *C. antarctica* have also been studied and both classical fed-batch production and semi-continuous production have been studied. It was pointed out by Pfeffer *et al.* 2006 that the hydrophilic ester in the cell culture broth reduces the activity of the enzymes significantly. It was also shown that the highest activity of the purified enzyme is found at temperatures as high as 50 and 70 °C in neutral pH (Pfeffer *et al.*, 2006). Again, these processes of production and purification were developed for active and purified enzymes. Therefore, enzyme activity needs to be studied during the process conditions, which might vary. Furthermore, optimisation of lipase activity needs to be within the parameters necessary for VLP production. These are the few notable points for co-expression of lipase enzymes to remove downstream fouling products in vaccine manufacturing processes.

1.4 Expression of heterologous proteins in *P. pastoris*

P. pastoris expression system is not only used for therapeutic protein but also used for expression of various other heterologous proteins like lipases. Large classes of lipases are expressed and studied in *P. pastoris* under various inducible as well as constitutive promoters (Huang *et al.* , 2014; Valero, 2012). Alcohol oxidase I (AOX 1) promoter is a remarkably strong and tightly regulated by methanol, is the most popular promoter that is used for expression of heterologous proteins in *P. pastoris*. Though P_{AOX1} promoter has favourable properties and industrial importance its transcriptional regulatory mechanism has not been clarified. Fine-tuning of heterologous gene expression is necessary for maximization of protein expression level. And developing an efficient strategy for optimizing promoter activity should be based on a better understanding of regulatory mechanism of promoter (Yang *et al.*, 2018). Strong promoters such as P_{AOX1} can produce up 22g/L intracellular heterologous proteins without hampering the cellular metabolism. It is also reported that P_{AOX1} induction requires the

complete lack of a repressing carbon source and the presence of the inducer methanol, the current regulatory model involves three regulatory states. Catabolite repression suppresses expression if a repressing carbon source is present (Cregg *et al.* , 1989; Vogl *et al.* , 2013). This means that the promoter is repressed still in presence of alternative carbon source and as soon as the repressing carbon source such a glucose is depleted the promoter P_{AOX1} reaches a state of de-repression. This property can be used as a synthetic switch for selectively producing heterologous protein of interest under different carbon sources. HBsAg has also been produced under other promoter's system like glyceraldehydes-3-phosphate dehydrogenase (GAP) promoter, P_{GAP1} as shown in the study done by Vassileva *et al.* , 2001.

1.4.1 Alternative *P. pastoris* promoters – P_{ENO1} and P_{THI11}

Like the strong promoters AOX1 and GAP catalyses the other novel promoters that are also used for the expression of heterologous protein production (Vogl *et al.* , 2013; Vogl and Glieder, 2013). In *Saccharomyces cerevisiae*, there are two genes, ENO1 and ENO2, coding for enolase that share 98% amino acid similarity and 95% identity, and it is shown to have functions of glycolysis using the enzyme enolase and has an unique function in *S. cerevisiae* (Kornblatt *et al.*, 2013; Mcalister and Holland, 1982). Enolase 1 promoter, P_{ENO1} is classified as a constitute promoter and it is triggered in presence of glycerol and is supressed in the presence of methanol or sorbitol (Uemura *et al.* , 1985; Ahmad *et al.* , 2014). P_{ENO1} is defined as a carbon source dependent promoter and shows activity in all carbon sources (Mecklenbräuker, 2011; Stadlmayr *et al.* , 2010). The strength of the promoter is about 20-70% of that of the GAP promoter. As these novel promoters do not use the conventional methanol carbon sources they become relatively lucrative for the use of dual expression system (Çalik *et al.* , 2015). P_{ENO1} is fundamentally different from P_{AOX1} as P_{ENO1} functions as constitute promoter and not as an inducible promoter.

Another novel promoter like the P_{ENO1}, and the vitamin-sensitive promoter THI11, P_{THI11} is characterized as a constitutive promoter and a vitamin sensitive promoter respectively. This can be controlled independent of the carbon, nitrogen or phosphor sources. (Landes *et*

al. , 2016; Stadlmayr *et al.* , 2010). The thiamine biosynthesis gene under the P_{THI11} does not show high transcript levels at low specific growth rate, but it exhibits interesting regulatory properties dependent on the availability of thiamine in the growth medium (Stadlmayr *et al.*, 2010). The promoter is derived from the gene sequence involved in the biosynthesis of thiamine and under non-repressing conditions this promoter has a constitutive expression pattern with growth rate dependent product formation. Due to this, the promoter can be used to repress the formation of the production with exogenous addition of thiamine into the feed media. The repressible promoters are of interest as they can be used to create negative feedback mechanism where the promoter can be repressed in the presence of a specific inducer (Delic *et al.* , 2013). P_{THI11} is fundamentally different from P_{AOX1} and P_{GAP1} commonly used promoters as P_{THI11} functions as repressible promoter and not as an inducible or a constitutive promoter.

1.4.2 Dual production of heterologous recombinant protein strategies in *P. pastoris*

Dual promoter has been used in other studies to produce α-amylase and phytase under GAP and AOX1 promoters (Parashar and Satyanarayana, 2016). In this study it was shown that the use of two different promoters is useful in producing higher titres of two other heterologous proteins in *P. pastoris*. The strategy of having two different heterologous proteins can also be used for the improvement of production process as well as increasing the titre. In recent reviews Öztürk *et al.* describes the double promoters has been used for the increasing the transcriptional capacity. It also has been indicated that it is essential to balance the need of two promoters and hence there is a need for the development of optimal fermentation conditions (Öztürk *et al.* , 2017). This strategy gives a distinct advantage of production of dual heterologous proteins under the same culture condition.

1.4.3 Cell engineering strategies for cell localization

When two heterologous proteins are expressed by the host cell, they are either secreted or are retained by the cell and expressed intracellularly. The intracellularly expressed proteins might give rise to toxicity in host organism like *P. pastoris* as protein of interest are retained by the cell. When two such heterologous proteins are intracellularly expressed by the host cell, a strategy could be to keep the proteins of interest in separate location of the cell, specific signal peptide for such cellular localization. This has been described by Mudassar Ahmad and his colleagues, 2014 in the Figure 1.8.

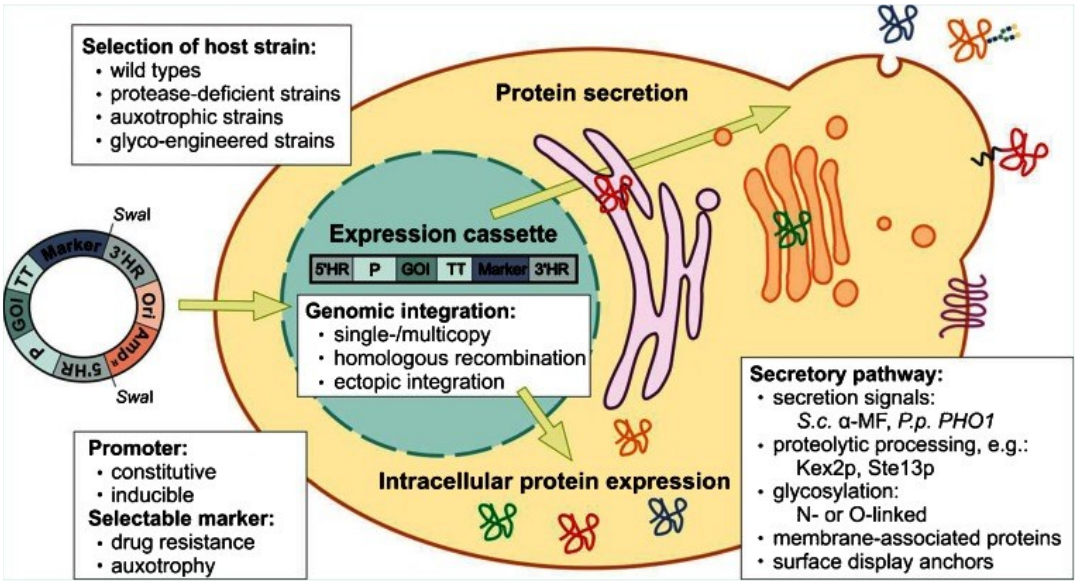


Figure 1.8: Cellular localization of recombinant proteins in *P. pastoris* (Ahmad *et al*., 2014).

The above Figure shows the general consideration for heterologous protein expression in P. pastoris. The gene of interest (GOI) is linearized prior to transformation in a plasmid of interest consisting of markers for integration. A representative promoter (P) and transcription terminator (TT) pair are shown. Depending on the marker the gene of interest is localized in cytosol or undergoes modification with proper signal sequence to intracellular or secretory expression. They can also be integrated into the cell membrane or be membrane anchoring.

In the present study three different locations of the host cell are targeted. These locations are cytosol, endoplasmic reticulum and cell surface of the host cell. The following

sections are a few examples where researchers have used these localization strategies for improved cell culture expression of recombinant heterologous proteins in yeast.

1.4.3.1 Cellular localization of heterologous protein in cytosol of *P. pastoris*

Heterologous protein expressed recombinantly in *P. pastoris* can be localized in the cytoplasm of the host cell. Previously studied by Wortmann *et al.* were the group had expressed green fluorescent protein (GFP) protein and localized it in the cytoplasm of yeast when both the GFP as well as human serum albumin (HSA) was expressed (Wartmann *et al.* , 2002). The GFP protein did not have any targeting sequence, hence the protein was expected to be accumulated in the cytoplasm of the transformants. Whereas the human serum albumin (HSA) containing the secretion sequence accumulated in the supernatant of the cell culture fluid.

Similar example can also be seen with the work done by Zhang *et al.*, where the transformants producing *Candida antarctica* lipase B was expressed with Glycosylphosphatidyl-inositol (GPI) anchor sequence. With number of transformants screen some showed retention of the *C. antarctica* lipase B in the cytoplasm of host *P. pastoris* cells (Zhang *et al.* , 2013). The transformants were confirmed with western blot analysis for the localization of the *C. antarctica* lipase B protein.

1.4.3.2 Cellular localization of heterologous protein in endoplasmic reticulum of *P. pastoris*

Signal peptides can be used for targeting proteins in specific regions of the host cell during the expression of recombinant proteins. This was shown by a study done by Schonberger *et al.* They described how the protein of interest, cloned in a eukaryotic system, is targeted to the secretory pathway and the periplasmic space of yeast. In this study they were able to model how the translocation from the nucleus into the endoplasmic reticulum modified the protein of interest with glycosylation before ultimately reaching the periplasmic space (Schonberger *et al.* , 1996). Similar to the cytosolic localization of the recombinant protein in *P. pastoris,* other cellular locations are also of interest. For the retention of expressed protein in the endoplasmic reticulum of the host cell, KDEL signal peptides are used. Medina-Godoy *et*

al. used the KDEL signal sequence tagged to 11S seed globulin pro-amaranthine protein to retain the protein of interest in the endoplasmic reticulum of *P. pastoris* (Medina-Godoy *et al.* , 2006).

1.4.3.3 Cellular localization of heterologous proteins in cell surface in *P. pastoris*

For the localization of heterologous protein on the cell surface of the *P. pastoris*, various tags and anchor sequences has been studied. Schonberger *et al.,* 1996 used the Glycosylphosphatidylinositol (GPI) anchor for surface display of the expressed protein. This expression and targeting did not affect protein function (Schonberger *et al.* , 1996). This study raises the possibility of having desired protein expression and trafficking into the periplasmic space using the secretory pathway. However, it has been reported that in the secretory pathway of proteins such as invertase enzymes the non-native luminal protein is degraded (Hong *et al.* ,1996). This suggests that thermodynamically unstable proteins having amino acid substitutions might be degraded by the internal translocation mechanism present in yeast cells. Thus, there is a need to have properly folded protein of interest into the targeted region of the yeast cells for it to be fully active and functional.

Expressed protein of interest can be localized on the cell surface. To have such localization protein internal repeats (PIR) has been added to the mature peptide sequence. This addition of the signal sequence in-turn facilitates the localization of the expressed protein on cell surface. A study by Moura *et al.* 2015 showed the method of displaying lipase on the surface of the yeast cells. They have compared two different types of signal sequences obtained from genes FLO9 and PIR1 which was used to develop the surface display strain. Another study by Pal Khasa *et al.* , 2011b. showed the method for isolating the signal sequence form PIR1 gene which is responsible for the signalling of the protein to the cell surface has been isolated and compared (Moura *et al.* , 2015; Pal Khasa *et al.* , 2011b). Enzymes like trehalose synthase has also been localised to the cell surface of *P. pastoris* as elucidated by Yang and his colleagues using the PIR1 protein as the signalling peptide (Yang *et al.*, 2017). The localization of

expressed heterologous protein, with the help of target sequences, on different parts of the cell has been carefully considered and studied and elucidated in the following chapters.

It is known that the Protein Internal Repeats, protein family with the conserved four-cysteine domain is found as a group of cell wall proteins which are anchored to the yeast cell wall using β1,3-glucan. It is likely that the amino acids around this sequence act as a localization signal to the bud scar which is formed during the yeast cell division (Sumita *et al.*, 2005). These PIR anchor proteins which are highly O-glycosylated can be released from the cell wall using mild alkaline conditions of (30 mM NaOH at 4°C overnight). These PIR anchor proteins are different in comparison to its counterpart GPI cell wall anchor proteins. The PIR proteins are linked to the cell wall components using β1,3-glucan. The overall linkage of the PIR proteins can be given as a structure which corresponds to Pir-(protein of interest)-Ser/Thr-O-chain←β1,3-glucan (Kapteyn *et al.*, 1999). Another study suggests that, PIR proteins are found in *Debaryomyces hansenii* and *Kluyveromyces lactis*, like *Saccharomyces cerevisiae*, *Candida albicans*, *Candida glabrata,* and to the mycelial fungi with putative sequences from *Blumeria graminis*, *Gibberella zeae*, *Neurospora crassa*, and *Magnaporthe grisea* containing an aditional cysteine and the conserved domain located in the N-terminal region of the proteins (De Groot *et al.*, 2005). The PIR proteins are localized inside the chitin ring of bud scars, this remains at the surface of the mother cell after completion of the budding process. It has also been reported that strength of the linkage between Pir1p and the cell wall depends on the number of repetitive sequence (AAAISQIGDGQIQATTKT) units of 18-19 amino acids which are highly conserved in PIR class of proteins (Sumita *et al.*, 2005).

In the present study, the heterologous protein expression is achieved using a signal sequence to guide the protein of interest in intracellular locations, and membrane attachment as shown in Figure 1.7. As described earlier the lip3 lipase from *C. rugosa* consists of a C-terminal functional domain and to have the functional domain away from the cell surface attachment the PIR tag sequence is expressed along with lipase where the PIR tag sequence is placed at the N-terminal region of the lipase.

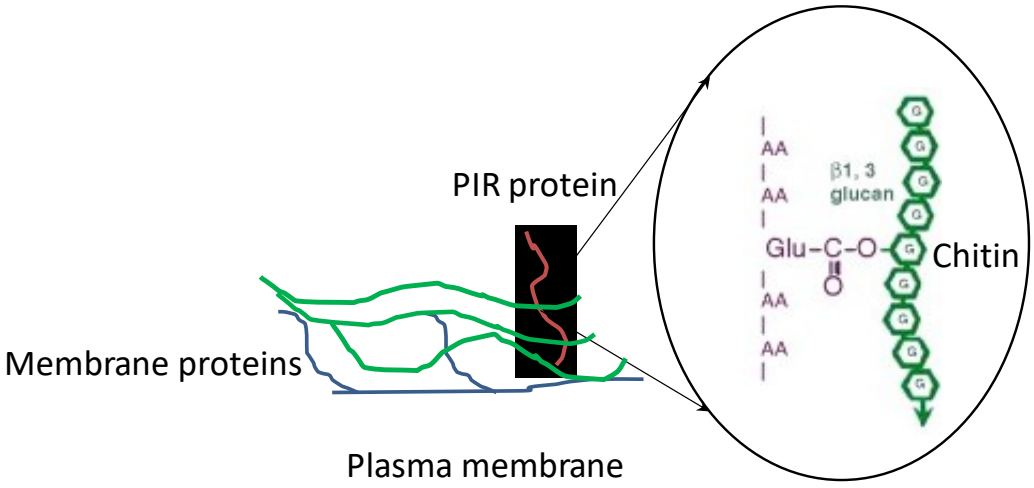


Figure 1.9: PIR attachment mechanism with β1,3-glucan (Gonzalez *et al*., 2009)
The schematic diagram shows the attachment residues of PIR protein (red line) with internal repeats that binds with β1,3-glucan in the chitin chain (green line) of the plasma membrane. Plasma membrane and the membrane proteins are shown in blue line.

1.5 Project significance and objectives

In the present manufacturing process for VLPs from yeast there are major difficulties in terms of recovery in the primary step. One of these difficulties for downstream processing and purification of VLPs, is due to the issue of fouling. The fouling agents are primarily from host cell lipid and DNA impurities. These contaminants should be removed to have better process recovery and yield of the product which will lead to lower cost production. Among the various strategies for removal of the impurities, enzymes that hydrolyse the lipids and DNA can be used. If they are produced within the host cells and are co-expressed, then it gives a unique opportunity for removal of the fouling agents.

Nevertheless, this approach needs further investigation to better understand the enzymatic mode of action and how this would affect the production process of VLPs. The removal of fouling agents such as neutral lipids from the yeast cells by using synthetic biology can also be achieved by creating host cell lines without the capability to synthesize lipids. This might create certain other issues as it might affect the cellular metabolism of the host cell. The development of a synthetic biology chassis can be used for production of VLP vaccines and

addresses downstream processing issues hence improving the cost and yield of the therapeutic product.

The ambition of the project is to study the performance of triacylglycerol lipids in typical bioprocess steps used for virus like particles production and to engineer the possible ways, of removing the host cell lipid impurities using expression of *C. rugosa* lip3 lipase in *P. pastoris*. To address this ambition there were four major objectives which are summarized as follows:

1. Chapter 3: Understanding the role of lipids in fouling during processing of intracellular VLP products. Model yeast-based process will be studied in a controlled manner representing a commercial VLP production. To remove the lipids from the process stream, exogenous lipase will be used which would in turn improve reduce the lipid fouling. A filter fouling test will be used to study the effect of fouling, along with quantification of lipid impurities to show removal of lipids by action of exogenous lipase.
2. Chapter 4: Design of a *P. pastoris* chassis for dual expression of HBsAg and *C. rugosa* lip3 lipase in *P. pastoris* cells. Design of this chassis will be done considering the localization of the *C. rugosa* lip3 lipase in three different cellular locations. The lipase will be expressed under two different promoters and HBsAg under AOX1 promoter. The design will also be done with V5 epitope tag which can be used for the detection of the expressed lipase. The genome integration of the constructed strains will be confirmed with antibiotic selection and polymerase chain reaction.
3. Chapter 5: To understand and characterize the novel strains that are created using the dual expression chassis. The expression of HBsAg producing strains in shake flask followed by bioreactor conditions will be tested. On the basis of the expression and functional characterization of the dual expression strains expressing both lipase and VLPs will be selected.
4. Chapter 6: The selected strains will be cultured to facilitate the production of heterologous proteins independently with different feed components. Independent production of lipase and VLP components will be then tested under controlled conditions

mimicking a bioreactor process. The work done to support the above objectives are described in the following chapters.

2 Materials and methods

2.1 Materials

2.1.1 Chemicals

All chemicals were purchased from Sigma-Aldrich (Gillingham, Dorset, UK) and of analytical grade unless otherwise specified. All the solvents used for the HPLC analysis were of HPLC grade.

2.1.2 In-silico design of constructs for *P. pastoris* strains

Pichia pastoris (*P. Pastoris*) expressing recombinant HBsAg under the control of AOX1 promoter and recombinant CRL lip3 under the control of P_{ENO1} promoter were used in this study (parent strain PPS9010 wild type was obtained from DNA2.0(ATUM)).

The HBsAg sequence was obtained from the GenBank database (J02205.1) and was synthesized by Eurogenetec and cloned into pJ902-15 (4252bp) shuttle vector. This pJ902-HBsAg (pJ9Sag) shuttle vector was then used for transformation of the gene into the parent strain PPS9010 wild type.

The *Candida rugosa* (*C. rugosa*) Lip3 sequence obtained from the GenBank database (X66006.1) along with the P_{ENO1} sequence (Uemura *et al.* , 1985) (1000bp upstream of the enolase gene), obtained from yeast genome database (www.yeastract.com), was synthesized and cloned into pJ902-HBsAg shuttle vector. The complete cassette containing P_{AOX1} sequence, HBsAg gene (Valenzuela Pablo, Gray Patrick, Quiroga Margarita, Zaldivar Josefina, Goodman Howard, 1979), P_{ENO1} sequence, P_{THI11} sequence and *C. rugosa* Lip3 sequence (Brocca *et al.* , 1998; Lee *et al.* , 1999; Pernas *et al.* , 2002) was then used for transformation into the parent PPS9010 wild type strain creating *P. pastoris* strain. In-silico designs for the plasmids were done using SnapGene software (from GSL Biotech; available at snapgene.com).

2.1.3 Construction of microfluidic device

Fabrication of the filtration device was done using SolidWorks CAD software (SolidWorks 2010, Dassault Systems SolidWorks, UK). Technical drawings as shown in the appendix was done and fabricated on polycarbonate sheets of 1.5mm using CNC micro-milling

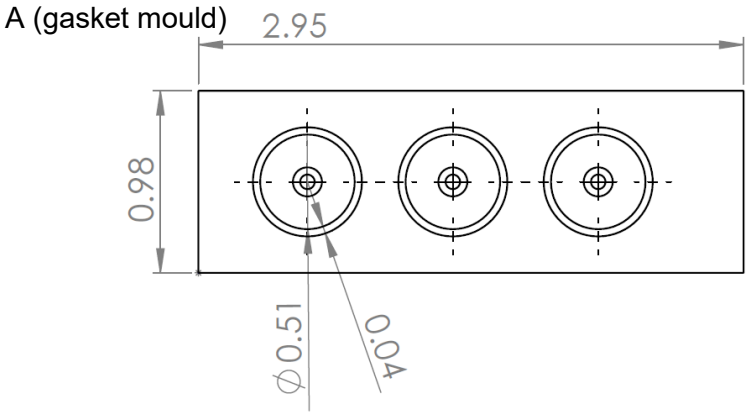
machine (M3400E, Folken Industries, USA) using 2-flute standard length end mills (Kyocera Micro Tools, USA). The G-code for the micro-milling machine was generated from the CAD files using MasterCam software (MasterCam X2, CNC Software, USA). There were two O-rings used in the device to prevent leakage and sealing of the filtration chamber. Structured poly (dimethylsiloxane) (PDMS) (Sylgard 184, Dow Corning, USA) parts were cast in moulds milled out of 5 mm thick Polycarbonate sheet. The monomer and curing agent were mixed at a 10:1 ratio (by weight), degassed in a vacuum desiccator to remove bubbles, poured into moulds, and degassed a second time. The mould was covered with a flat sheet of polycarbonate, clamped between aluminium plates, and cured at 90°C for 2 hours. Once cooled, parts were removed from the moulds and any excess PDMS trimmed away with a scalpel.

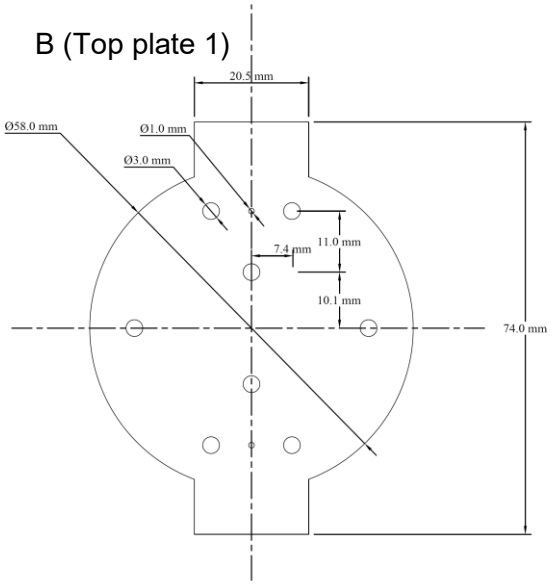
The method for making the PDMS O-rings was done as per previously described (Macown *et al.* , 2014). The device was then fitted with an array of screws which helped the membrane to remain in place also prevented any leakage. The inlet and the outlet were attached with Flangeless M6 Male connector with ferrule and peek tubing. A syringe pump was used to pump the feed material through the peek tubing into the device. The end of the syringe was attached with a 1/16 M6 male/female luer connector which was attached to the M6 Male connector with ferrule. A T-junction was used between the syringe pump and the inlet of the device. This was used to remove any air bubble in the line. The outlet at the other end also had the same M6 Male connector with ferrule and peek tubing connector. A beaker was used to collect the filtrate. A 13mm 0.45µm PES disc membrane from PALL was used as a filter in the device.

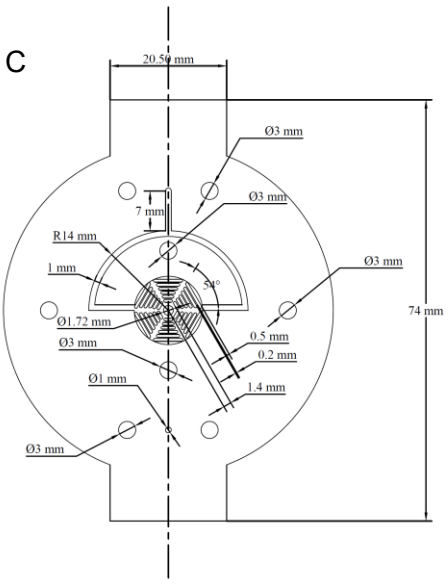
2.1.3.1 Microfluidic device design

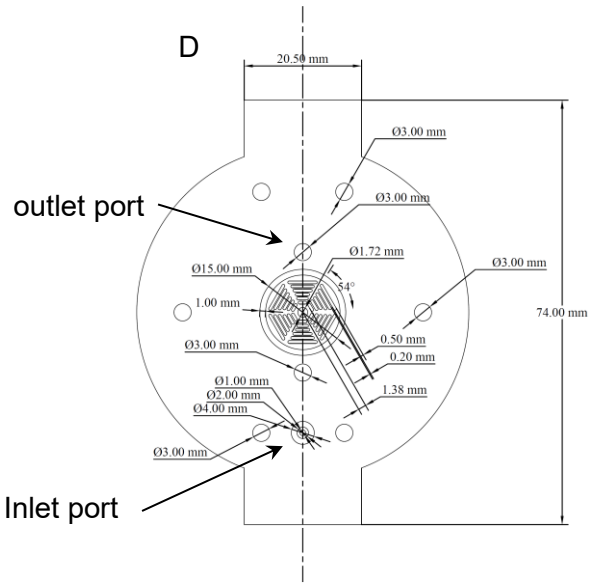
The following designs of microfluidic device dimensions given in inches. The gasket mould shown in Figure 2.1(A) was used to create the gaskets that were placed between the upper and the lower plates of microfluidic device. The parts of the device is shown in Figure

2.1(B-F). After the assembly of the microfluidic device the inlet and the outlet ports were used for attachment of a PEEK tubing for pumping the feed liquid as shown in Figure 2.2.









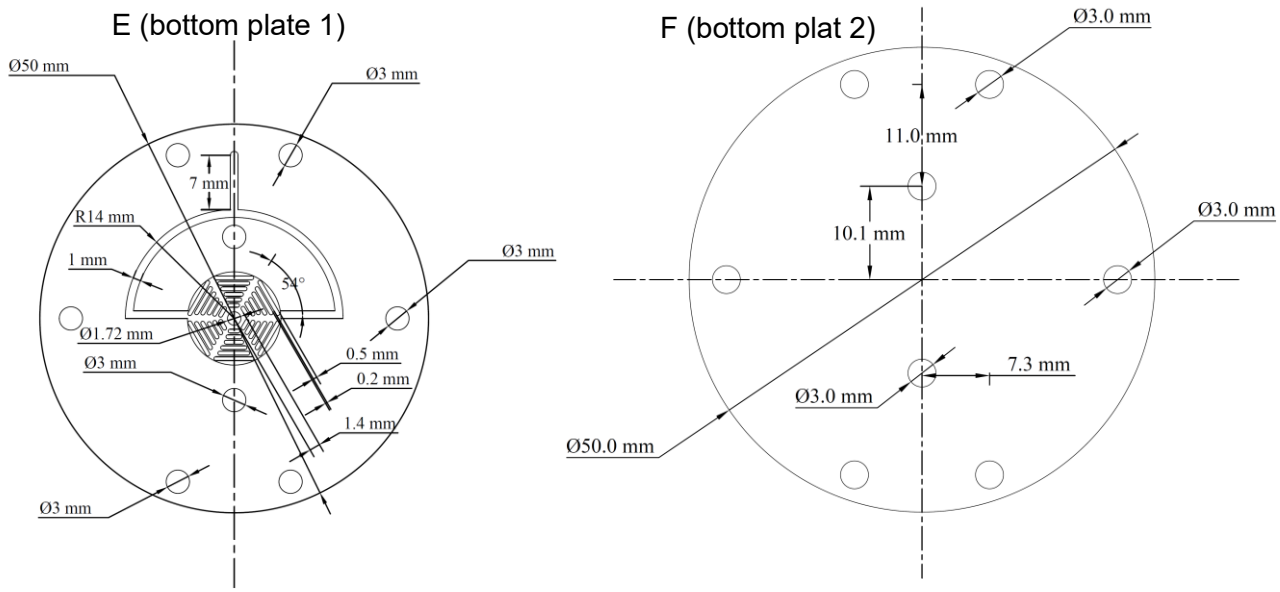


Figure 2.1: Gasket mould and the layers of the microfluidic device bottom plate to monitor membrane fouling under confocal microscope.

(A)The gasket mould was used for construction of O-rings for the microfluidic device which were used in between the upper plate (C) and the lower plate (D) for the outer ring. the inner smaller O-ring and the bigger O-rings were used in between the same plates to seal the gap produced by the membrane and to prevent leakage. The plates (B) and (F) were used as the top most and the bottom most plates. They were used for attachment of the screws in the outer edges and the inter two sides. The (C), (D) and (E) plates had hexagonal grids in the middle of the plate where the membrane is places and to support the membrane. The top half plates (C) and (D) had a flap on the two sides, this was designed specifically to fit in the Z-plate of a confocal microscope. The membrane is places in the centre of the assembly with the O-rings and hexagonal grid as supports and screws were used to fit all the layers together. The inlet and the outlet areas of the where the inlet and outlet tubing is attached for fluid flow is shown in plate (D).

After the assembly of the microfluidic device the plate could be mounted on the Z stage of the confocal microscope for imaging of the membrane fouling. Feed containing oleic acid and florescent BODIPY (493nm-503nm) dye (0.0001μg/mL) which binds specifically to the TAG was pumped into the microfluidic device. The tagged TAG when fouling the membrane could then be imaged using a confocal microscope. The confocal microscope images could be collected where the fouling of the membrane could be shown to occur in

stages and fouling of the membranes could be further elucidated. The fitting of the microfluidic device on the confocal microscope stage is shown in Figure 2.2.

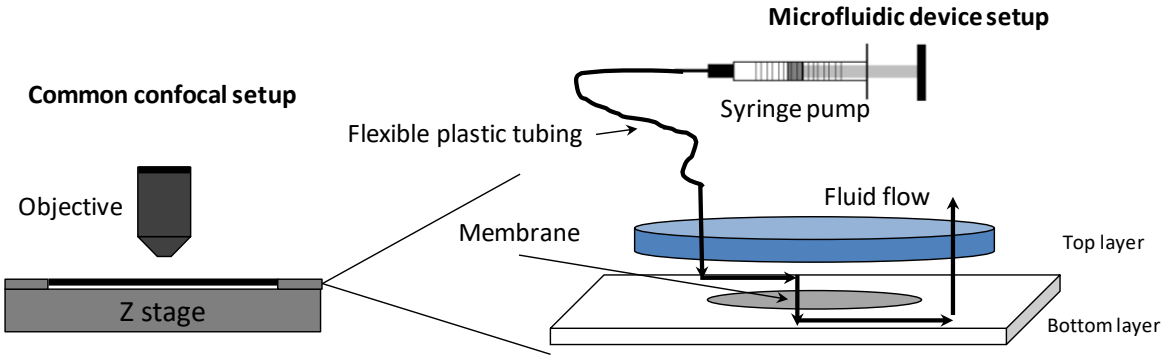


Figure 2.2: Confocal microscope microfluidic chip setup for study of dead-end filter fouling

Confocal microscope setup was fitted with the microfluidic device with 0.45µm PES membrane. The microfludic device was fixed on the z stage of the confocal microscope in such a manner that the membrane can be studied on the Z-axsis. The microfluidic device (Appendix I) had two main layers with the membrane residing between the two layers as shown in the schematic diagram. The P. pastoris cells were grown in YPD media overnight at 30°C, 250rpm before harvest and homogenization in 1x PBS medium. The harvested cells were centrifuged, and the supernatant was mixed with BODIPY (493nm-503nm) dye (0.0001µg/mL) before it is passed through the membrane using a syringe pump. The confocal micrscope was focused using an 10x air objective and the image was taken on the Z plane.

2.2 *P. pastoris* cultivation

2.2.1 Pichia pastoris cell bank

Cell bank preparation was done by plating the *P. pastoris* cells on YPD (yeast peptone dextrose) agar plates and grown at 30°C for 24 hours. The colonies were inoculated in 5mL of YPD media in 50mL flacon tubes and grown in the same conditions for another 24 hours. Two baffled shake flasks containing 50-100mL of YPD was then inoculated with 1mL from this overnight grown culture. One of the flasks was used for monitoring of the growth every 2 hours. These flasks were incubated at 30°C and agitated at 250rpm in an orbital shaker. When

the OD_{600nm} of the cell culture reached mid-exponential phase (OD_{600nm} ~20) 30mL of sterile glycerol was added to the shake flask and mixed well. The resulting mixture was then aliquoted into 1mL cryo-vials and stored at -80°C. Working cell banks were prepared using the same procedure for liquid culture by inoculating 1mL of the Master Cell Bank in 250mL of YPD medium in a baffled shake flask. The resulting culture was mixed with sterile glycerol and aliquoted in cryo-vials as mentioned above.

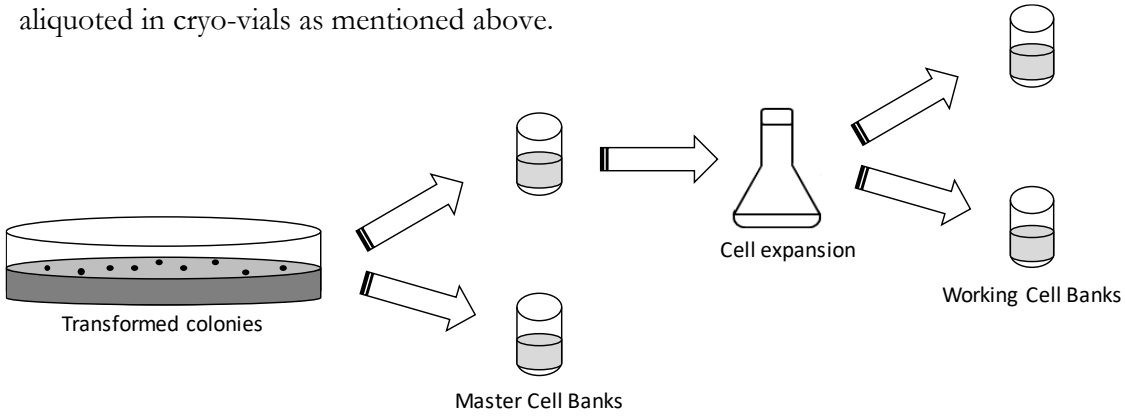


Figure 2.3: Growth and expansion of transformed *P. pastoris* for cell bank preparation
The above Figure shows the transformed colonies were grown on YPD agar plates containing of 1000µg/mL of zeocin. The colonies were picked and grown in 50mL YPD media containing 1000µg/mL of zeocin to make master cell banks. Initial experiments were done using one set of these working cell banks the rest were stored at -80°C as glycerol stocks. The working cell banks were then further expanded in fresh sterile YPD media and glycerol stocks were prepared from these expanded cells. The working cell bank obtained from the expanded cell were used in further experiments.

2.2.2 Shake flask fermentation

Yeast extract was obtained from Merck and bacterial peptone from BD Difco labs. 40% sterile filtered dextrose stock was prepared for the YPD media. Glycerol and other culture media were prepared from Sigma Chemicals. Culture media was prepared from protocol given for EasySelect™ *Pichia* Expression Kit, with 1x YNB (yeast nitrogen base) final concentration and 0.02% Biotin per litre added to each culture media. 200 g of D-glucose in 1000mL for 20% Dextrose, 5mL of methanol with 95mL of water for making 5% Methanol. 100mL of glycerol with 900mL of water was used for making 10% Glycerol. 132mL of 1 M K_2HPO_4,

868 mL of 1 M K_2HPO_4 was used to prepare 1M potassium phosphate buffer, pH 6.0. 364.4 g sorbitol in 1L of water was used for 2M sorbitol-based media. PBS Buffers tables (Gibco, UK) were used for washing and re-suspending the *P. pastoris* cells. The culture conditions were followed as per the standard molecular biology techniques (Green and Sambrook D.W., 2012).

2.2.3 Bioreactor fermentation

2.2.3.1 Large scale 20L fermentation

For the cultivation of the *P. pastoris* strain containing HBsAg gene under P_{AOX1} a starter culture was generated by inoculating 1mL of working cell bank in 500mL baffled shake flask containing 50mL of YPD medium. The fermenter was steam sterilized after filling it with 7.5L of basal salt medium. The fermenter was then supplemented with PTM_1 medium and then inoculated with the cell culture. The starter culture was incubated for 24hours at 30°C. The initial cell density of OD_{600nm} ~1 was attained. This cell culture was then transferred aseptically into a 2L baffled shake flask containing 500mL of YPD medium followed by incubation under the same conditions till OD_{600nm} ~20 was achieved. 0.25L of the culture was inoculated into a 30 L BIOSTAT C plus (Sartorius, UK) fermenter with a working volume of 13L. The fermentations were run according to the Invitrogen protocol for *P. pastoris* fermentation (Invitrogen Corporation, 2002). *P. pastoris* cultured at 30°C, a pH of 5.0 and a dissolved oxygen level of 30% via a cascade control system was maintained using BIOSTAT C plus bioreactor software. This cascade control system increased the impeller speed from 400rpm to 1000rpm in order to increase the oxygen transfer to the fermentation broth. To supplement the fermentation broth with oxygen at maximum impeller speed, gas blending was used at a rate of 1vvm. Antifoam addition was controlled at a fixed flowrate. 17.5% (w/v) Ammonium hydroxide solution was used as base and 14% (v/v) orthophosphoric acid was used for controlling the pH of the fermentation broth.

At the end of the initial glycerol batch phase the carbon source was seen to be depleted. This was indicated by a DO spike, that is where the DO briefly goes to 100% and growth stalls

for a short duration of time. After the initial batch glycerol phase of 22 hours, it was grown to high cell density with 50% (w/v) glycerol feed containing 12mL of PTM_1/L, at a constant flowrate of 18.15 mL/hr/L for four hours. The induction of the HBsAg was carried out by feeding the fermentation broth with 100% methanol containing PTM_1. This medium was fed into the culture continuously for the production phase after the end of the 4 hours glycerol feed till the end of the fermentation (72 hours). This methanol containing medium was initially fed at a lower flowrate of 3.6 mL/hr/L for 4 hours followed by 7.3 mL/hr/L for another 4 hours before the feed rate was set at 10.9 mL/hr/L. This methanol feed rate was set for the next 72 hours, after which the fermentation broth was harvested.

2.2.3.2 Small scale 250mL fermentation

For small scale bioreactor an initial overnight starter culture was cultivated in YPD medium. 7 ml of from this culture was used to inoculate 0.15 L of the same modified defined media (YNB supplemented with sorbitol) in an Eppendorf DasGip (Eppendorf, UK) 0.25L bioreactor, employing the 'SMG' cultivation strategy. A dissolved oxygen cascade was employed to maintain a minimum 30%, it utilised an increase in agitation rate (400-6500rpm) along with the ability to blend oxygen if required. 17.5% (w/v) ammonia solution and 14% (v/v) orthophosphoric acid was used to maintain the pH at 6.0. The culture was maintained at a temperature of 30°C throughout the culture. After 46hrs the culture had an additional 50mL of YNB Medium supplemented with methanol to start the production of HBsAg. The culture was grown till 66hrs and then had another 50mL of YNB medium supplemented with glycerol added. This induced the production of V5-CRL lip3 enzyme under P_{ENO1} promoter.

The fermentation was carried out with similar steps as described in section 2.2.3.1 apart from the fermentation and feed media. The feed medium was fed with bolus injections in place of continuous feeding with peristaltic pump as used during the 20L large scale fermentation.

2.3 Analytical techniques

2.3.1 Cell disruption

2.3.1.1 Homogenization

For homogenization the *P. pastoris* cells where produced in 5L shake flasks in YPD media. After 50 hours growth the cells were centrifuged at 10,000xg for 10 mins followed by re-suspension in 1x PBS (Phosphate buffer saline) pH 7.4 (10% solids) (w/v). The suspended yeast cells were then homogenized using a Gaulin Lab 1000 homogenizer (APV Gaulin, UK) at 500bar pressure with 5 passes. The method for homogenization was followed as previously described (Bracewell *et al.* , 2008). The homogenized material was then centrifuged at 10,000xg for 20 min at room temperature

2.3.1.2 Sonication

The strains were grown overnight in 10mL YPD using 50mL falcon tubes. They were then centrifuged and re-suspended in 0.5mL of 25mM Tris-Cl pH 7.5 and sonicated using a SoniPrep. The disruption was done using a SoniPrep sonicator with 6 secs on and 10 secs off for 3 cycles with 10,000x amplitude. After disruption 500mM EDTA -1/100 to achieve 5mM and 0.1% triton X-100 was added (Bláha *et al.* , 2018)

2.3.2 Yeast homogenate characterization

2.3.2.1 Ultra-scale down for shear stress

The Ultra-scale down (USD) rotating disc shear device, made by the biochemical engineering department at University College London, was used in recent publication describing the shear stress on cell culture materials (Chatel *et al.* , 2014). The modular device has a single chamber to hold the sample. This chamber is fitted with a rotating disc. The rotation of the disc creates shear stress at the edge of its blade. There is a controller fitted with the device which controls the speed, ranging from 0 rpm to 18000 rpm. This corresponds to an energy dissipation of 0 to 106 W. Kg^{-1} and a shear rate of 0 to 106 s^{-1}. The USD device was loaded with yeast homogenate, and samples sheared at different rates were subsequently

clarified at a range of centrifugation speeds. This gave clarification curves for a range of shear stresses. It was found that there is minimal effect on the clarification due to shear stress. This indicates that there is no or little effect on the particulates in the homogenate from the level of shear stress created in the USD device. The centrifugation speeds used for the analysis in comparison to the continues centrifuge is shown in the following table 2.1.

Table 2.1: Centrifugation speed for equivalent lab scale to industrial scale centrifuge
The centrifugation speeds were wet using a 2mL small scale centrifuge considering its rotor dimensions. The equivalent 50mL $V.t\Sigma^{-1}$ was calculated which was then fixed with 50mL volume and fixed lab scale rotor. The equivalent $L.hr^{-1}$ flow rate was calculated based on the $V.t\Sigma^{-1}$ value obtained from the lab scale centrifuge. The centrifugation speeds were fixed for clarification experiments done in lab scale.

50mL lab scale centrifuge				2mL small centrifuge				$Q_{pathfinder}$ (L/hr)
$V/t\Sigma_{lab}$	volume (mL)	Time (mins)	rpm	V/tΣ*50mL	volume	Time (mins)	rpm	
1.07E-07	50	5	2976	1.12E-07	2	1	6600	136.9
3.10E-09	50	20	8590	3.17E-09	2	16	9800	3.9
6.20E-09	50	20	6074	6.34E-09	2	8	9800	7.8
1.24E-08	50	20	4295	1.27E-08	2	4	9800	15.5
2.58E-08	50	20	2976	2.54E-08	2	2	9800	31.0
5.17E-08	50	20	2104	5.29E-08	2	1	9600	64.7

2.3.2.2 Particle size distribution

The Mastersizer 3000 (Malvern Instruments, UK) was used to determine the particle size distribution of yeast homogenate samples to assess cell disruption by determining average cell size and cell debris. The Mastersizer uses static light scattering, which is a technique for measuring the size of particles that pass through a laser beam and scatter the light at an angle that is directly related to their size. During operation a refractive index of 1.5 was assumed for *P. pastoris* cells and the dispersant was assumed to be water. Samples were added dropwise to 120 mL ultra-pure H_2O in a Hydro 3000 MV dispersion unit until a light obscuration of 15% was reached based on optimization of multiple sample at different concentrations. This range was selected to ensure that there was no multiple scattering and statistically significant result

was obtained. The measured scattering was then plotted to show the distribution of particle sizes in the sample.

2.3.2.3 Clarification using centrifugation

The centrifugation of yeast homogenate and enzyme treated samples was completed using an Eppendorf centrifuge 5415R with rotor F45-24-11. Sigma theory was applied to extrapolate to large scale clarification conditions, using six different centrifugation speeds at laboratory scale, which corresponded to the high to low flow rate range in a disc-stacked centrifuge (pathfinder, PSC1).

2.3.2.4 Dead-end filtration setup

The yeast samples were passed through the dead-end syringe filter using a syringe pump. A T-piece was connected to feed inline to measure the pressure build up. A dead-end absolute pressure sensor attached to data acquisition (DAQ) system for recording the pressure build-up. The pressure was recorded from the pressure difference obtained with the absolute pressure sensor attached in the feedline. The pressure difference is considered zero as atmospheric pressure. Feed samples were passed through a 0.45μm PES disc membrane fitted in a disc membrane holder from Sartorius. The cut off for the pressure build up due to cake formation was kept at 3 bar max. Total volume filtered was then used for the calculation of percentage throughput.

2.3.3 Lipid analysis using liquid chromatography analysis

2.3.3.1 Lipid extraction

Lipid extraction was done after the homogenization of the yeast cells. The yeast homogenate was clarified using centrifugation at 10,000xg for 10 mins at room temperature. The sample supernatant was mixed with solvent in the ratio of 1:3 (17:1; $CHCl_3$: MeOH; v/v), in glass test tubes. The Mixture was then vortexed using a vortex mixer for 5-10 mins at 1800 rpm. After vortexing, water with equal volume to the volume of the sample as per the Bligh-Dyer method (Bligh and Dyer, 1959) was added to the mixture. This was then centrifuged at 5000xg for 5 minutes at room temperature. As expected, the lower phase consisting of the

chloroform held the neutral lipid fraction and the upper phase held the phospholipids. Cell debris formed the middle layer. The lower layer was pipetted out into a fresh glass tube and dried using a GeneVac vacuum centrifuge (GeneVac, UK). The dried samples were re-dissolved in 200μL of solvent (1:2; $CHCl_3$: MeOH; v/v) and 2 μL of this sample was injected in to the HPLC-ELSD for analysis.

2.3.3.2 High performance liquid chromatography with ELSD detector

Triacylglycerol was extracted from yeast homogenate using the Bligh and Dyer method as mentioned earlier in section 2.3.3.1. The extracted lipid was then put through the Agilent 1260 HPLC using Zorbax reverse phase 300SB-C8 Solvent Saver Plus 3.5μm, 3.0 x 100 (Cat No. 861973-306) and lipids were detected using an Agilent 1200 ELSD detector. The lipids were separated based on their polarity. The method for analysis was developed in house and is a modified method from vegetable oil analysis using HPLC-ELSD (Bullock, 2010a).

After the homogenization step the sample supernatant was then mixed with solvent in the ratio of 1:3 (17:1; $CHCl_3$: MeOH; v/v)(850mL (1266.5g)of $CHCl_3$ with 50mL (39.6g) of MeOH) (Ejsing *et al.* , 2009) in glass test tubes. The Mixture was then vortexed using a vortex mixture for 5-10 mins at 1800 rpm. After vortexing the mixture as per Bligh Dyer method, water with equal volume to the volume of the sample was added to the mixture. This was then centrifuged at 5000xg for 5 minutes at room temperature. It was found that the lower phase consisting of the chloroform had the neutral lipid fraction and the upper phase had the phospholipids. The HPLC was run using standard Agilent HPLC running methods.

Briefly, the sample was injected with equal volumes and run with a linear gradient with decreasing concentration of methanol. The linear gradient conditions were set with buffer A (methanol /acetic acid/ water) (750:250:4) and buffer (B) (acetonitrile/MeOH/acidic acid/ IPA) (500:375:4:122) at 5-70 in 14 min, 70-95 % in next 6 min, hold 3 min, 95-5 % B for 3 min. and the ELSD settings were set at Nebulizer: 50 °C, Evaporator: 27 °C, Gas: 1.4 SLM. The flowrate was kept at 0.34 mL/min throughout the run, for a total run time of 24 mins.

2.3.3.3 Cell culture sugar analysis using HPLC

For the cell culture analysis a Dionex HPLC system (Camberley, UK) with a Bio-Rad Aminex HPX-87H reverse phase column (300×7.8 mm2, Bio-Rad Labs., Richmond, CA, USA), controlled by Chromeleon client 6.60 software was used for the separation and analysis of all the carbon sources namely sorbitol, glycerol and methanol (Rios-Solis *et al.* , 2015). The standard curves were prepared using the solutions used in the cultivation experiments. The identification of the peaks based on retention time as obtained after analysing the standards using the same HPLC method. For the quantification of the samples the area under the curve was calculated after baseline subtraction

2.3.4 Purification of HBsAg and *C. rugosa* lipase

2.3.4.1 HBsAg - VLP purification

The purification of HBsAg was done using multiple steps including hydrophobic interaction chromatography followed by anion exchange and size exclusion chromatography. The ÄKTA Avant system was used for all purification steps.

2.3.4.1.1 Hydrophobic interaction chromatography (HIC)

The cells obtained from the culture were washed and re-suspended in 1x PBS buffer at a concentration of 10% wcw/mL. The re-suspended cells where then homogenized using a Gaulin lab40 homogenizer at 500bar with 5 passes. The homogenized yeast sample was then centrifuged at 10,000rpm for 10 minutes at room temperature.

A 5mL HiTrap Butyl-S 6 FF (Cat. No. 17-0978-14) from GE was used for purification of HBsAg. 3M $(NH_4)_2SO_4$ (ammonium sulphate) stocks were prepared for the buffer preparation. Stocks of 1M Na_2HPO_4 (Cat. No. 71645) (di-sodium hydrogen phosphate) and 1M NaH_2PO_4 (Cat. No. S9638) (mono-sodium hydrogen phosphate) were prepared in distilled water. 20mM $NaPO_4$ (sodium phosphate) buffer was prepared by titration of the two stock buffers to pH 7.0. These buffers were used for preparation of equilibration and elution buffers. The appropriate volume of 3M $(NH_4)_2SO_4$ was used to prepare 0.6M $(NH_4)_2SO_4$ in 20mM $NaPO_4$. 10mM $NaPO_4$ along with 30% IPA (Cat. No. P/7507/17) (Iso-propyl alcohol) was used for

stripping the column. 0.5M NaOH (Cat. No. 06203) (sodium hydroxide) was prepared and used for CIP of the column before storage in 20% ethanol (EtOH) (Cat No. 51976).

The supernatant of the clarified homogenate was then reconditioned using 3M $(NH_4)_2SO_4$ (Cat. No. A2939) to give a final concentration of 0.6M in 25mM $NaPO_4$ and loaded on to the pre-equilibrated HiTrap column at pH 7.0. A step gradient was used to elute the HBsAg from the column. The column was stripped using buffer containing 30% IPA followed by CIP and storage of the media in EtOH.

2.3.4.1.2 Ion-exchange chromatography

The HIC eluted sample was then dialysed overnight using 30mL (total volume) Slide-A-Lyser® 10kDa MWCO (Cat. No. 66830, Thermo Scientific, UK) dialysis membrane in 20mM Tris-HCl pH 8.0 buffer. The dialysed sample was then loaded on to the Q Sepharose FF anion exchanger column (17-5156-01, GE, UK) for further purification.

For Anion exchange purification step using prepacked 5mL HiTrap Q Sepharose FF was used. Stocks of 1M Tris base (Cat. No. T1503) pH 8.0 was prepared using concentrated hydrochloric acid. 25mM Tris-HCl binding buffer was prepared from appropriate volume of Tris-HCl stock. 25mM Tris-HCl containing 0.5M NaCl (Cat. No. S7653) (sodium chloride) elution buffer was prepared.

The protein of interest was eluted on a linear gradient of 20 column volumes (CV) to 25mM Tris-HCl buffer containing 1M NaCl. 0.5M NaOH prepared and used for CIP of the column before storage in 20% EtOH.

2.3.4.1.3 Size exclusion

The eluted fraction from the anion exchange column was then concentrated and buffer exchanged with 1x PBS buffer (Cat. No. 18912-014, Gibco, UK) 1 tablet dissolved in 1L of ultrapure water) using a 0.5mL 10kDa MWCO Vivaspin 500 PES spin concentrator (Cat No. VS0102, Sartorius, UK).

The obtained sample was then then injected into a Superpose™ 6 10/300 GL column (Cat No.17-5172-01, GE, UK). The elution peak was fractionated and analysed for the presence of

HBsAg using western blot analysis having anti-HBsAg antibody as described in section 2.3.6.2. The eluted peak was also compared with the commercial gel filtration standards (Cat No.151-1901, BioRad) on the basis of their retention time and BioRad product insert information.

2.3.4.2 *C. rugosa* lip3 Lipase Purification

For purification of *C. rugosa* lip3 lipase hydrophobic interaction chromatography was used. 5mL HiTrap Butyl-S 6 FF (Cat. No. 17-0978-14, GE, UK) was used for purification of *C. rugosa* lipase (V5-CRL lip3). The appropriate volume of 3M $(NH_4)_2SO_4$ were used to prepare 0.5M $(NH_4)_2SO_4$ in 25mM Tris-Cl pH 7.0. 10mM CHAPS buffer was used for eluting the protein of interest from the column. 0.5M NaOH was prepared and used for CIP of the column.

The supernatant of the clarified homogenate was then reconditioned using 3M $(NH_4)_2SO_4$ to give a final concentration of 0.5M $(NH_4)_2SO_4$ and loaded on to the pre-equilibrated HiTrap column at pH 7.0. A linear elution gradient over 20 column volumes was used to elute the *C. rugosa* lipase (V5-CRL lip3) from the column. But there was no significant peak obtained during the linear gradient step. The column was then stripped using 10mM CHAPS buffer and a peak containing the *C. rugosa* lipase (V5-CRL lip3) was obtained during this step. The column was then washed with 0.5M NaOH before storage in 20 % EtOH solution.

2.3.5 Microscopy

2.3.5.1 Transmission electron microscopy

The HBsAg purified samples, obtained after a two-step purification, were concentrated using a 10kDa concentrator and were buffer exchanged with 5mM HEPES buffer pH 7.5. For transmission electron microscopy the VLP were negatively stained and the following steps were employed.

A 400-mesh copper carbon/form var coated grid was held by forceps. 2 μL of sample was applied onto the grid surface and left on for 2-3 minutes, then drawn off from the edge of the grid with Whatman filter paper.

The sample was then quickly washed by placing onto a drop of 20mM phosphate buffer pH 7.3 for 10 seconds, taking care not to allow the grid to dry between drawing off the sample and placing it, sample side down, on to the drop of buffer. Excess buffer was drawn off from the edge of the grid, as in the previous step. The grid was stained immediately by placing onto a drop of 1% uranyl acetate in distilled water for 30 seconds.

Excess stains were then drawn off from the edge of the grid with filter paper and the grid allowed to air-dry for several minutes before observation. The grids were then imaged using a Jeol1010 TEM and images recorded using a Gatan Orius camera at set magnifications. The microscope used for the imaging was Phillips CM12 with an ATM camera system.

2.3.6 Western blot analysis

The western blot analysis for lipase and HBsAg was done using following immunoblotting methods.

2.3.6.1 SDS-PAGE analysis

NuPAGE® Novex 4-12% Bis-Tris 10 well 1 mm pre-cast gel kits from Invitrogen (Paisley, UK) were used for the analysis of HBsAg and V5-CRL lip3 expressed in *P. pastoris* to provide insight on the protein production and host cell impurities. Samples were prepared in 1mL centrifuge tubes with NuPAGE® LDS Sample Buffer (4x) 2.5µL and NuPAGE® Reducing Agent (10x) 1µL for reducing samples. The samples were then made up to 30µL with ultra-pure water. Running buffer was prepared by diluting 20x MES (2-(N-morpholino) ethanesulphonic acid) buffer in reverse osmosis (RO) water to a final volume of 1L. NuPAGE® Antioxidant-15mL 0.5mL for each litre of MOPS running buffer was used while running the gel under reducing conditions. SeeBlue® Pre-stained Protein Standard (4 to 250 kDa) (Thermofisher, UK) was used for the reference protein ladder. The proteins gels were stained for 15 mins with InstantBlue™ Ultrafast protein stain after the gels were run followed by overnight washing in ultra-pure water. The gels were imaged using Amersham Imager 600 imager.

2.3.6.2 Chemiluminescence western blot

For western blot analysis 4mL of *P. pastoris* culture cells were centrifuged at 10,000xg for 5 mins to remove the culture supernatant and then re-suspended in 0.5mL of disruption media consisting of 50mM MOPS, 1mM 4-benzenesulfonyl fluoride hydrochloride (AEBSF), 5mM DTT, with pH adjusted to 7.5 using 1M NaOH. After disruption 500mM EDTA -1/100 to a final concentration of 5mM and triton X-100 to 0.1% were added (Bláha *et al.* , 2018). A SoniPrep sonicator was used for disruption, as described in section 2.3.1.2. The obtained samples where then loaded on to a 4-16% Bis-Tris gel and run using MOPS buffer as described in 2.3.6.1. The gels were run at 200V for 50 mins and transferred onto a nitrocellulose membrane using an Xcell MiniBlot II module (Invitrogen Corporation, 2002). For dot blot analysis the membranes were spotted with 1µL of samples after boiling the samples in reducing media for 10mins at 70°C.

Membranes were blocked using 5% Skimmed milk in 1x Tris-buffered saline (TBS) buffer containing 0.1% Tween 20 (TBST). The pH of the TBST was pre-adjusted to 7.5. Membranes were kept at room temperature for 60 mins on an orbital shaker.

The blocking media was then replaced with 2.5% skimmed milk in 1x TBST buffer containing the primary antibody. The gel was incubated in the primary antibody in 2.5% skimmed milk media under agitation at 2-8°C overnight. The primary antibody was a rabbit polyclonal to the Hepatitis B Virus Surface Antigen (Ad/Ay) (HRP) (Cat. No. ab20878, Abcam). The primary antibody was dissolved in buffer containing skimmed milk with 1000x dilution volumetrically. The membrane was then washed with 1x TBST 3 times for 5 mins each followed by a 15 mins wash in 1x TBS media. As the primary antibody was HRP conjugated, secondary antibody was not required for obtaining the chemiluminescence. The membrane was developed using 4 mL of Bio-Rad's Clarity Western ECL substrate.

Detection was performed using automated exposure setting on an Amersham Imager 600. As a standard 0.025µg of Hepatitis B Surface Antigen (HBsAg), recombinant subtype adw (Cat. No. 5015.005, Aldevron, US), was used.

2.3.6.3 Fluorescent western blot

The immunofluorescent blotting was used for V5-CRL lip3 detection. The sample preparation was as described in above section 2.2.2 and homogenized followed by clarification using centrifugation before the western blot analysis. Post transfer of membrane and blocking with 5% skimmed milk in 1x TBST buffer, a mouse monoclonal anti SV5-Pk1 primary antibody (Cat. No. ab27671, Abcam, UK) in 2.5% skimmed milk in 1x TBST buffer was used. After the overnight incubation in the primary antibody the membrane was washed with 1x TBST 3 times for 5 mins each followed by 15 mins wash in 1x TBS media.

The membrane was then incubated with goat anti-mouse IgG H&L (Alexa Fluor® 488) secondary antibody (Cat. No. ab150113, Abcam, UK) in 2.5% skimmed milk in 1x TBST. After 1 hour of incubation the membrane was washed with 1x TBST 3 times for 5 mins each followed by 15 mins wash in 1x TBS media.

The V5 peptide from Abcam (Cat. No. ab15829) was used as a standard for quantification. The visualization of the bands were done using Amersham Imager 600 and the bands were quantified using ImageJ (Schindelin *et al.* , 2012) and Origin (OriginLab, Northampton, MA).

2.3.6.4 DOT-BLOT analysis

Dot blot analysis for quantification of HBsAg and V5-CRL lip3 expressed protein used the same steps as already described above section 2.2.2. The samples were reduced using the NuPAGE® Sample Reducing Agent (10X) (Cat. No. NP0004, Invitrogen™, UK) and boiled at 70°C for 10 mins before spotting the dots on to the nitrocellulose membrane.

The blocking of the membrane followed by use of primary and secondary antibody was as per the western blot analysis explained in section 2.3.6.2 and 2.3.6.3. For quantification the intensity of the dots where then measured using the Amersham 600 gel imager and analysed using hill fitting algorithm in Origin (OriginLab, Northampton, MA) program

2.3.7 Lipase method development

2.3.7.1 Colorimetry analysis for lipase activity

The activity of commercial lipase from *C. rugosa*, *R. oryzae* and *P. fluorescens* was tested with 4-nitrophenyl laurate as a substrate. The standard curve was created with 4-Nitrophenol. Activity was measured over time at 460nm using a TECAN plate reader. The experiment was done as per previously published (Ruiz *et al.* , 2004) using a microwell plate. Briefly, the lipase assay buffer was made with 55.56mM phosphate buffer pH 8.0 with 1.33% Triton X 100. This buffer was filtered through a 0.22µm PES filter. The stocks of the 25mM 4-nitrophenyl laurate substrates and 25mM 4-Nitrophenol standard where prepared in isopropanol. The substrate was serially diluted from 2mM to 0.625mM in the lipase assay buffer premix.

The standard was also diluted from 1M to 7.813µM. 100µL of each standard was dispensed in the microwell plate and used for standard curve measurement. 50µL substrates were dispensed and the plate was kept at 37°C for 10minutes before measurement. Along with this plate another plate was kept which had only 100µL of sample. 50µL of the sample was then pipetted into the first plate having the substrate. The sample and the substrate were mixed using 2sec orbital shaking before taking the reading. The reading was then analysed for kinetics of the enzyme. A total of 10 reads per well were taken over a 30-minute period with 10 cycles. A Lineweaver Burk plot was obtained from the kinetics data. Lipase activity, K_m and V_{max} was calculated from the Lineweaver Burk plot. Specific activity of the enzyme was calculated as activity is equal to number of enzyme units per mL of reaction mixture per milligram of protein.

2.3.7.2 Lipase activity using zymogram

The activity of the lipase was studied using zymogram analysis of V5-CRL lip3 activity. The strains where grown overnight in 10mL YPD using 50mL falcon tubes. They were then centrifuged and re-suspended in 0.5mL of 25mM Tris-HCl pH 8.0 and sonicated using Soni-Prep as described in section 2.3.1.2. The Zymogram was developed after running the samples in a 4-16% BisTris SDS PAGE gel under non-reducing condition (Laemmli, 1970). The

imaging using Amersham gel imager was done after soaking the gel in 25mM Tris-HCl pH 8.0 containing 2.5 Triton X 100 for 45mins in RT on an orbital shaker. Followed by 2 washes of 30 minutes each, with 25mM Tris-HCl pH 8.0. Then the substrate 2.5mM MUB in 50% MeOH with 25mM Tris-HCl pH 8.0 buffers was spread on the gel, which was incubated at 37°C on an orbital shaker and visualized using an Amersham Gel imager under UV-transillumination (λ_{ex} 312nm).

2.3.8 Molecular biology

2.3.8.1 Strains and plasmids

The genes that were used in the present study were synthesised and cloned into plasmids by Eurogenetec. These synthesized plasmids where then used for transfection of the *P. pastoris* wild type strain to generate strains containing HBsAg and *C. rugosa* lip3 gene. The transformation was carried out with well-known molecular biology techniques as explained in section 2.3.8.2. The details of the plasmids used and the strains generating from these plasmids are listed in the below table.

Table 2.2: *P. pastoris* strains and plasmids

The table shows strains created corresponding to promoters' present containing HBsAg gene and localization of C. rugosa lipase gene for each strain. The wild type strain used in this study is named as PPS9010-WT. The cloning vector used was pUC57 and P. pastoris shuttle vector used was pJ902-15. The strains are created with the same shuttle vector backbone identified as **J9**. *HBsAg is under P_{AOX1} and the strain generated using P. pastoris wild type is named as PPS9010-J9Sag strain. HB***sAg** *gene sequence is present in all the strains and is identified with '***S***'. The lipase gene from C. rugosa are present under P_{ENO1} and P_{THI11} promoters. The plasmids and the strains containing P_{ENO1} sequence are identified with '***E***', and once containing P_{THI11} sequence are identified with '***T***'. The lipase is localized at different locations of the cell, which are identified with '***LC***' for localization in cell cytoplasm, endoplasmic reticulum identified with '***Lkdel***' and outer surface of the cell identified with '***LS***'.*

Plasmids types	**Plasmids Names**	**Strain types**	**Strain names**
P. pastoris shuttle vector	pJ902-15	*P. pastoris* parent strain (wild type)	PPS9010-WT
Shuttle vector containing HBsAg gene sequence	pJ9Sag	*P. pastoris* strain with HBsAg gene	PPS9010-J9Sag
Cloning vector	pUC57	*P. pastoris* strain with HBsAg gene, ENO1 promoter sequence and *C. rugosa* lipase gene (Cell surface localization)	PPS9010-J9SEATLS
Cloning vector with *C. rugosa* lipase gene (Cytoplasm localization)	pUC57-LC	*P. pastoris* strain with HBsAg gene, ENO1 promoter sequence and *C. rugosa* lipase gene (Cytoplasm localization)	PPS9010-J9SEATLC
Cloning vector with *C. rugosa* lipase gene (Cell surface localization)	pUC57-LS	*P. pastoris* strain with HBsAg gene, ENO1 promoter sequence and *C. rugosa* lipase gene (Endoplamic recticulum localization)	PPS9010-J9SEATLkdel
Cloning vector with *C. rugosa* lipase gene (Endoplamic recticulum localization)	pUC57-Lkdel	*P. pastoris* strain with HBsAg gene, THI11 promoter sequence and *C. rugosa* lipase gene (Cell surface localization)	PPS9010-J9STATLS
Cloning vector with ENO1 promoter sequence	pUC57-EAT	*P. pastoris* strain with HBsAg gene, THI11 promoter sequence and C. rugosa lipase gene (Cytoplasm localization)	PPS9010-J9STATLC
Shuttle vector with ENO1 promoter sequence and HBsAg gene sequence	pJ9SEAT	*P. pastoris* strain with HBsAg gene, THI11 promoter sequence and *C. rugosa* lipase gene (Endoplamic recticulum localization)	PPS9010-J9STATLkdel
Shuttle vector with ENO1 promoter sequence, HBsAg gene sequence and *C. rugosa* lipase gene (Cytoplasm localization)	pJ9SEATLC		
Shuttle vector with ENO1 promoter sequence, HBsAg gene sequence and *C. rugosa* lipase gene (Cell surface localization)	pJ9SEATLS		
Shuttle vector with ENO1 promoter sequence, HBsAg gene sequence and *C. rugosa* lipase gene (Endoplamic recticulum localization)	pJ9SEATLkdel		
Cloning vector with THI11 promoter sequence	pUC57-TAT		
Shuttle vector with THI11 promoter sequence and HBsAg gene sequence	pJ9STAT		
Shuttle vector with THI11 promoter sequence, HBsAg gene sequence and *C. rugosa* lipase gene (Cytoplasm localization)	pJ9STATLC		
Shuttle vector with THI11 promoter sequence, HBsAg gene sequence and *C. rugosa* lipase gene (Cell surface localization)	pJ9STATLS		
Shuttle vector with THI11 promoter sequence, HBsAg gene sequence and *C. rugosa* lipase gene (Endoplamic recticulum localization)	pJ9STATLkdel		

2.3.8.2 *P. pastoris* transfection

Bacterial cell transformations were performed using standard molecular biology techniques (Green and Sambrook D.W., 2012). Briefly, after the constructs had been made, they were linearized and integration into the genome of PPS9010 wildtype *P. pastoris*. For linearization, the constructs were cut with SacI-HF restriction enzyme and transformed into freshly prepared electro-competent cells (Lin-Cereghino *et al.* , 2005). In short, the shuttle vector was grown in DH10α *E. coli* strain for propagation with overnight growth in low salt LB media. Plasmid DNA was isolated using the Qiagen mini-prep kit. The genome integration gave rise to two P_{AOX1} sites and P_{AOX1} terminator sequence. After transfection the strains were grown in zeocin containing YPD plates for selection of transformant. The transformants where plated on 1mg/mL zeocin YPD agar plates as well as 0.2mg/mL the plates were kept at 30°C for 2-3 days. The confirmed colonies grew in 1000µg/mL and 200µg/mL zeocin. Confirmation of transformation was done using colony PCR.

2.3.8.3 Polymerase chain reaction and agarose gel electrophoresis

The samples were mixed with 6x gel loading dye and loaded on a 0.8% agarose gel containing GelRed™ stain (Biotium, UK) and made in 1x TBE. The confirmed colonies grew in 1000µg/mL and 200µg/mL zeocin. They were picked and were boiled in distilled water. The DNA of interest was then amplified using polymerase chain reaction (PCR). PCR was done with 1 cycle of denaturation at 98°C for 30 sec. This step was followed by denaturation at 98°C for 10 secs then 25 cycles of annealing at 60°C for 30 sec, and extension for 2min at 72 °C. At the end of the 25 cycles of annealing and extensions, final extension was done for 5min at 72 °C. At the end of the cycle a hold at 4°C for 4 hours was performed. The colonies were boiled in 20µL of distilled water and were mixed with 6x gel loading dye and ran on a 0.8% agarose gel to confirm the size of the amplified gene according to the gel running instructions from NEB. The agarose gel was run at 100V for 45 mins. with the molecular weight markers used were 1kb HyperLadder™. The size of the HBsAg gene (0.72kb) and CRL lip3 gene (1.5kb) was confirmed by the colony PCR as visualised under UV light.

3 Exogenous use of lipase on yeast homogenate for host cell lipid degradation

3.1 Introduction

Lipid fouling of the membranes and resins during downstream processing can, not only give rise to process related issues like back pressure and decreased flux rates hence increasing the total process time, but also affects the dynamic binding capacities in resins. Being hydrophobic in nature lipids can even alter the ligand properties of the chromatography resins. These give rise to inconsistent elution profiles and can causes a major issue in reproducibility of the purification process (Burden *et al.* , 2012; Jin *et al.* , 2010). Thus, it is pivotal that removal of lipid is needed in the primary recovery operations of the high value products like HBV from yeast homogenate.

In this chapter an attempt is made to understand the use of lipase on complex colloidal substance as a yeast homogenate, in this case homogenate obtained from *P. pastoris* cells. To understand these following questions are addressed in the present chapter.

1. What are the effects in treating heterogeneous mixtures like yeast homogenate with lipase?
2. Can exogenous lipase be used for the removal of lipid impurities?

To address the above questions, it is important to understand the properties of colloidal mixture like yeast homogenate. During homogenization of yeast cells, they are disrupted and cellular components like DNA, protein and cell organelles are released into the mixture along with the cell components debris. During regular bioprocessing of yeast removal of these cellular components is done using clarification by centrifugation and filtration. The cell debris needs to be removed before filtration as they foul the membranes and processing such material becomes difficult. But even after high speed centrifugation there are large numbers of suspended solids that are found in the supernatant of the yeast homogenate. As described earlier along with cell debris there are large quantities of fatty material released in the yeast lysate during homogenization. These fatty materials in the yeast lysate do not pellet down with high speed centrifugation and remains suspended in the supernatant. To test the condition *P.*

pastoris was grown as described in chapter 2, section 2.2.2 and re-suspended in 1x PBS (phosphate buffer saline) at the concentration of 10% wcw. mL^{-1}. These cell cultures were not induced with methanol and they did not produce HBsAg VLPs. The suspended *P. pastoris* (10% w/v) was then homogenized using 1200bar for 3 passes using a Gaulin lab 40 homogenizer and centrifuged at 10,000xg for 10 mins. The fatty material is seen after centrifugation as shown in Figure 3.1. The generic flow chart shown in Figure 3.2 is considered as the most common steps used for processing of intracellular yeast products. As described above, after homogenization and clarification using high speed centrifugation, off-white soft white fatty layer on top of a more packed white layer of remaining cell debris of *P. pastoris* cells. In the present chapter an attempt is made to use commercially available lipase from different microorganisms on the *P. pastoris* homogenate. The use of such exogenous lipase will reduce the lipids that are released during homogenization and consequently it may help in reduction of matrices and membrane fouling. After the selection of the microbial lipase dead-end filter fouling is studied with treated homogenate (exogenous lipase treated) mixture in comparison to the untreated yeast homogenate to show the effect of using such lipases on membrane filters while filtering lipid rich process material.

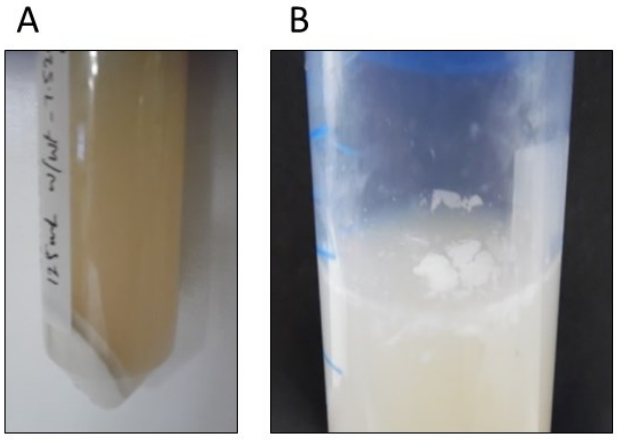


Figure 3.1: Clarified supernatant and fatty material from *P. pastoris* homogenate
The above images show suspended particles and fatty material formed in the soluble fraction after the homogenization of yeast. (A) Yeast suspended in phosphate buffered saline (10% wcw/mL) was homogenized at 1200bar for 3 passes using a gaulin lab 40 homogenizer. The homogenized sample was then centrifuged using a fixed angle centrifuge

at 10,000xg for 10 mins. There are two distinct layers of pellet seen in the centrifuged sample. The upper layer consisted of soft fatty material and the lower denser solid layer. (B) The above image is the same centrifuged yeast homogenate sample as described earlier with a black background. The image is taken in such a manner that the white fatty material is visible against the darker back ground.

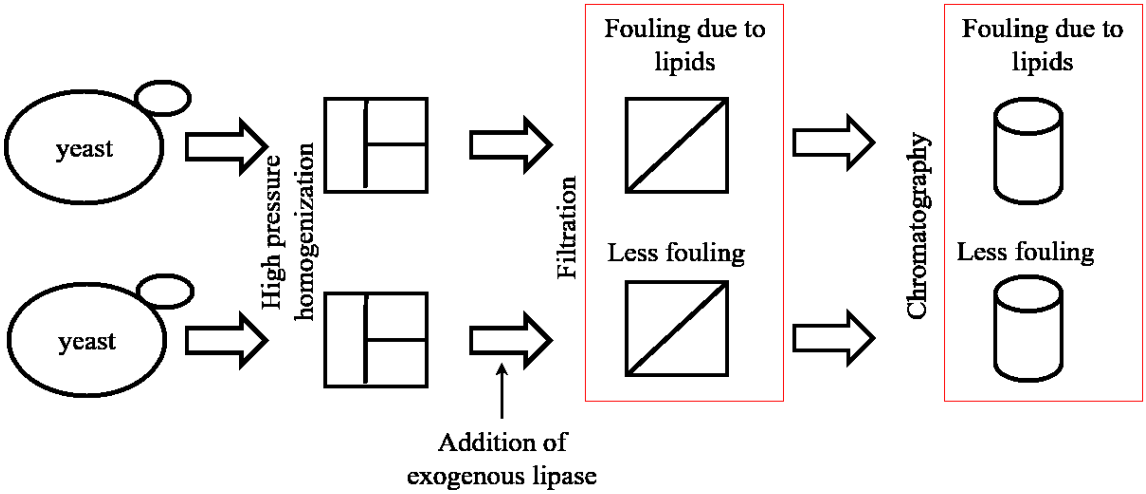


Figure 3.2: Flow chart underlining the addition of exogenous lipase for lipid removal
Yeast producing intracellular products are homogenized for their extraction. These yeast homogenates are then clarified using centrifugation or filtration before purification using chromatography. The lipid impurities released during homogenization fouls the filtration membranes and matrices used during purification steps. The above flow chart shows the exogenous addition of lipase will act on lipid impurities in turn reducing the fouling of membranes and matrices.

3.2 Homogenization of *P. pastoris* and characterization of lipids

3.2.1 Lipid extraction and classification using HPLC-ELSD

Lipid extraction from biological material is well known in the literature. The material in question is directly homogenized in presence of chloroform and methanol (Bligh and Dyer, 1959; Knittelfelder and Kohlwein, 2017a; Schneiter and Daum, 2006). The homogenate mixture is then separated using centrifugation into two distinct layers, neutral lipid containing chloroform layer and the non-lipid or methanol miscible layer. In the present study certain steps were taken to isolate and extract specific class of lipids as shown in Figure 3.3. The yeast

cell where first homogenized using a gaulin homogenizer (lab 1000) with three discreet passes (Jin *et al.* , 2010). Without the presence of chloroform as it might not only extract the soluble lipids but can also break down the lipids in the cell membrane. In an industrial process the yeast cells are homogenized and separated from the cell debris using centrifugation techniques (Gurramkonda *et al.* , 2009; Hardy *et al.* , 2000).

Due to this reason the yeast cells were first homogenized in 1x PBS buffer and centrifuged. The centrifugation was done using benchtop centrifuge which corresponds to the industrial scale centrifuge. The extract of the lipid is optimized on the basis of maximum extraction of TAG classes of lipids which are found to foul the downstream membranes and matrices (Bracewell *et al.* , 2008; Burden *et al.* , 2012; Siu *et al.* , 2006). From the above studies it has been indicated that the major lipid impurity obtained from yeast homogenate is triacylglycerol. Triacylglycerol (TAG) belongs to a set of 21 major classes of lipids found in yeast. These common lipids obtained shown in a study done on *S. cerevisiae* (Ejsing *et al.* , 2009).

In the study done by Ejsing *et al.* 2009 showed that the ratio of chloroform and methanol is changed to 17:1 from the conventional 3:1 (chloroform: methanol) has higher extraction of lipids in comparison to the conventional method. Using this mixture about 99% TAG has been extracted. This ratio of chloroform methanol mixture was used to extract the lipids from the clarified supernatant of *P. pastoris* homogenized cells. Even though the composition of the solvents for lipid extraction was known, the mixing time needed to be optimized for maximum extraction of lipid from the *P. pastoris* homogenized cells. This optimization was done to attain maximum efficiency of lipid extraction from the yeast homogenate. Anthracene was used as an indicator for calculation of extraction efficiency. After optimization of the protocol, 10mins of vortexing at 1800 rpm using a benchtop vortex mixture was sufficient to extract the needed lipid from the sample. This extraction process included vortexing the mixture of solvents and yeast homogenate.

The vortexed sample was centrifuged and the lower chloroform phase consisting of the lipids where separated using a glass pipette. Care was taken so that there is no portion of the undissolved middle layer consisting on cell debris, DNA, proteins, and the methanol phase was pipetted along with the chloroform phase. The chloroform phase is then dried using a lab scale solvent dryer (Genevac EZ-2 Series Personal Evaporator). The extracted and dried lipids with an extraction efficiency of 70% were then analysed using HPLC-ELSD and quantified using lipid standard curves.

3.2.2 Analysis of homogenized yeast using HPLC-ELSD

The detection and analysis of lipids from yeast is done using various HPLC methods. Two major class of these methods involved the use of normal phase buffers for separation of specific classes of lipids (Knittelfelder and Kohlwein, 2017b). Other methods used reverse phase buffer for the separation of lipid classes (Olsson *et al.* , 2014; Perona and Ruiz-Gutierrez, 2003). The reverse phase chromatography is used for the separation of the lipid molecules and detection is done by evaporative light scattering detectors (Ross *et al.* , 2011). Other methods are also developed for the detection and analysis of lipids such as charged aerosol detection (Khoomrung *et al.* , 2013). In the present study the extracted lipids are analysed on Agilent 1260 HPLC system using reverse phase HPLC column attached with an evaporative light scatter detector as shown in Figure 3.3. The HPLC run condition were modified from Bullock, 2010b and optimized with in-house system and column. The retention time of the commercial standards of fatty acids where used for identification of peaks obtained from the extracted yeast lipids. These peaks were then quantified using standard curves of the commercial standards obtained from the same HPLC-ELSD method. Standard curve of the Trioline, oleic acid, ergosterol was done using this method. The standard curves had statistical p value of less than 0.01 and regression value of 0.998%. The same method was used to analysis of all the fatty acid and triacylglycerol's from the yeast homogenate samples.

Figure 3.3 panel (C) shows the typical yeast homogenate lipids that were analysed after lipid extraction. The peaks were well resolved and shows separation of fatty acids, ergosterol and triacylglycerol among other major fatty acids. In panel (D) shows two HPLC traces that were obtained after lipid extraction, *P. pastoris* were grown under shake flask conditions and under bioreactor conditions and were superposed for comparison. Under stress conditions and elongated fermentation conditions, when *P. pastoris* is grown in a fermenter the triacylglycerols are degraded into monoacylglycerols. So, the intensity of the triacylglycerols species reduces in the sample obtained from the fermenter. In contrast the when the same *P. pastoris* cells are grown in shake flask, more amount of triacylglycerols are seen and less monoacylglycerols which is indicates that the cells are not under stress and accumulates large fatty acids like triacylglycerols in comparison to monoacylglycerols.

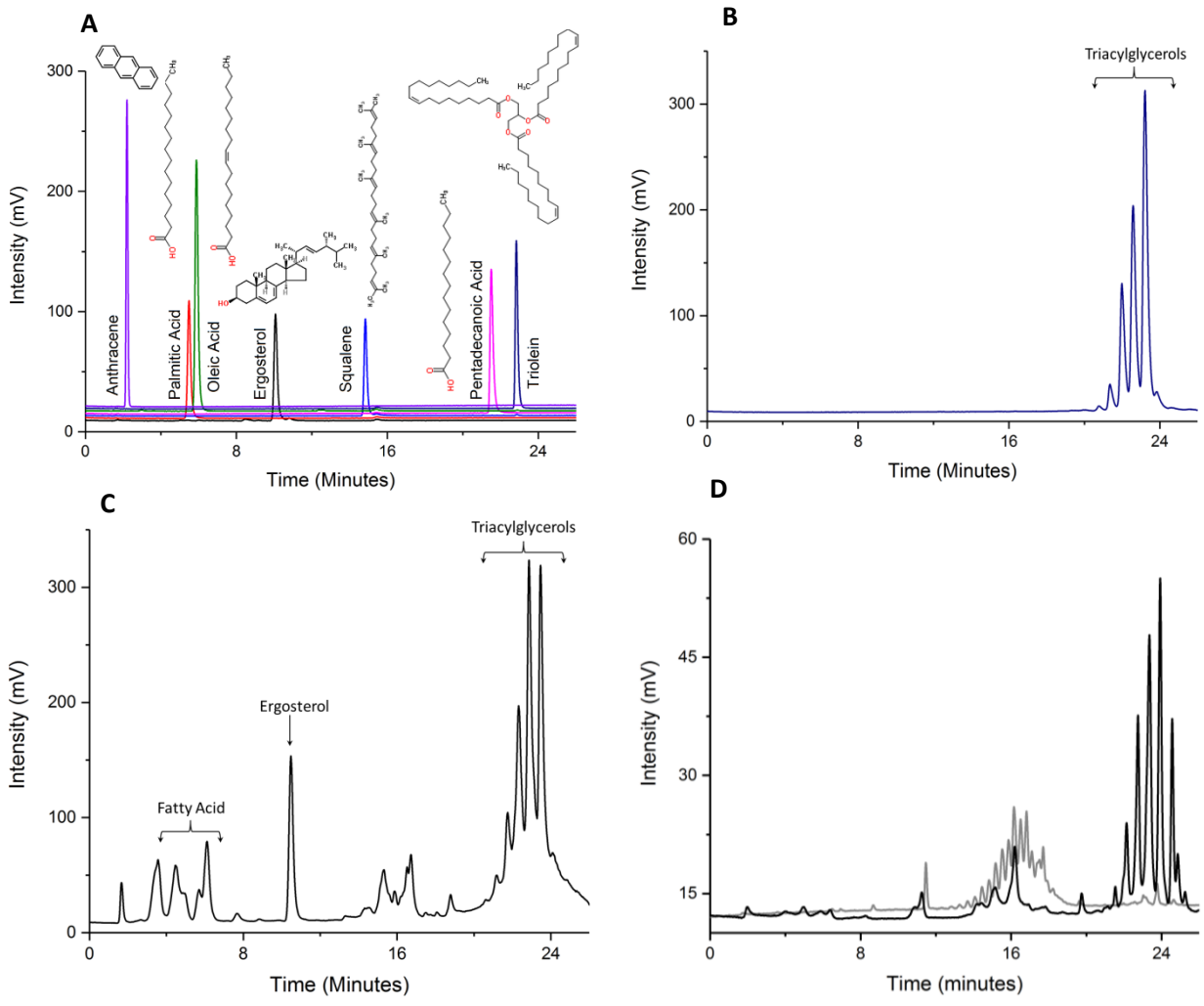


Figure 3.3: HPLC chromatographic profile for lipid analysis from *P. pastoris* homogenate

(A) Over lay of standards y-axis analysed using RP-HPLC with Zorbax C8 column. Lipids were eluted using a linear gradient at a flow rate of 0.34 mL/min and analysed using an ELSD detector. The commercial standards were run with 0.5mg/mL concentrations. Using the method described above. (B) Mixture of triacyglycerides found in rapeseed oil. It is seen that all the triacylglycerol's are resolved and clusters together while eluted from the column. (C) A typical yeast homogenate there the peaks are identified as fatty acid, Ergosterol and triacylglycerol's as three major groups of lipids analysed using the RP-HPLC method with ELSD detector. The retention time is comparative to the standards run using the same method. Not all the peaks obtained from the crude yeast homogenate are identified using this method. (D) The lipid profile also changes at different stages of the cell culture. The lipids obtained from yeast grown in shake flask for 48 to 72 hours (black) shows more triacyglycerides (TAG) and less amount of monoacyglycerides (MAG). In case of bioreactor production (gray)monoacyglycerides increases in amount at the end of 72 hours with methanol induction showing cell stress and increased metabolism hence less TAGs are detected in comparison to MAGs.

To identify the groups of fatty acids and quantify the degradation of fatty acids by use of lipase commercial standards were analysed using the same HPLC-ELSD method. In panel (A) the commercial single chain fatty acid standards namely oleic acid (0.5mg/mL), palmitic acid (0.5mg/mL), and pentadecanoic acid (0.5mg/mL) along with squalene (0.5mg/mL), ergosterol (5mg/mL) and three chain fatty acid triolein (0.1mg/mL) where analysis. In panel (B) a mixture of triacylglycerols which is found in rapeseed oil was analysed. It shows that all the different variety of triacylglycerols are obtained in the end of the chromatographic run. Based on triacylglycerols and fatty acid retention time obtained from the commercial standards, the peaks from the *P. pastoris* homogenate were identified. Some peaks were not identified and are not labelled. For the identification of these classes of fatty acids more advanced techniques such a mass spectrometry is needed to be done. The HPLC profile obtained from the extracted lipids samples shown in panel (D) was seen to be lower than the previously obtained result from other shake flask experiment shown in panel (C). This was due to the fact that new detector was used for this analysis in the later part of the study and this detector gave lower response to the same concentration of lipids in the sample. The difference seen in the trioline

peak profile, panel (B) and (C) is since they are obtained from different culture materials. The buffers and standards used for the HPLC-ELSD assay has been listed in chapter 2, section 2.3.3.2. In short, the buffer A composed of methanol/water/acetic acid (750:250:4) was run in a linear gradient with buffer B composed of Acetonitrile/methanol/IPA/acetic acid (500:375:125:4).

3.3 Use of lipase for removal of lipids from yeast homogenate

C. rugosa lipase (0.1mg/mL) was added exogenously to yeast homogenate to find the effect of lipase on the lipids seen after homogenization. It was found that the lipase treated sample show no formation of fatty layer with the solid pellet of the cell debris. To test the amount of fatty material, present in the supernatant the clarified yeast homogenate was passed through a 0.45µm PES (polyethersulfone) disc membrane filter. The effect of lipase is visible as the fouling layer reduced in the treated sample as shown in Figure 3.4 The action of lipase was also tested at different centrifugation speeds. It was seen that the percentage solids remaining in the solution in comparison to treated and untreated yeast homogenate was substantially different.

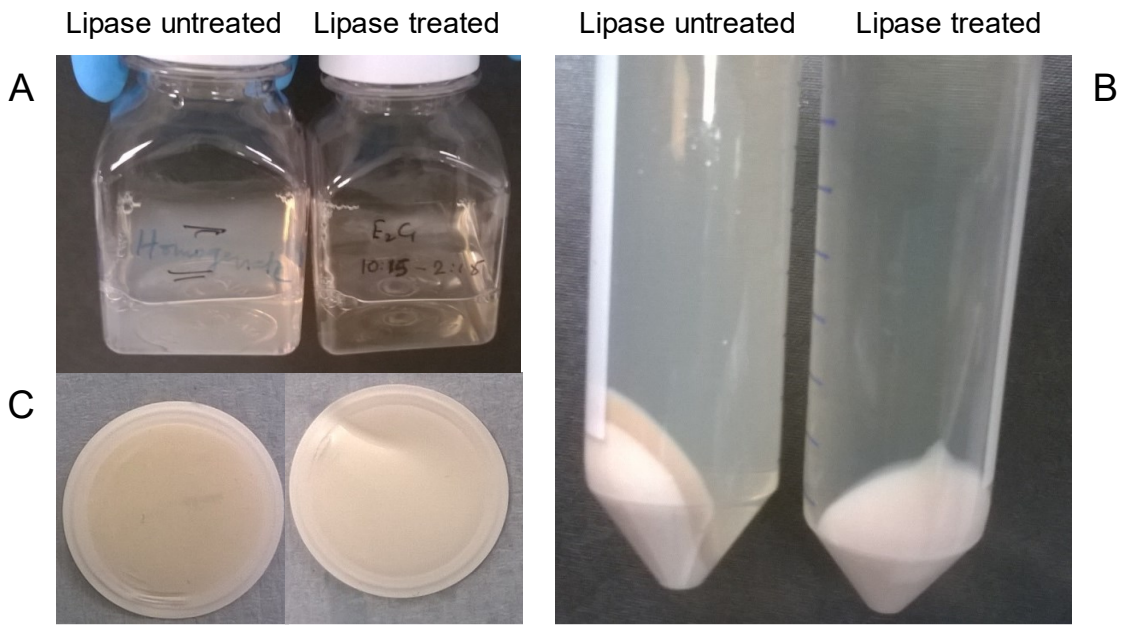


Figure 3.4: Action of *C. rugosa* triacylglycerol lipase on *P. pastoris* homogenate

The above images show suspended particles in the soluble fraction after the homogenization of P. pastoris. P. pastoris homogenate was suspended in phosphate buffer saline (10% wcw/mL) and was homogenized at 1200bar for 3 passes using a gaulin lab 40 homogenizer. The homogenized sample was then centrifuged using a fixed angle centrifuge at 10,000xg for 10 mins. (A) Centrifuged supernatant of P. pastoris homogenate with a black background. (B) The pellet formed two distinct layers after the. The upper layer consisted of off-white soft white fatty layer and the lower denser solid layer. (C) the supernatant was then filtered using 0.45µm disc filter and the treated sample did not show any fouling layer on the filter as it was seen with the untreated sample.

3.4 Types of lipases and their use on yeast homogenate

Triacylglycerol lipases are a class of lipase (EC 3.1.1.3) which uniquely target the ester bond in triglycerides in presence of water giving rise to a diacylglycerol and a carboxylate. These are essential enzymes found in most lipid degradation metabolic pathways (Grillitsch and Daum, 2011). In the present study six different commercially available triacylglycerol lipases are studied, namely lipase from *A. niger*, *C. antarctica*, *Burkholderia sp.*, R. *oryzae*, *Pseudomonas fluorescens* and *C. rugosa*. The yeast cell where first homogenized using a Gaulin homogenizer (lab 1000) with discreet passes (Jin *et al.* , 2010). These enzymes where added to the yeast homogenate before and after clarification.

The treated homogenate was then incubated for two hours at 37°C and mixed using an orbital shaker at 180rpm. After two hours the lipid was extracted and analysed using HPLC as described above sections 3.1 and 3.2. The concentration of triacylglycerol (TAG) considerably decreased in comparison to the untreated homogenate. The enzymes were compared at four different concentrations 0.1, 0.01, 0.001 and 0.0001 mg/mL final concentration of enzyme in the yeast homogenate. The lipase at 0.0001 mg/mL showed no effect and was not considered for further experiments. The best performing enzymes were from bacterial species *C. rugosa*, *R. oryzae* and *P. fluorescens* and they were then compared head to head as shown in Figure 3.5. At higher concentration of enzyme (0.1mg/mL) the total TAG concentration reduced from 40.5mg/L to just 3.2mg/L as obtained from the HPLC-ELSD quantification.

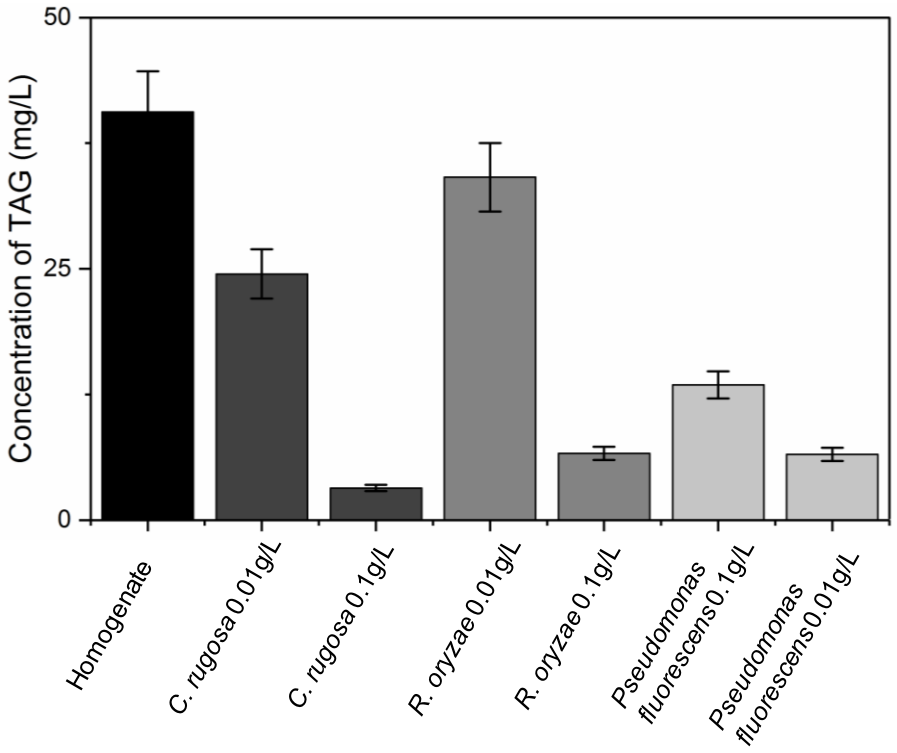


Figure 3.5: Concentration of triacylglycerol in *P. pastoris* homogenate after lipase treatment

Yeast homogenate was treated with lipases from different bacterial species to see the lipid degradation in yeast homogenate. The samples were incubated with 0.1 and 0.01mg/mL with respective enzymes. Where the enzymes were added endogenously in the yeast homogenate (10% wcw/mL) and incubated at 37° C for two hours under stirring condition. The lipids from the samples are then extracted using Bligh-Dyer method (Bligh and Dyer, 1959) as described in chapter 2, section 2.3.3.1, for total lipid extraction and using RP-HPLC with zorbax C8 column. Lipids were eluted using a linear gradient at a flow rate of 0.34 mL/min and analysed using an ELSD detector. The error bars represent standard deviation for each data point (SD) (n=3).

3.4.1 HPLC-ELSD study for degradation of triacylglycerol.

The enzymes were also tested for activity using 4-nitrophenyl laurate (4nPL) as a substrate (Ruiz *et al.* , 2004). It was found that the activity of the enzyme increases with increase in pH from pH 6.0 to pH 8.0. Optimum activity of the enzyme was found at pH 8.0. The effect of lipase from *C. rugosa* has been studied in various different solvent and aqueous conditions(Tenkanen *et al.* , 2003) (Chang *et al.* , 2006) (López *et al.* , 2004) and the optimum

pH is found between pH 5.0 and 7.0. Though, these studies have been done with pure aqueous buffer or near pure mixtures containing only the substrate, in the present study the lipase of interest is the lipase that has high activity in yeast homogenate. In aqueous buffer containing 4-nitrophenyl laurate substrate *P. fluorescens* was found to be most active. But while comparing them in yeast homogenate commercial *C. rugosa* lipase was found maximum removal of TAG as shown in Figure 3.5. The formation of fatty acid was also quantified from the yeast homogenate treated with *C. rugosa* lipase as shown in Figure 3.6. The enzyme was dissolved in 1x PBS. The stock solution was made with a concentration of 10mg/mL. The lipase was added to the sample and incubated for 2 hours at 37°C at room temperature. A final concentration of enzyme was kept at 0.1 mg/mL. Lipid was extracted from the sample after incubation was analysed using HPLC-ELSD. By centrifugation of this material, it is seen that % clarification of the material improves when treated with lipase. The homogenate was then tested with and without treatment of lipase. This was done to find if the % throughput during filtration is a function of lipase or just centrifugation.

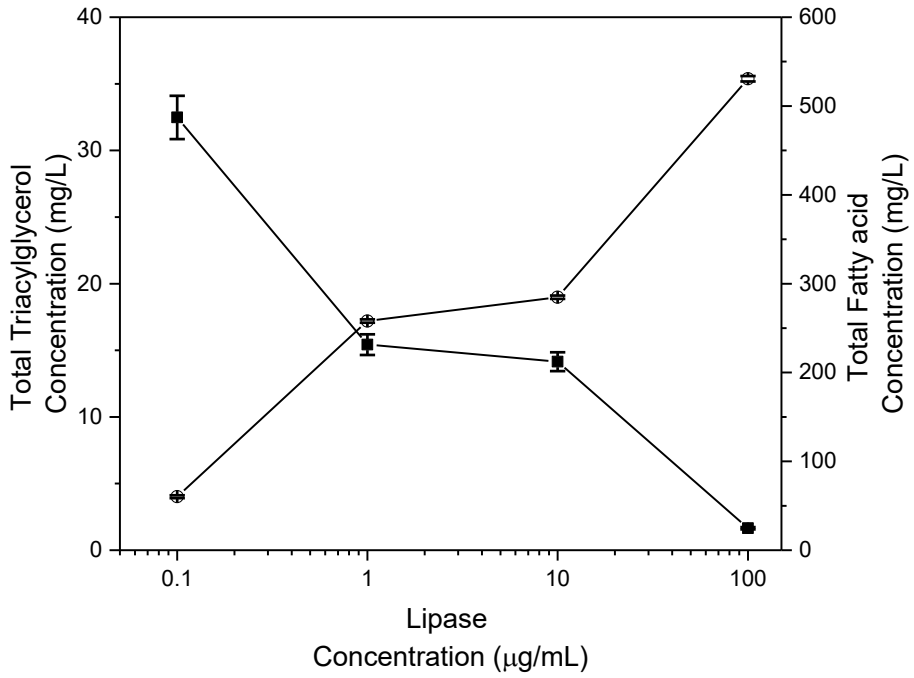


Figure 3.6: Degradation of triacylglycerol and formation of fatty acid in *P. pastoris* homogenate when treated by *C. rugosa* triacylglycerol lipase

The above Figure shows reduction of Triacylglycerols and the formation of Fatty acids when analysed using RP-HPLC-ELSD. The yeast homogenate was treated with Lipase from Candida rugosa at concentration ranging from 0.1mg/mL to 0.0001mg/mL. The samples where then kept at 37 °C for 2 hours at 180rpm constant stirring. The neutral lipids are then extracted using Bligh-dyer method for total lipid extraction and using RP-HPLC with zorbax C8 column. Lipids were eluted using a linear gradient at a flow rate of 0.34 mL/min and analysed using an ELSD detector. The graph shows the reduction of total triacylglycerol's (substrate) with increasing concentration of lipase. In the same sample the total fatty acids (product) concentration increases with increasing concentration of enzyme. The error bars represent standard deviation for each data point (SD) (n=3).

The effect of lipase can also be seen in the percentage solids remaining. When homogenate is treated with the lipase at 0.1mg/mL and clarified there was decrease in percentage solids remaining seen studied using OD_{600nm} of the clarified supernatant. Briefly, optical density of the untreated homogenate reduced from an OD of 50 to 16. Were as the sample when treated with lipase and then clarified the optical density of the supernatant was found to be OD ~ 3. This is a strong indicator that there is an effect of lipase and removal of suspended colloidal lipids.

Thus, adding the lipase might help in the clarification of high lipid load material like yeast homogenate. To select the triacylglycerol lipase which can be used to remove the triacylglycerol's in yeast homogenate, 6 different triacylglycerol lipases from different bacterial and fungal species were selected. From these lipases the best performing enzymes were found to be the one from fungal species C. rugosa and bacterial species of P. florescence shown in Figure 3.6. The lipase from C. rugosa degrades 80% of the TAG from the yeast homogenate. Lipase from C. rugosa Type VII, ≥700 unit/mg solid was taken for analysis.

3.4.2 Lipase activity using 4-nP substrates

The activity of commercial lipase from *C. rugosa*, and *P. florescence* was tested with 4-nitrophenyl laurate as a substrate. This was done to find higher active lipase which can be exogenously used on the triacylglycerol lipids in yeast homogenate. The standard curve was

created with 4-nitrophenol. Activity was measured over time at 460nm using a TECAN plate reader. The experiment has been done before using microwell plates which was elucidated by Ruiz *et al.* , 2004 in his work. Briefly, the lipase assay phosphate buffer at pH 8.0 was made with 1.33% Triton X 100. The substrate and standards were prepared in the assay buffer premix. 100µL of each standard was dispensed in the microwell plate and used for standard curve measurement. 50µL substrates were dispensed and the plate was kept at 37°C for 10minutes before measurement. 50µL of the sample was used for measuring the enzyme kinetics. The sample and the substrate were mixed using 2sec orbital shaking before taking the reading. From the plots the specific activity of the enzyme was calculated as activity is equal to number of enzyme units per mL of reaction mixture per milligram of protein (units/mg/mL). Though the activity of the enzyme is higher than *C. rugosa* against 4-nitrophenyl laurate substrate, it was found to have almost similar effect in yeast homogenate as shown in the previous section.

The use of *P. fluorescens* lipase and *C. rugosa* lipase might have similar effects when co-expressed along with other heterogeneous proteins. It is found that the activity of *C. rugosa* triacylglycerol lipase VIII is active at both 37°C as well as room temperatures. Its activity reduces but does not depleted completely at pH 7.0. In case of lipase from *Pseudomonas fluorescens,* the activity is below detection (BD) at pH 7.0. The enzyme activities are compared at different temperatures and pH against para-nitrophenyl laurate substrate. The degradation of the ester bond in the substrate gives rise to the nitro phenol, which is detected at 460nm wavelength.

Table 3.1: Lipase activity of *C. rugosa* and *P. fluorescens* by colorimetry analysis
P. fluorescens activity with 4-nitrophenyl laurate was found to be below detection (BD) when analysed in comparison with C. rugosa with buffer at pH 7.0. It showed high activity when the assay was done with pH 8.0. The assay was done with triplicate of each sample which it gave a standard deviation from 0.55 to 0.65 in case of P. fluorescens lipase and C. rugosa lipase gave a standard deviation from 1.56 to 2.45. Even though C. rugosa lipase

did not show very high activity, but it was considered active at a broad range from pH 7.0 to 8.0.

Lipase activity with 4-nitrophenyl laurate (µmole/min/mg)

	37°C	26°C	
C. rugosa	8.70 ± 2.45	3.29 ± 1.95	pH 7.0
	6.27 ± 1.65	6.41 ± 1.56	pH 8.0
P. fluorescens	BD	BD	pH 7.0
	195.47 ± 0.65	110.39 ± 0.55	pH 8.0

3.5 Analysis of *P. pastoris* homogenate during lipase treatment

3.5.1 Effect of heat on *P. pastoris* homogenate

The aim of the study was to find the effect of temperature at 37°C with increased cycles of homogenization on the crude yeast homogenate during lipase treatment. To study the effect of homogenization and differentiate its effect from the increased temperature, the yeast cells were suspended in PBS at 10% wcw/mL and homogenized using gaulin lab 40 homogenizer with discreet passes (cycles). As the number of cycles increases the percentage of smaller particle in the homogenate. The increased homogenization cycle increases the percentage of particles having size less than 1µm whereas the percentage of particles ranging from 1µm to 10µm reduces. It is also observed that these smaller particles form bigger size particles when incubated at 37°C for 2 hours under stirring conditions at 180 rpm as shown in Figure 3.7. After the incubation at 37°C the smaller particles in the supernatant were not detected. This showed that there is a significant effect of increased temperature on the P. pastoris homogenate. The heat reduces the amount of submicron particles and creating larger particles in the mixture. This can have different effects on the fouling of membranes when such material undergoes filtration using dead-end filter membranes. The incubation at 37°C may have effect on yeast homogenate particles and causing them to aggregate together to form

larger particles. This may be a reason for increase in large particles. as shown in the Figure 3.7 panel (B).

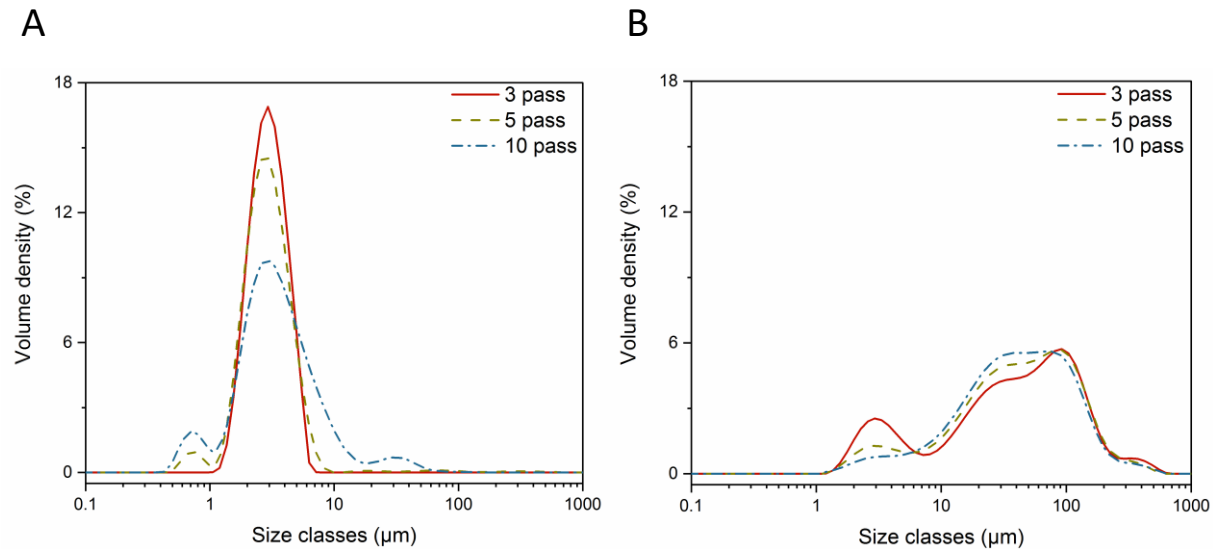


Figure 3.7: Particle size distribution of *P. pastoris* homogenate after heat treatment.

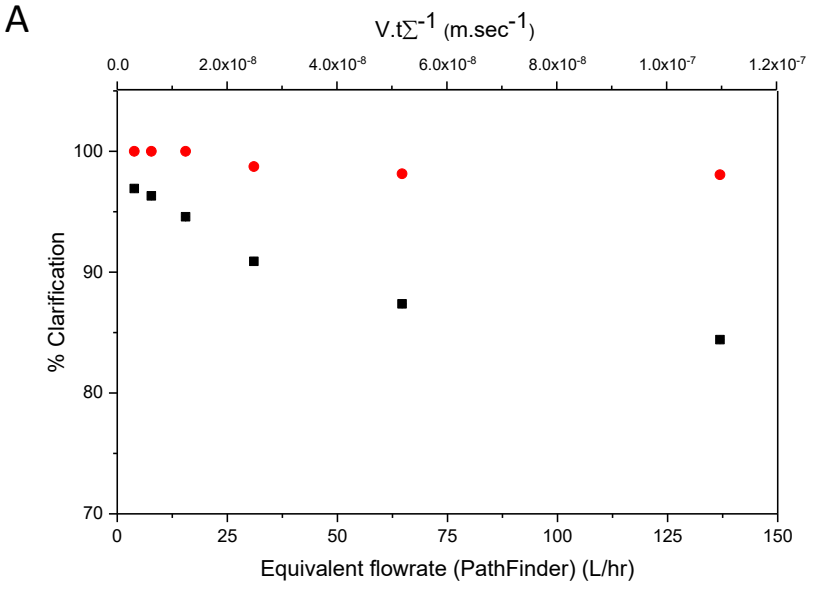
The above Figures show the particle size distribution obtained after P. pastoris homogenization at 500bar with multiple passes and when it is treated with heat treatment. The P. pastoris were grown in YPD medium using the method earlier described in chapter 2 section 2.2.2. (A) Red line shows the distribution of particles between 1µm and 10µm after homogenization with 1 discreet pass at 500bar pressure. The green dash line represents the particle distribution after 3 passes and the blue dash dot line represents the particle distribution after 5 passes. As it can be seen from the above Figure as the number of passes increases so does the small particles below 1µm and at higher homogenization cycles there is also an increase in larger particle size. (B) The same samples when kept in at 37°C for 2 hours and shaken at 180 rpm on an orbital shaker, the smaller particles form clumps of larger size particles. The red line shows that even after 1 cycle yeast cells clump together at a temperature of 37°C. Same is seen with samples undergone with 3 cycles and 5 cycles of homogenization.

3.5.2 Clarification of *P. pastoris* homogenate using ultra-scale down device

P. pastoris homogenate are clarified to remove large particle and the supernatant is used for filtration. To study the industrial scale continuous centrifugation a scale down system is used in this study. Here a fixed angle centrifuge is used with fixed volume of 50mL. The

theoretical $Vt^{-1}\Sigma$ values for the equivalent flow rate were calculated and converted into rpm that is used in the fixed angle centrifuge. This centrifugation speed was then used on the yeast homogenate samples with cycles with 0, 1, 3, 5, 7, and 10 passes. The supernatant was then analysed based on OD_{600nm} and the percentage remaining solids were then calculated. The % solid remaining and the clarification percentage was calculated for each cycle of homogenisation and plotted as shown in Figure 3.8.

As described in the earlier section there is a large effect of heat on the yeast homogenate samples, similar observations were recorded after calculation of percentage solids remaining. As the number of cycles of homogenization increased so did the submicron particles which could not be centrifuged at high centrifugation speeds. By incubating the samples with heat and shaking at 180rpm the clumps formed as seen earlier are easier to centrifuge and can be clarified. This was confirmed with the percentage solids remaining with and without incubation of the yeast homogenate at 37°C. This reduction of the submicron particles indicated that centrifugation at lower flow rate can be used for clarification of high percentage of submicron particles from the 37°C incubated and stirred feed solution. The difference in percentage solids remaining of treated and not treated samples is shown in Figure 3.9.



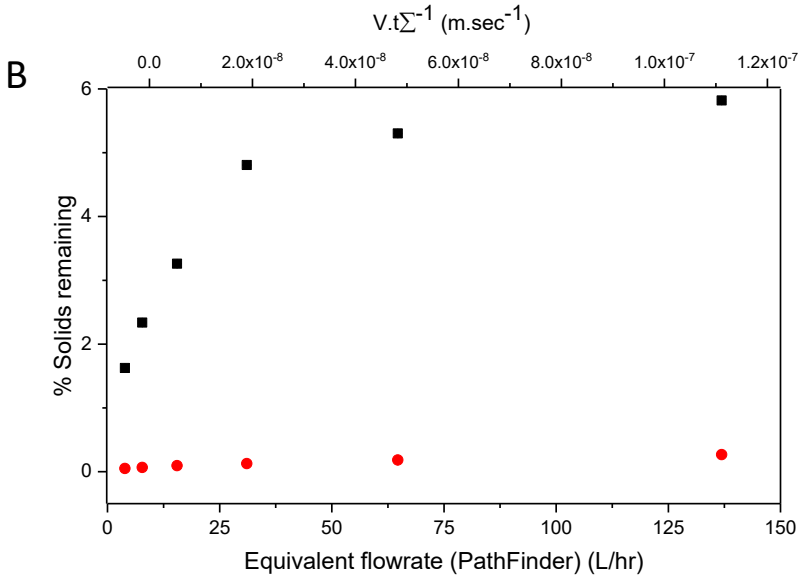


Figure 3.8: Lab scale clarification of *P. pastoris* homogenate equivalent to continuous centrifuge

The above Figures show the effect of exogenous addition of C. rugosa lipase (0.1mg/mL) to yeast homogenate. The experiment for centrifugation is done as described in chapter 2. (A) Lipase treated yeast homogenate (red dots) and clarified at different centrifugation speeds. This is compared with the untreated yeast homogenate (black squares) which shows higher percentage of suspended solids in the clarified supernatant. (B) The percentage clarification obtained with the corresponding percentage solids remaining in the clarified supernatant.

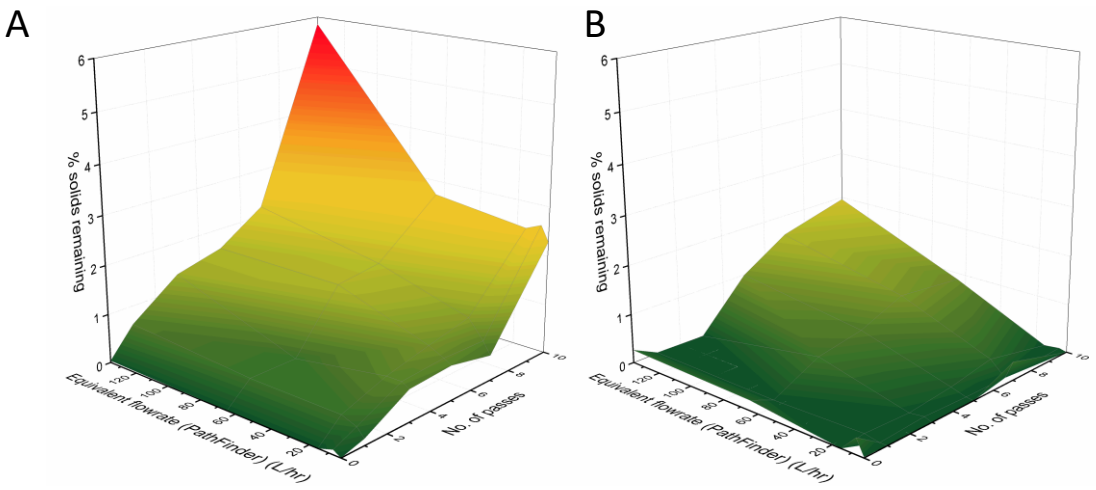


Figure 3.9: Solids remaining from *P. pastoris* homogenate after clarification

The above Figures show percentage solids remaining in yeast homogenate after centrifugation corresponding to the number of passes during homogenization. (A) 10% (wcw/mL) yeast cells are suspended in PBS and is homogenized with multiple passes at

500bar using Gaulin lab 40 homogenizer. Using the lab scale centrifuge equivalent flow rate to the pathfinder is selected and remaining suspended solids based on optical density (OD_{600nm}) was compared. Higher flowrate with a greater number of passes had larger percentage of solids remaining. (B) Same samples when treated with heat (37°C on orbital shaker at 180 rpm for 2 hours) gave much lesser percentage solids remaining with a greater number of passes as well as at higher equivalent flow rate.

3.5.3 Effect of heat on particle size distribution in of yeast homogenate

Yeast homogenate was studied using particle size distribution as described in chapter 2, section 2.3.2.2. To study the effect of heat along with centrifugation yeast homogenate was subjected to 37°C for 2 hours and shaken at 180 rpm on an orbital shaker as shown in Figure 3.10. It was observed that after centrifugation submicron particles are suspended in the supernatant of the yeast homogenate with most of the cell debris removed as pellet. The remaining sub-micron particles are observed to form larger size particles when incubated at 37°C for 2 hours under agitation. To check the effect of centrifugation the supernatant of the centrifuged yeast homogenate was repeatedly exposed to heat treatment (37°C). In Figure 3.10 (C and D) shows that remaining sub-micron particles form larger particles after the treatment. It was then concluded that the removal of cell debris with the help of centrifugation does not contribute to the formation of large particles on heat treatment. The smaller particles form the larger aggregates on heat treatment. This phenomenon of large particle formation after heat and agitation including the centrifugation gives an added advantage to the improve the clarification performance of the yeast homogenate.

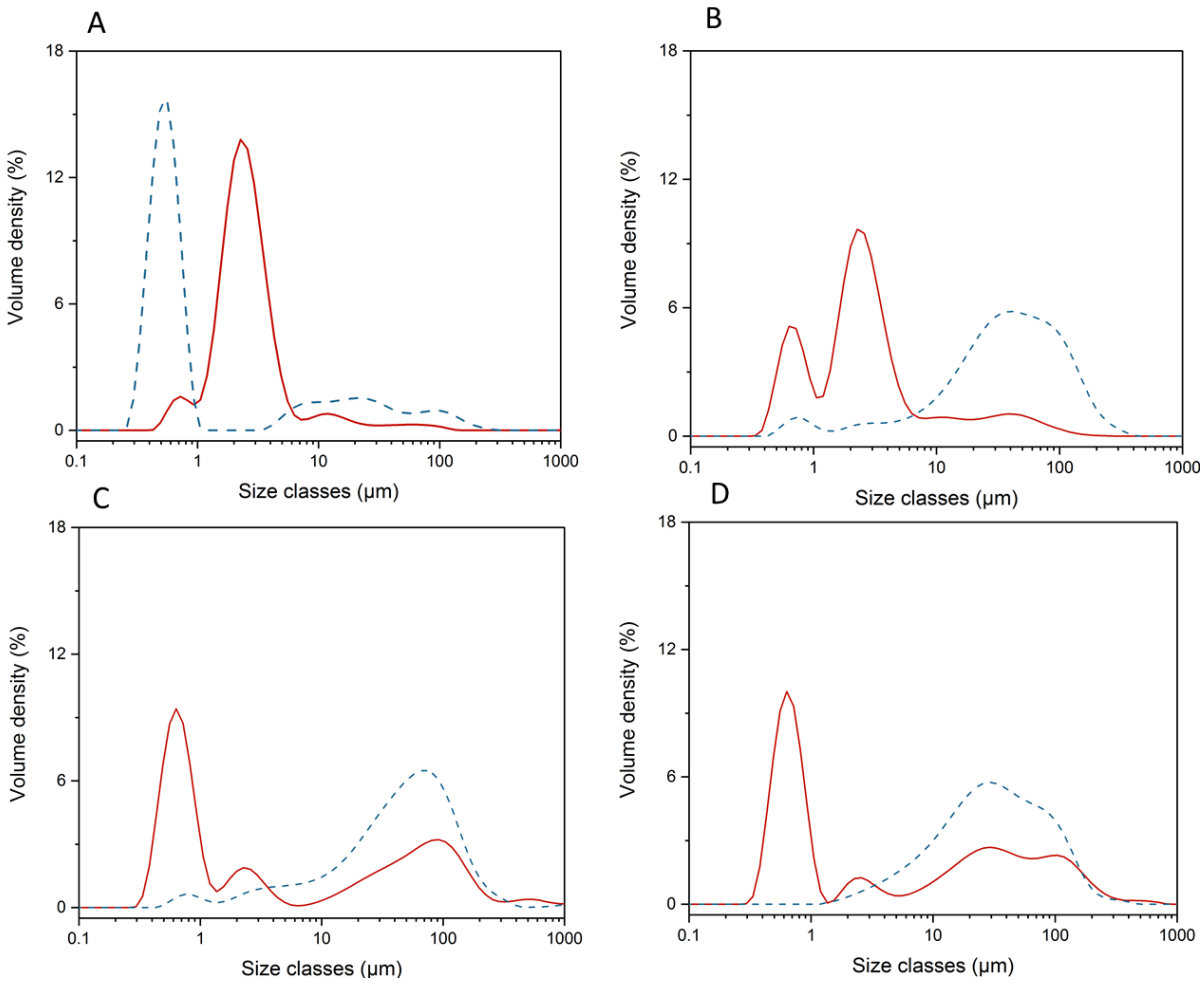


Figure 3.10: Particle size distribution of clarified and heat-treated *P. pastoris* homogenate.

(A)Red line shows the particle size distribution obtained after yeast homogenization at 500bar with 5 discreet passes. Only submicron particles (blue dash lines) are seen suspended in the samples after centrifugation at 600xg for 20 mins. (B) The yeast homogenate supernatant (red line) showed submicron particles. This supernatant sample was heat treated and kept at 37°C for 2 hours with shaking at 180 rpm on an orbital shaker. When the treated supernatant sample was analysed it showed particles of large size (blue dash lines) in place of submicron particles. (C) and (D) The treated supernatant was clarified again using centrifugation at 600xg for 20 mins. After clarification the resultant supernatant showed some remaining large size particles and submicron particles. This resulting sample was then treated again with heat and agitation resulting in same effect where the submicron particles (red line) formed large size particles (blue dash lines).

3.6 Fouling during filtration of *P. pastoris* homogenate

3.6.1 Filter fouling test using dead end filtration setup

For the test of fouling that occurs during filtration a simple filtration setup was made as shown in Figure 3.11. The yeast homogenate is used a sample which is loaded on to the 10mL syringe and fitted on to a syringe pump. This syringe pump is then attached with a PEEK tubing to disc membrane filter holder. The connection between the disc membrane filter and the syringe pump is done using a T-junction. An absolute pressure sensor is attached to the end of this end of the T-junction. This pressure sensor is then attached with a digital recorder with the help of a data acquisition (DAQ) system. The recorder can record the pressure different applied on the pressure sensor and the atmospheric pressure. The obtained values are measured as the difference of pressure per unit time. The filtrate generated from the filter is then measured using a calibrated weight balance.

The obtained measurements are recorded in terms of filtrate volume and pressure rise during the filtration. An absolute board mount pressure sensors 0-500 psi straight O-Ring interface (Honeywell Cat No 40PC100G) is attached using a T-junction at feed side of the filter. A syringe pump is used to pump the yeast homogenate at the constant flux of 100LMH. The feed pressure is measured using the absolute pressure sensor and difference in the pressure is calculated with retented pressure as atmospheric pressure. This filtration setup was used for clarification of lipase treated and untreated *P. pastoris* homogenate. The method for sample preparation used in these experiments is prepared as per the flow chart given in Figure 3.12.

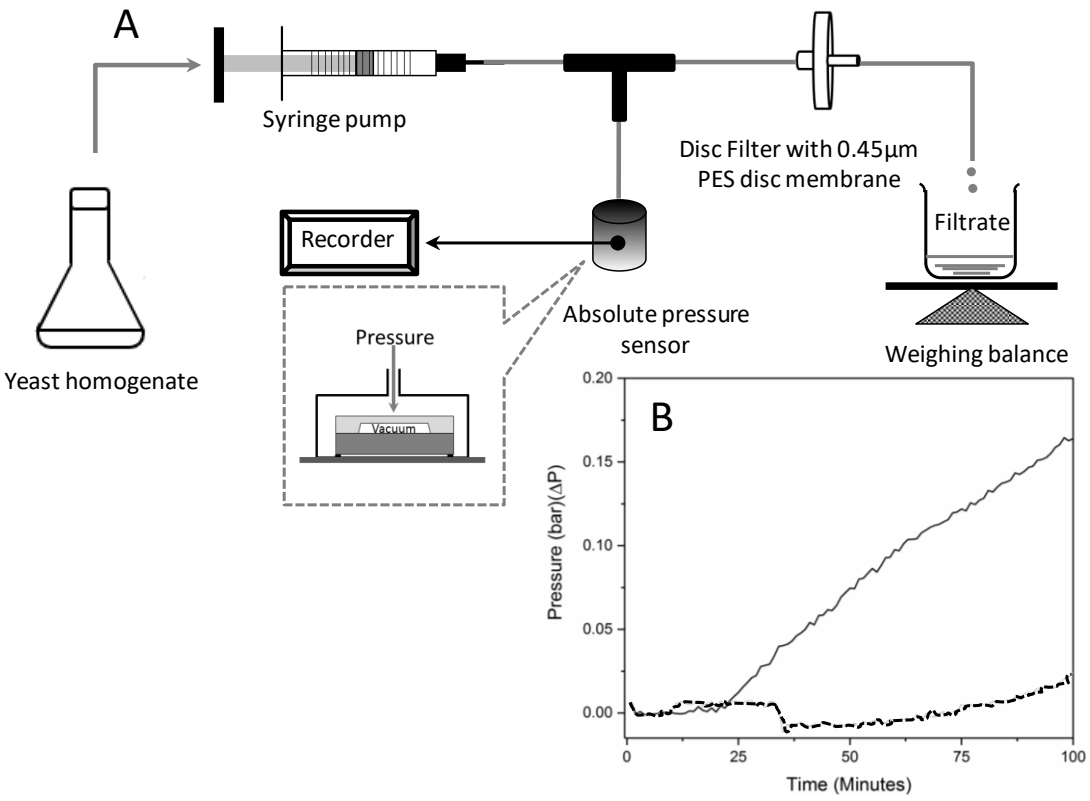


Figure 3.11: Filtration setup for *P. pastoris* homogenate

Schematic diagram for filtration setup is shown in the above Figure. Polyether sulfone disc filter membrane of 0.45µm was used to study the dead-end filter fouling. (A) In the above setup yeast homogenate was loaded in a 10mL syringe and using a syringe pump, it was pumped into the filter housing at 100LMH. Dead-end pressure sensor was attached to the line using a T-piece. The pressure readings were recorded using a DAQ system connected to a computer. The filtrate obtained was then measured using a container on a digital weighing balance. Filtration experiment was repeated three times for measure of error. Each run was done using a fresh membrane filter. (B)The graph shows the pressure reading obtained from the absolute pressure sensor attached to the filtration setup using the T-junction. The black line represents the pressure rise when clarified yeast homogenate supernatant is passed through the filter. The black broken line represents the enzyme treated and clarified yeast homogenate passed through the same setup. The flow rate chosen for this experiment was 10LMH and corresponding flow rate (0.05 mL/min) was set on the syringe pump for the dead-end filtration.

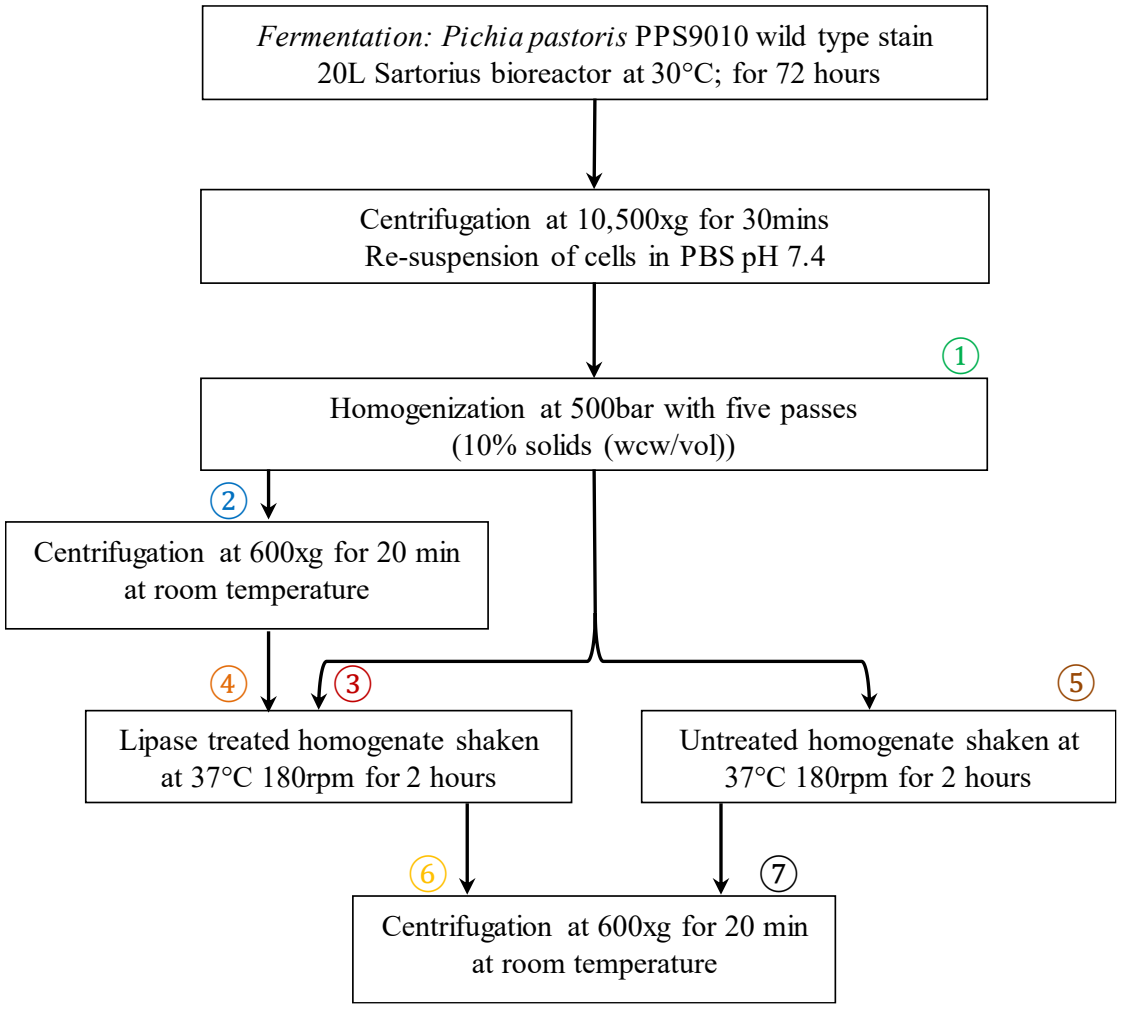


Figure 3.12: *P. pastoris* homogenate sample preparation for filter fouling test

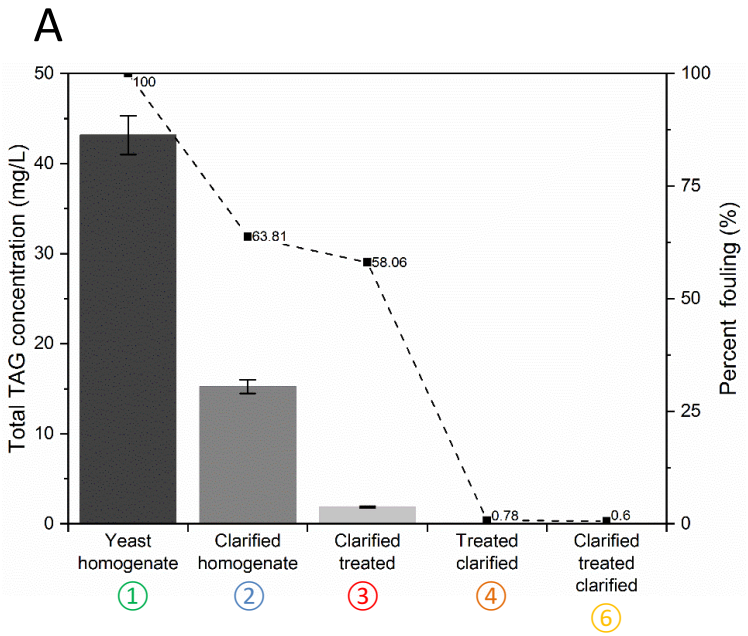
The flow chart shows the sample preparation procedure before yeast homogenate was applied to dead end filtration using a 0.45µm polyethersulfone disc membrane filter at 100LMH. The lipase treated, and untreated samples are clarified before testing the filter by centrifugation at 600xg (comparable to pathfinder continuous centrifuge at 60L/hr) for 20mins at room temperature. The five samples that are analysed are ① yeast homogenate, ② clarified homogenate, ③ P. pastoris homogenate treated with lipase and ⑤ P. pastoris homogenate untreated with lipase. ④Clarified homogenate treated with lipase. ⑥ clarified homogenate treated with lipase and clarified again. ⑦Homogenate untreated with lipase and clarified.

3.6.2 Use of exogenous lipase in *P. pastoris* homogenate

C. rugosa lipase VIII was selected based on the previous experiments and lipase treated *P. pastoris* homogenate was used for filter fouling test. In this experiment the effect of heat

treatment as well as the action of lipase was tested using filtration and percentage clarification. The *C. rugosa* lipase (0.1mg/mL) treated samples were centrifuged at 600xg for 10 mins and samples with and without treatment were analysed. The enzyme treated yeast homogenate (1% wcw/vol in 1x PBS) were passed through a dead-end filter and the filtrate was collected in a vessel for throughput measurement as shown in the schematic diagram Figure 3.11. The effect of the centrifugation and lipase treatment was hard to separate as the effect of temperature would mask the effect of lipase treatment. The treated and clarified samples showed reduced fouling and increased throughput during filtration. But this effect cannot be completely attributed to the degradation of lipids as seen by the action of lipase through HPLC analysis.

The filter used in this study is a 0.45µm PES disc membrane. The open pore side is kept towards the feed side. Cut-off pressure was kept at 43 PSI (2.96 bar). It is found that the homogenate at higher concentration would foul the membrane before quantifiable data can be obtained. The pressure rise in the untreated and centrifuged homogenate was less than the homogenate sample which was not centrifuged. The *C. rugosa* lipase (0.1mg/mL) treated and clarified homogenate was found to have much less pressure build up indicating clearer feed. This shows a positive effect on clarification on the yeast homogenate after the treatment of lipase as shown in the following Figure 3.13.



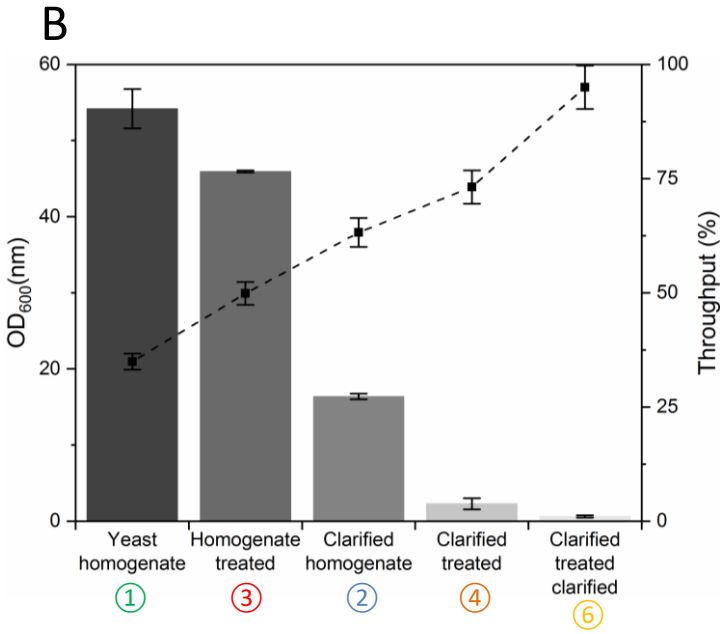


Figure 3.13: Lipase treatment on clarified and non-clarified *P. pastoris* homogenate

The above Figure shows the effect of lipase on removal of triacylglycerol (TAG) and corresponding reduction in fouling of a 0.45µm PES (Polyethersulfone) disc filtration membrane during dead end filtration at 100LMH. The treated samples underwent incubation in 37°C for 2 hours. (A) Lipase treatment was done on yeast homogenate and was analysed for total TAG and percentage fouling (solid square connected with dash) occurred during dead end filtration. Yeast homogenate shows maximum concentration of triacylglycerol (TAG). The yeast homogenate was centrifuged at 600xg for 10 mins and is denoted as clarified homogenate. The samples which are treated with C. rugosa lipase (0.1mg/mL) before and after the clarification show reduced percentage fouling along with reduced TAG. The error bars represent standard deviation for each data point (SD) (n=3). (B) As described earlier the samples are tested with solids remaining and corresponding percentage throughput (solid square connected with dash) obtained after filtrations of the samples. Homogenate treated (not clarified) was tested which gave the same amount of total TAG, percentage fouling and had very little change in OD as well as percentage throughput obtained after filtration. The error bars on percentage throughput represent 5% standard error taken as for each data point (n=2).

3.6.3 Fluroscent microscopy for membrane fouling

3.6.3.1 Dynamic fouling mechanism of filtration membrane using microfluidic device and confocal microscopy.

The filter membrane fouling can be studied using confocal microscope as fouling of chromatography beads were studied and shown in study by Burden *et al.*, 2012. Here the filter membrane needs to be fouled using the lipids, ideally from yeast homogenate and stained using lipid binding BODIPY dye. To visualize the filter membrane, it is necessary to have the filter membrane fitted on the plate of the microscope. To have such a fitting a microfluidic device was constructed, the device construction and design is described in chapter 2 section 2.1.3.1. The complete setup with the microfluidic device and the details of the fabrication and construction of the microfluidic device is given in chapter 2 section 2.1.3. The completely assembled microfluidic filter holder was used to fit a filter membrane on a confocal microscope z-stage and can be helpful in studying the dynamic fouling of the filter membrane. Fluorescent dye such as BIODIPY for lipids was used to track the fouling mechanism of biological impurities. This microfluidic device with the filter membrane was specifically designed to study the study filter membrane fouling, during the fouling process. As the fouling was not visible higher resolution techniques like confocal microscopy was used. The device was constructed in collaboration with Dr. Nikolay Dimov and Dr. Nicolas Zita.

The study of dynamic fouling of filter membrane using fluorescent confocal microscopy did not produce desirable results due to quick fouling and back pressure created during the fouling of the membrane. The fouling of the membrane was much faster than expected along with flow and fluid dynamics were not set up to the optimal conditions. This created difficulties in reproducing the results and fouling the membrane in a consistent manner. For imaging the fouling and flow dynamics needed to be done. Due to these difficulties another approach was taken where the fouled membrane was sectioned using cryo-sectioning techniques and visualized under fluorescent microscopy described in the next section 3.6.3.2.

3.6.3.2 Membrane lipid fouling study using fluorescence microscopy.

Having studied the lipid fouling during the primary recovery operation for VLP products from yeast homogenate in-depth analysis of interaction of these impurities. Different approaches can be taken namely visualization of the fouled membrane stained with fluorescent dye under microscope after 10μm cryo-sectioning. The possible method of studying fouling mechanism, as described by Lu *et al.*, 2015, is where ceramic membranes are fouled using water and oil emulsions. These membranes are then cross sectioned and studied under scanning electron microscopy. The use of scanning electron microscope is expensive and time consuming. Other methods of studying fouled membranes could be by use of dye which binds to the foulant and could be visualized. To test this theory, which is to study lipid fouled membrane using a lipid binding dye, few experiments were under taken.

Briefly, the *P. pastoris* (PPS9010-wild type) cells were grown in YPD overnight and then homogenized in 1x PBS buffer. 10mL of the centrifuged homogenate supernatant was passed through an 0.45μm PES disc membrane till there was back pressure seen up to 3 bar pressure. Two different dyes, Nile red and BODIPY dye were used to study the lipid fouling on membrane. Nile red was found to non-specifically bind to the membrane on the filtrate side as well as the retained side as shown in Figure 3.14. The Nile red stains the membrane on both sides even though the membrane was use in dead end filtration mode, hence the fouling should be only on one side. When visualized using ET-DS-Red (TRITC/Cy) filter both ends of the filter is illuminated. The use of BODIPY dye is found to be more sensitive and shows the fouling of the neutral lipids to the polyethersulfone membranes (PES) after filtration of yeast homogenate as shown in Figure 3.15. Membrane stained with BODIPY dye showed clear lipid fouling layer corresponding to single side brightly stain part of the membrane in comparison to the membranes stained on both sides with Nile red dye. During the fouling the flux decreased and source of the decreasing the flux was considered due to the lipids in the feed material. Further removal of lipids and the effect of lipase is described in the following section 3.7.

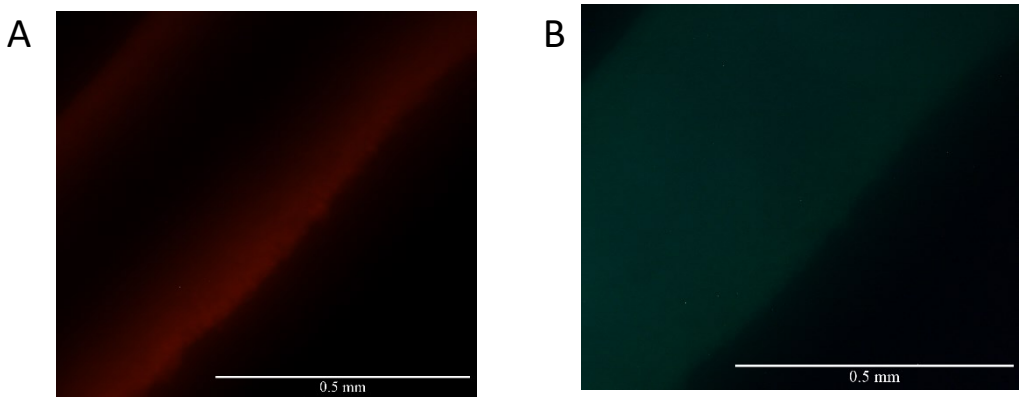


Figure 3.14: Fluorescent microscopy using Nile red for membrane lipid fouling

The image was taken with the help of Nikon fluorescent microscope after the membrane was strained with nile red dye. The fouled membrane was stained using 0.0001mg/mL Nile red in PBS for 10 mins. The membrane was then vertically solidified in OCT (cryo-binding media) in a container and mounted on the cryo-tome. 10µm sections were obtained using the cryo-tome which where then used for visulization of the fouling due to lipids. The lipids (A) were visualized using ET-DS-Red (TRITC/Cy) filter (excitation: 530-560nm emission: 590-650nm) and the membrane (B) was visualized using ET-GFP (FITC/Cy2) filter (excitation:450-490nm emission: 500-550nm)

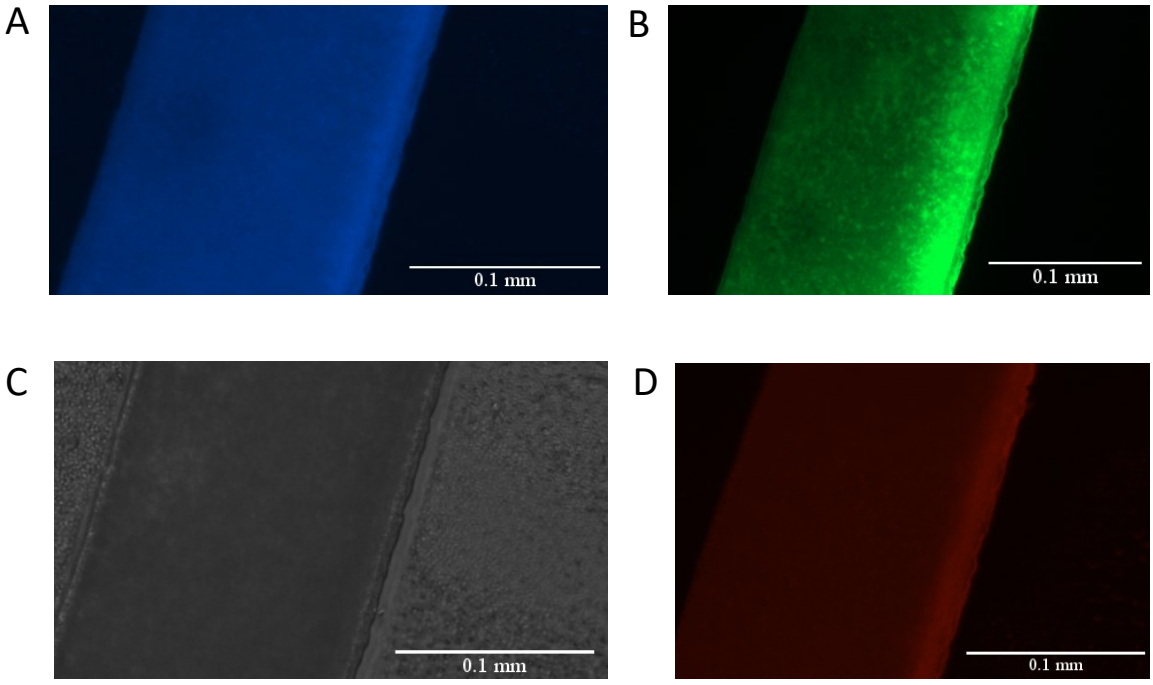


Figure 3.15: Fluorescent microscopy using BODIPY dye of polyethersulfone (PES) lipid fouled filter membrane with nominal pore size

The images were taken with the help of nikon fluorescent microscope after the membrane was strained with BODIPY dye (excitation: 493nm and emission: 503nm). The BODIPY

dye (0.0001μg/mL) was mixed with the homogenate supernantant such that it binds with the neutral lipids which fouls the membrane. The membrane was then vertically solidified in OCT (cryo-binding media) in a container and mounted on the cryo-tome. The membrane (A) were visulaized using DAPI filter (excitation:357nm and emission: 447nm) and the lipids (B) was vialized using GFP filter (excitation: 470nm and emission: 525nm) which highlights the fouled lipids (C) The texas red was used to visualized the filter memrbane without any stain. (D) The fouling was also visulized at a higher exitation, emession range using texas red filter (excitation: 585nm and emission: 624nm).

3.7 Effect of lipase on filterability of a dead-end filter

3.7.1 Constant flux filtration for lipase treated *P. pastoris* homogenate

For the constant flux filtration, a filtration setup having dead end filter with 0.45μm polyether sulfone disc membrane filter was used as shown in Figure 3.11. Sample preparation was done using *P. pastoris* homogenate which was centrifuged at 10,000xg for 20 minutes before filtration evaluation as described in chapter 2, section 2.3.2.3 and section 2.3.2.4. Briefly, the sample preparation was done as shown in the following Figure 3.16. The samples were clarified before the treatment was done to ensure the removal of large particles. After the clarification, the *P. pastoris* homogenate was treated with exogenous addition of *C. rugosa* lipase VIII with a final concentration of 0.1mg/mL and kept at 37°C for 2 hours under 180rpm orbital mixing. The homogenate treated with lipase and not treated with lipase both were processed identically and were then clarified using centrifugation at 8960xg for 20 minutes at room temperature. The second centrifugation also ensured that there are no cell debris which might contribute to the filter fouling and only suspended fatty material consisting major of the lipids remain in the solution along with other cellular materials. The filtration study was done at constant flow rate for comparison of treated and untreated sample as shown in previously reported techniques in literature (Field *et al.* , 1995; Hlavacek and Bouchet, 1993).

The above-mentioned method was employed for all filtration analysis and pressure along with cumulative filtrate volume was measured as shown in

Figure 3.17. It was found that at lower constant flux the untreated sample and treated sample shows the same filterability and % throughput at the end of filtration after 25 minutes. With higher flux rate the clogging starts to become more evident and less volume of permeate is obtained at the end of filtration. At 800LMH the untreated sample gave 20% less filtrate compared to the treated *P. pastoris* homogenate. This suggests that at a concentration of 0.1mg/mL lipase can be used for the removal of lipids from the *P. pastoris* homogenate and has a positive effect at higher flux rate which can be used for improvement for downstream processing of *P. pastoris* based lipid rich feed materials.

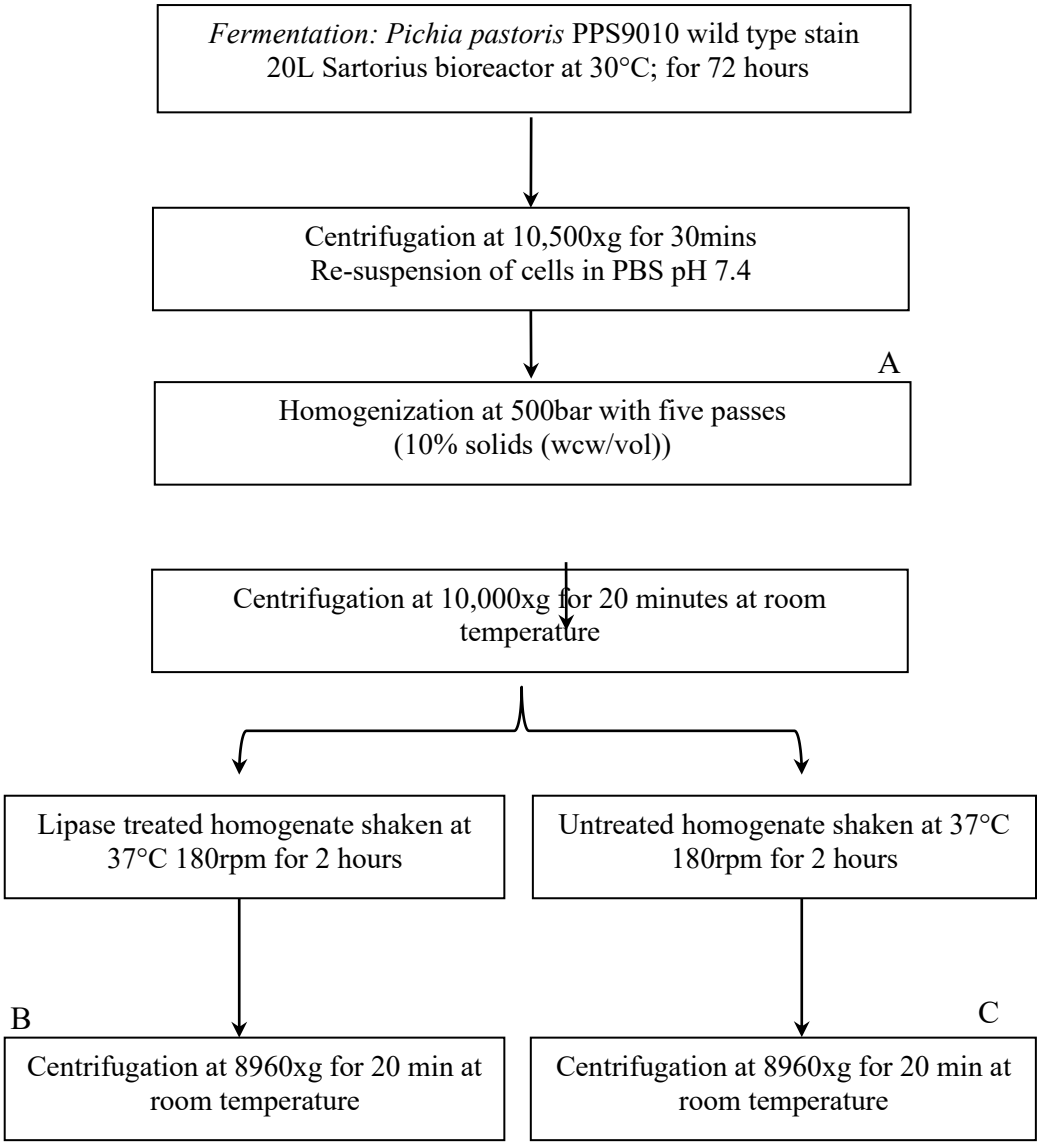


Figure 3.16: *P. pastoris* homogenate sample preparation for filterability test

Sample preparation procedure before yeast homogenate was applied to dead end filtration using a 0.45µm polyether sulfone disc membrane filter at constant flux. The P. pastoris

homogenate is centrifuged at 10,000xg for 20 minutes before lipase treatment. This is to ensure the removal of debris. The lipase treated, and untreated samples are clarified before testing the filter by centrifugation at 8960xg (comparable to pathfinder continuous centrifuge at 3 L/hr) for 20mins at room temperature.

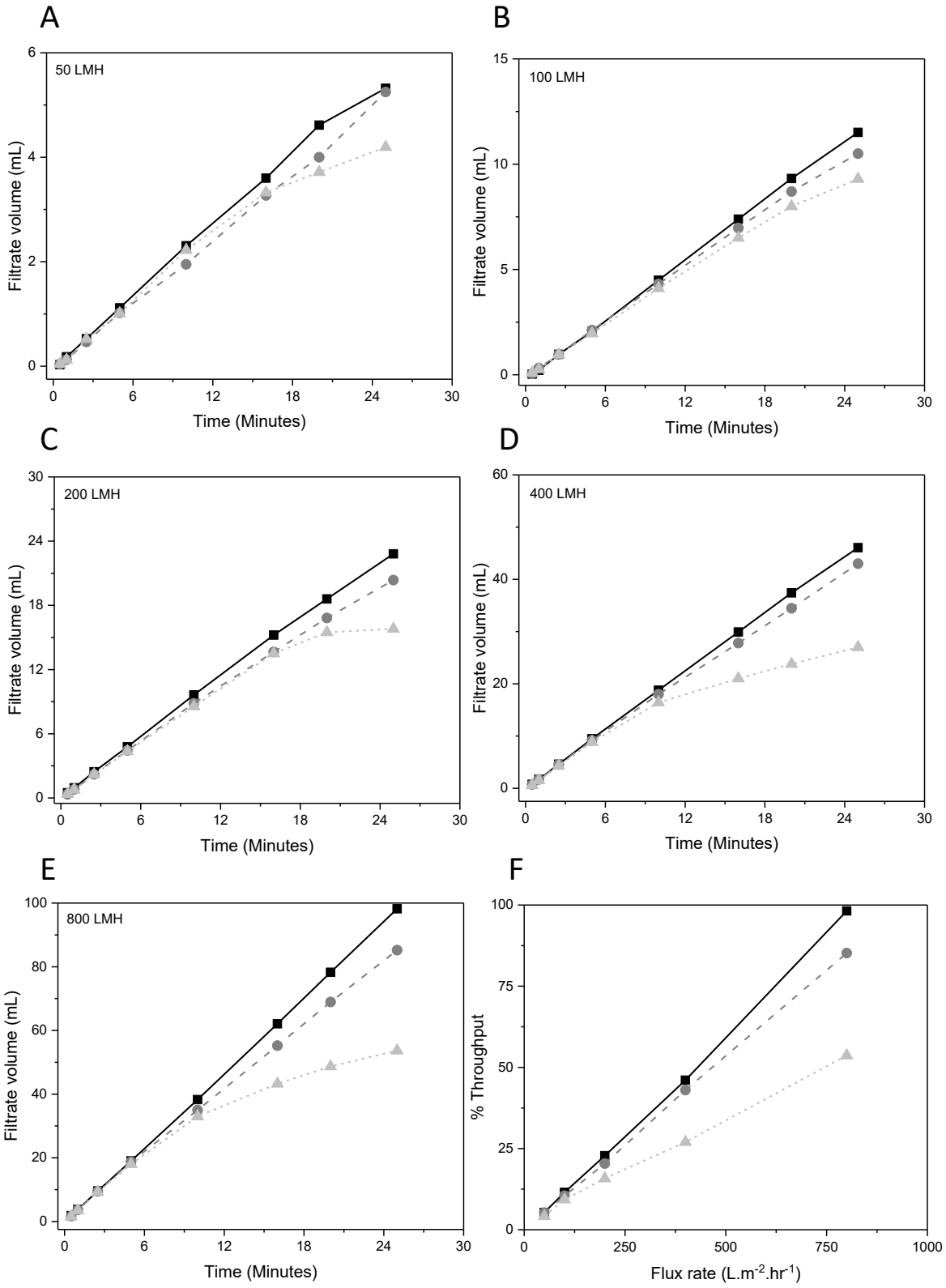


Figure 3.17: Dead-end filtration of clarified treated and untreated *P. pastoris* homogenate

P. pastoris cells were grown and homogenized as mentioned earlier in Figure 3.14. the first centrifugation is done to ensure removal of all the cell debris. The treated sample is P. pastoris homogenate that is treated with C. rugosa lipase (0.1mg/mL) and kept at 37°C for 2 hours under orbital shaker at 180rpm. The untreated sample is P. pastoris homogenate that is not treated with lipase but kept at 37°C for 2 hours under orbital shaker at 180rpm. After centrifugation at 8960xg for 20 mins only suspended materials including lipids remained in the clarified supernatant. This clarified supernatant is passed through a dead-end 0.45µm filter for 25 mins under constant flux. (A) Cumulative volume of filtrate obtained from dead-end filtration using treated P. pastoris homogenate (dark grey solid circles) in comparison to untreated homogenate (light grey solid triangles) at 50LMH constant flux. The filtrate obtained from 1x PBS pressure is shown as solid black squares. At the lower flux less, amount of filtrate is obtained at the end of 25 mins. (B), (C), (D), and (E) shows the increased flux influences the untreated P. pastoris homogenate. At higher flux the membrane fouls quickly and over time the total volume of untreated homogenate filtrate was 50% less than the C. rugosa lipase treated P. pastoris homogenate. (F) Comparison shown in terms of percentage throughput obtained after filtration of the treated and untreated homogenate at increasing flux. The untreated P. pastoris homogenate showed about 20% less throughput in comparison to treated P. pastoris sample.

3.7.2 Fouling during filtration of *P. pastoris* homogenate

P. pastoris cells were grown as mentioned in the previous and homogenized as previously mentioned in chapter 2, section 2.3.1.1. To test the effect of lipase removing triacylglycerol from *P. pastoris* homogenate, samples were prepared as described in Figure 3.14. Briefly, *P. pastoris* homogenate is treated with *C. rugosa* lipase (0.1mg/mL) and kept at 37°C for 2 hours under orbital shaker at 180rpm. *P. pastoris* homogenate that is not treated with lipase but kept at 37°C for 2 hours under orbital shaker at 180rpm is denoted as the untreated sample shown in Figure 3.16 (C). Before filtration test the samples were clarified using fixed angle centrifugation at 600xg for 20 mins. The samples were then subjected to filtration with 0.45µm polyethersulfone disc membrane filter at a flux of 100 LMH. As shown in the Figure 3.18, the pressure increases immediately after starting the filtration processes in case of untreated and unclarified homogenate.

The clarified homogenate (untreated) (Figure 3.18: dark grey triangle), when filtered gives higher pressure in comparison to clarified and treated homogenate (Figure 3.18: light grey inverted triangle). When these pressure curves were linearized the best fit was found according to the equations explained by Hlavacek and Bouchet, 1993. Using the following equation where P_o is the initial pressure measured by PBS, σ is clogging coefficient, ϵ is the porosity of the membrane which is considered as 70%, A is area of the membrane 0.0041m^2, V is volume (mL) of filtrate.

$$\Delta P = \Delta P_o exp \frac{\sigma V}{\epsilon A}$$

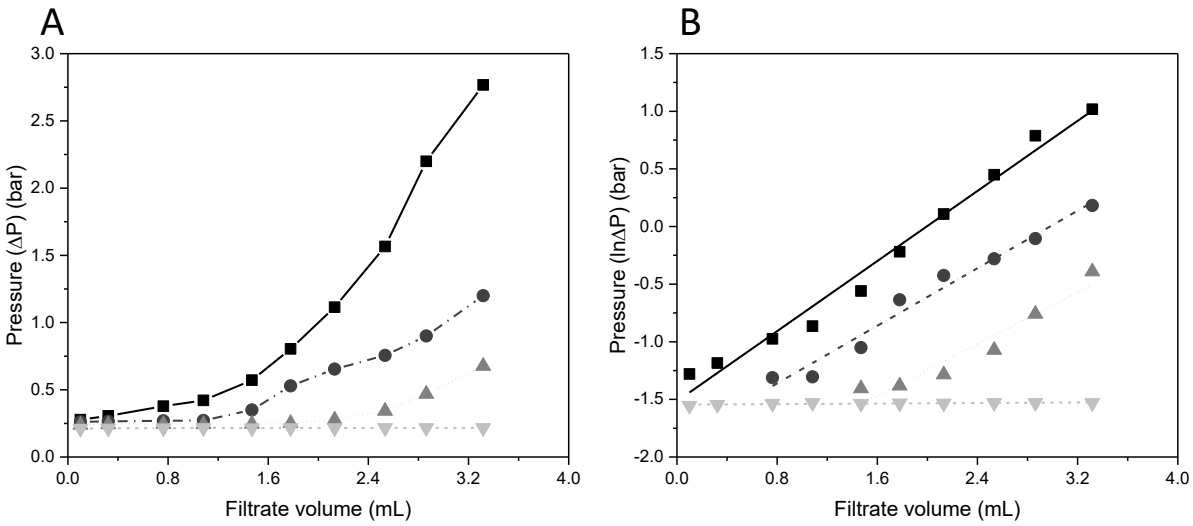


Figure 3.18: Increase in pressure during dead-end filtration of treated and untreated *P. pastoris* homogenate

To study the increase in pressure due to filter fouling, the yeast homogenate obtained as described in Figure 3.14 were homogenized at 500bar with five discreet passes. The yeast homogenate was clarified using fixed angle centrifugation at 600xg for 20 mins before subjecting the samples to filtration with 0.45µm polyethersulfone disc membrane filter at 100LMH (A) Pressure curves from clarified yeast homogenate samples with P. pastoris homogenate (black squares) in comparison to untreated homogenate (Figure 3.14 (C)) (dark grey solid circles) and treated homogenate (Figure 3.14 (B)) (grey solid triangles). The pressure obtained from 1x PBS pressure is shown as inverted light grey triangles. (B) Linearized form of the pressure per unit volume of filtrate shows the difference between the fouling obtained from homogenate and under treated/untreated conditions.

3.8 Conclusion

During the processing of heterologous protein, in this case HBsAg VLPs, host cell impurities play a major role in downstream process fouling. The use of benzonase for removal of host cell DNA is well established in the literature and is commercially used. Here we try to see an analogous approach to remove the lipid impurities. The cell culture used for these experiments did not produce HBsAg VLPs, only wild type *P. pastoris* was used in the experiments. With the help of the above experiments the following can be concluded.

1. The difficulties in the use of exogenous lipase in yeast homogenate were studied and heat incubation needed for lipase activity is seen to play a major effect on clarification performance.
2. Exogenous lipase (0.1 to 1mg/mL) can be used for the removal of lipid impurities as seen with HPLC analysis.

Lipase from *C. rugosa* used in this study shows a positive effect on removal of lipid impurities during processing of yeast homogenate. The action of lipase needs to be studied carefully as there is a substantial effect seen on the yeast homogenate due to heat incubation. It is known that different class of lipase acts on different lipids classes. Triacylglycerol lipase used in this study is specific for the triacylglycerol found as the major foulant during downstream operations. The removal of substantial amount of lipids shows the positive action of lipase during bioprocessing and can be used for improvement of downstream processing of therapeutics like VLPs. As seen from this chapter *C. rugosa* lipase was found to be best performance in comparison to all the microbial lipases tested on yeast homogenate. The exogenous addition of lipase degraded lipids and removed substantial amount of lipid impurities released from yeast homogenate. The issues that might be faced for the design and co-expression of two heterologous proteins in the *P. pastoris* are discussed in the following chapters.

4 Design and construction of *P. pastoris* strains to establish independent transcriptional control of transgenes and the effect of intracellular localisation on the function of an exogenous lipase.

4.1 Introduction

Cell disruption for extraction of intracellular VLPs gives rise to host cell impurities like DNA, protein, and lipids. It is shown in previous studies (Burden *et al.* , 2012; Jin *et al.* , 2010) that these impurities adhere to the downstream process membranes and matrices creating difficulties in maintenance and smooth operation of downstream processes. Exogenously added enzymes can reduce the in-processes impurities like lipids. But there can be another approach where the lipase is co-expressed along with the heterologous protein of interested in the host cell. For the co-expression of two heterologous proteins it is essential to design and construct strains that will be able to express both the proteins without affecting the normal growth and expression of the host organism.

A strategy of employing different promoters which control the expression of the two heterologous proteins as described by Parashar and Satyanarayana, 2016 can be used. This system of dual expression has a unique advantage where the expression of heterologous proteins can be controlled in a staggered manner with the help of inducible promoters in *Pichia pastoris (P. pastoris)*. This property of promoter control helps in expression of heterologous protein in a single expression system.

To do so the following questions are addressed in the present chapter.

1. Can *P. pastoris* have two heterologous protein produced sequentially under influence of two different promoters?
2. Can a dual expression cassette be used to integrate a potentially cyto-toxic protein, in this case *Candida rugosa* lip3 lipase, under the influence of promoters like P_{ENO1} and P_{THI11} and HBsAg under P_{AOX1} into the genome of *P. pastoris*, without disrupting its growth?

To answer these questions, *P. pastoris* strain consisting of HBsAg gene under P_{AOX1}, was constructed in the first instance. The HBsAg expression cassette was then sub cloned with *C. rugosa* lip3 lipase tagged with the simian virus V5 epitope tag sequence (V5-CRL lip3) under

P_{ENO1} and P_{THI11}, creating a dual expression cassette. The V5-CRL lip3 was constructed with the signal peptides for localization of the target protein at three different regions of the cell. This dual expression cassette was then transfected into the *P. pastoris* genome. With the above combination of promoters and cell localizations, six different strains along with one strain containing only HBsAg gene under P_{AOX1} were generated. The nomenclature used to identify these strains is elucidated in the following table 4.1.

The plasmid backbone used, is identified with 'J9' (J902-15) and the strains containing HBsAg was named 'S'. The strains containing the promoters P_{ENO1} and P_{THI11} are identified with 'E' and 'T' respectively. The strains containing the lipase gene having signal peptide that retains the protein in cytoplasm, endoplasmic reticulum and surface is identified with 'LC', 'Lkdel' and 'LS' respectively. For ease of identification of the strains with specific localization sequence that retains the protein in cytoplasm, endoplasmic reticulum, and surface, are also termed as CYTO, KDEL and SURF respectively.

Table 4.1: Seven strains constructed in this study

*The table shows strains created corresponding to promoters present and localization for each strain. The strains are created with the same plasmid backbone identified as J9. AOX1 promoter is inducible in presence of methanol. HBsAg is under AOX1 promoter and named as PPS9010-J9Sag strain. HB**sAg** is present in all the strains and is identified with '**S**'. Whereas lipase from C. rugosa is present under ENO1 and THI11 promoters. The promoters are ENO1 identified with '**E**', THI11 identified with '**T**'. The lipase is localized at different locations of the cell, namely cytoplasm (CYTO) identified with 'LC', endoplasmic reticulum (KDEL) identified with 'Lkdel' and outer surface of the cell (SURF) identified with 'LS'.*

Localization of lipase	AOX1-HBSAg	AOX1-HBSAg-ENO1-Lipase	AOX1-HBSAg-THI11-lipase	Strain name	Second name
Endoplasmic recticulum	✓			PPS9010-J9Sag	Sag
		✓		PPS9010-J9SEATLkdel	KDEL
			✓	PPS9010-J9STATLkdel	
Cytoplasm		✓		PPS9010-J9SEATLC	CYTO
			✓	PPS9010-J9STATLC	
Cell surface		✓		PPS9010-J9SEATLS	SURF
			✓	PPS9010-J9STATLS	

4.2 Expression of heterologous proteins in *P. pastoris*

4.2.1 V5 epitope tagging and cellular localization of *C. rugosa* lipase in *P. pastoris*

C. rugosa lipase has been industrially produced and is extensively studied (Domínguez De María *et al.* , 2006; Pernas *et al.* , 2002). *C. rugosa* triacylglycerol Lip3 lipase (Lee *et al.* , 1999) that is chosen in the present study has been previously studied and cloned (Fickers *et al.* , 2011; Lotti *et al.* , 1993; Lux *et al.* , 2007). As it was shown in chapter 3 section 3.7 that lipase from *C. rugosa* can be used for removal of lipid impurities from yeast homogenates. It is known from the crystal structure of lipases that at the catalytic site there is a well-defined lid as well as a hydrolase domain which take part in the catalytic reaction as discussed earlier in chapter 1, section 1.3.3.1. As seen from the crystal structure fatty acid molecule interacts near to the catalytic active site. This suggested that there is a conformational change required for interfacial activation (Ericsson *et al.*, 2008). The schematic illustration of the location of the lipase active site near the C-terminal end of the protein has been discussed in chapter 1, section 1.3.3.1. Considering the location of the active site in the C-terminal region of the lipase, the tags used for easy detection as well as the signal sequences used for localization of the lipase was added at the N-terminal region of the protein.

In previous studies, it has been reported that lipases can be immobilized on the cell surface. The activity of the immobilized lipase depends on the position of its active site. Liu *et al.* described the immobilization of lipase done by using the N-terminal region as well as the C-terminal region (Liu *et al.* , 2014). It is known that the C-terminal region of *C. rugosa* lipase effects its activity (Hung *et al.* , 2011). Due to these reasons in the present study the mature peptide was cloned with simian virus V5 epitope tag in the N-terminal region of the protein to keep the V5 tag as far away from the active site. V5 tagging used for easy detection is placed as far away from the active site as possible so that it does not interact with the active site.

The V5 tagged *C. rugosa* lipase was expressed using three different signal peptide sequences. As described in table 4.1, the lipase was expressed in the cytosol (CYTO), surface bound (SURF) and endoplasmic retained (KDEL). Each of these variants had different signal peptides, PIR signal sequence (MYRNLIIATALTCGAYSAYVPSEPWSTLTPDASLESALK DYSQTFGIAIKSLDADKIKRE) in case of SURF strains (Pal Khasa *et al.* , 2011a) and, KDEL sequence in case of KDEL strains. There was no signal sequence for the cytoplasmic retained strains. All the clones had the signal sequence upstream of the V5 tag. After the addition of the signal sequence and the V5 tag sequence to the lipase mature gene, the complete gene sequence was added downstream of the promoter sequence namely the P_{ENO1} and the P_{THI11} for the construction of J9SEAT and J9STAT variants respectively.

4.2.2 Promoters for expression of two heterologous proteins in *P. pastoris*

For the expression of heterologous proteins in *P. pastoris* glucose, glycerol, and methanol are used as carbon sources of choice for induction, whereas sorbitol is less preferred as a carbon source of choice. In the present study P_{ENO1}, P_{THI11} and P_{AOX1} are chosen for the expression of heterologous proteins. Among these promoters P_{ENO1} is a constitutive promoter which shows expression in both glucose as well as glycerol but is inhibited in presence of ethanol and methanol. Whereas, P_{THI11} is identified as a repressible promoter whose activity is repressed in presence of thiamine in the culture media. P_{AOX1} is a strong inducible promoter which is induced in presence of methanol and repressed in presence of glycerol. It is also seen that the activity of these promoters P_{ENO1}, P_{THI11} and P_{AOX1} is not affected by use of sorbitol as the carbon source. The strategies used in the present study for the regulation of heterologous protein expression are tabulated in the following table 4.2.

Table 4.2: Predicted induction and repression of promoters in this study

The table shows the carbon source need for the induction of promoters. As described above the P_{AOX1} is induced strongly in presence of methanol and is repressed by glycerol. P_{ENO1} is a constitutive promoter expressed in presence of glycerol. From the literature it is known that P_{ENO1} is repressed in the presence alcohol like methanol and ethanol (Stadlmayr et al. , 2010). P_{THI11} is a repressible promoter and is repressed in presence of thiamine in the medium (Landes et al. , 2016).

	Glycerol	Methanol	Thiamine
P_{AOX1}	Repressed	Induced	-
P_{ENO1}	Induced	Repressed	-
P_{THI11}	Induced	Induced	Repressed

4.3 Design strategy for localization of heterologous protein in *P. pastoris*

Most cell culture strategies involve production of a single protein under a single promoter. Novel promoters described earlier gives an innovative option for the expression of more than one protein under single cultivation process. Strain with *C. rugosa* lipase (V5-CRL lip3) under P_{ENO1} along with HBsAg under P_{AOX1} was consideration for construction.

Along with the cell culture strategies the expression of the protein in different localization of the cell is also important. As described earlier HBsAg is shown to be produced under P_{AOX1} and is localized in the endoplasmic reticulum (ER). The cell localization of this antigen is not changed during the construction of dual expression strains. The localization of the V5-CRL lip3 is done in such a manner that it would not get expressed in the same location of the cell as the HBsAg. To do so there were three different cell locations that were chosen namely endoplasmic reticulum retained, cytoplasm retained, and cell surface displayed. All the constructs where generated by Eurogentec using DNA synthesis methods.

4.3.1 Design strategy for localization of heterologous protein in cytoplasm of *P. pastoris*

To localize the heterologous protein in cytoplasm the native secretion sequence was removed from the 5' end of the lipase gene while cloning. In place of the secretion sequence V5 epitope sequence was added which is later used for detection of the lipase protein using anti-V5 antibodies during western blot analysis. Along with the addition of the V5 epitope tag (GKPIPNPLLGLDST), AscI and SalI restriction sites were added in the 5' end and 3' end of the gene sequence respectively. These unique sites were selected for easy in cloning these gene into synthetic constructs used for transformation of the *P. pastoris* cells. This strategy is elucidated in the following Figure 4.1.

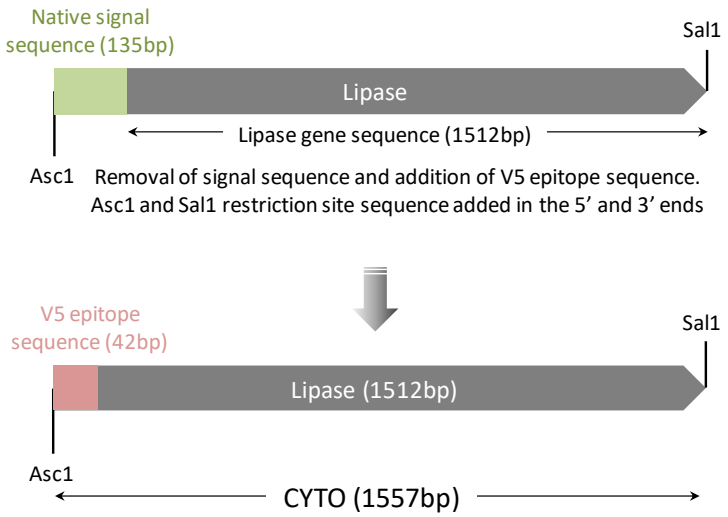


Figure 4.1: Construction of *C. rugosa* lipase gene with V5 epitope tag for cytoplasm localization

C. rugosa lip3 lipase (GenBank ID: X66006.1) gene sequence was added with AscI restriction site at the 5' end and the SalI restriction site at the 3' end. For the localization of C. rugosa lip3 lipase in the cytoplasm of the P. pastoris cell, the secretion signal sequence (MKLALALLLIASVAAAPTAKLANGDTITGLNAIINEAFLGIPFAE) from the native gene sequence was removed. V5 tag sequence (GKPIPNPLLGLDST) was added at the 5' end of the gene sequence of C. rugosa lip3 lipase.

4.3.2 Design strategy for localization of heterologous protein in endoplasmic reticulum of *P. pastoris*

The expression of *C. rugosa* lipase were done along with HBsAg while keeping in mind that HBsAg is expressed and localized in the ER region of the cell. In this strategy, the lipase was localised in the ER region with addition of KDEL sequence in the N-terminal end of the *C. rugosa* lipase gene (Medina-Godoy *et al.*, 2006). Along with the addition of the V5 epitope tag, AscI and SalI restriction sites were added in the 5' end and 3' end of the gene sequence respectively. This strategy is elucidated in the following Figure 4.2.

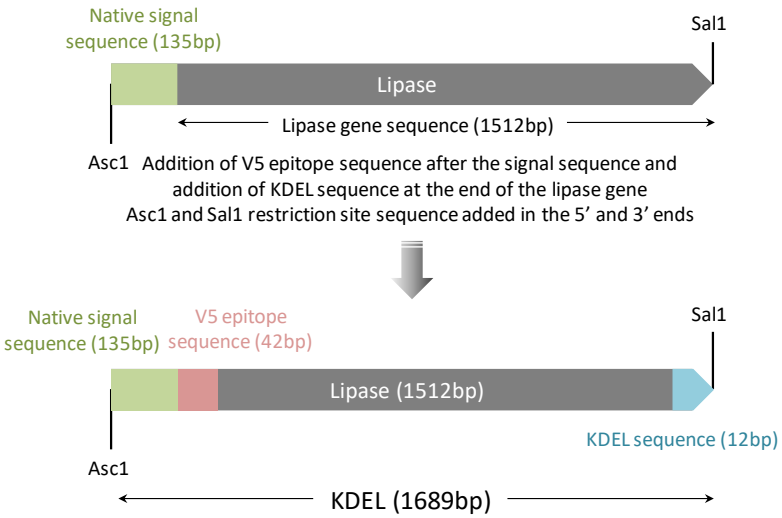


Figure 4.2: Construction of *C. rugosa lip3* lipase gene with KDEL sequence and V5 tag for endoplasmic reticulum retention

C. rugosa lip3 lipase gene (GenBank ID: X66006.1) sequence was added with AscI restriction site at the 5' end and the SalI restriction site at the 3' end. For the localization of C. rugosa lip3 lipase in the endoplasmic reticulum of the P. pastoris cell, the KDEL gene sequence was added at the 3' end of the lipase gene. The V5 tag sequence was added after the secretion sequence before the gene coding for the mature C. rugosa lip3 lipase peptide.

4.3.3 Design strategy for localization of heterologous protein with PIR signal sequence in cell surface of *P. pastoris*

In case of surface display PIR1 signal gene sequence (MYRNLIIATALTCGAYS

AYVPSEPWSTLTPDASLESALKDYSQTFGIAIKSLDADKIKRE) (Moura *et al.* , 2015; Pal Khasa *et al.* , 2011b) was added in the place of KDEL sequence to display the lipase protein on the cell surface. Along with the addition of the V5 epitope tag, AscI and SalI restriction sites were added in the 5' end and 3' end of the gene sequence respectively. This strategy is elucidated in the following Figure 4.3.

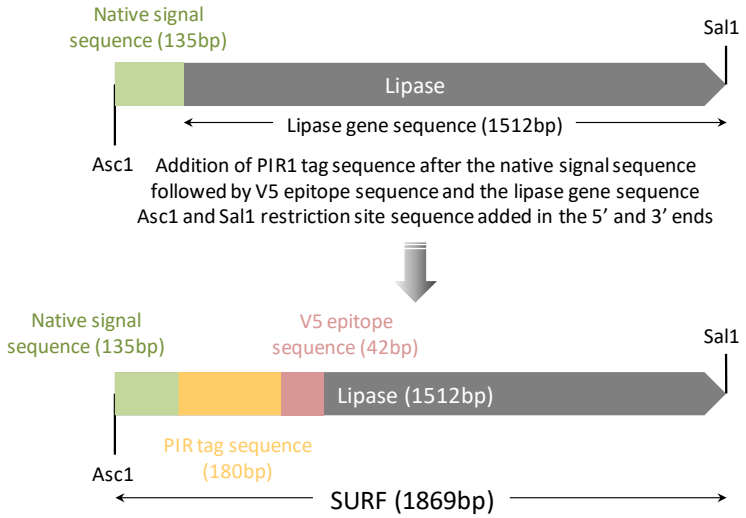


Figure 4.3: Construction of *C. rugosa lip3* lipase gene with PIR sequence and V5 tag for surface displayed strain

*C. rugosa lip3 lipase gene (GenBank ID: X66006.1) sequence was added with AscI restriction site at the 5' end and the SalI restriction site at the 3' end. For construction of the surface retention strategy of C. rugosa lip3 lipase on the cell surface of the P. pastoris cell, the PIR1 signal sequence (*MYRNLIIATALTCGAYSAYVPSEPWSTLT PDASLESALKDYSQTFGIAIKSLDADKIKRE*) was added at the 5' end of the lipase gene after the native signal sequence. The V5 tag sequence was added after the PIR1 signal sequence before the gene coding for the mature C. rugosa lip3 lipase peptide.*

In the present study, the *C. rugosa* lip3 native secretion sequence is added preceding the PIR1 N-terminal transmembrane domain which results into the soluble domain being present in the ER lumen and ultimately to the cell exterior surface. The expression of the PIR1 tagged CRL-V5 lip3 protein is shown in the following schematic Figure 4.4 (A). This strategy was considered following the work reported by Wang and his colleagues (Wang *et al.*, 2008), were they have used the alpha secretion signal sequence along with the PIR1 protein with the enhanced green fluorescence protein (EGFP) displayed on the cell surface. The alpha secretion

sequence is the most commonly used signal sequence for recombinant protein production. It consists of a 19-amino acid signal sequence followed by a 67-residue sequence containing three consensus N-linked glycosylation sites and a dibasic endopeptidase processing site as described in work done by Kurjan and Herskowitz, 1982. All the sequences contained the simian virus V5 tag sequence making it easy for detection. All the lipase open reading frames (ORFs) having V5 tag and other signal sequences contained two restriction sites AscI at the 5' end and SalI at the 3' end of the gene. The dual expression strategies were both the *C. rugosa* lip3 lipase along with HBsAg is further shown to specify the intended position of the lipase enzyme with respect to the HBsAg location according to the literature is shown in the following Figure 4.4.

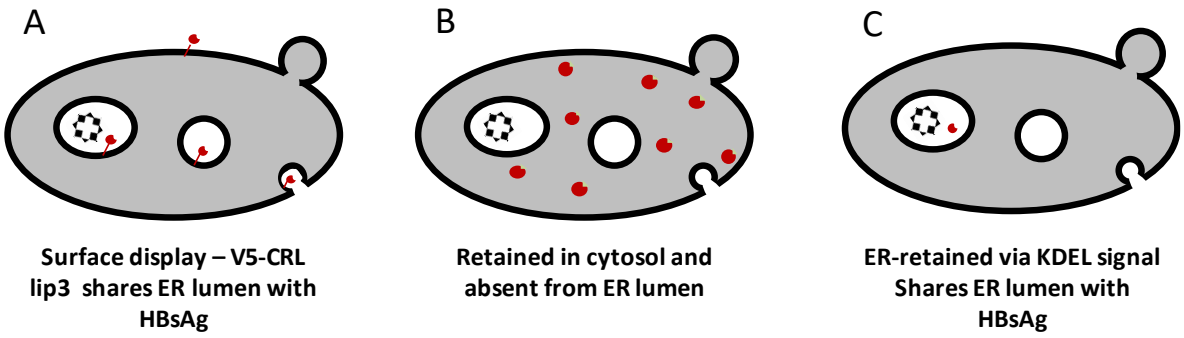


Figure 4.4: A Schematic diagram for cell localization of co-expression of HBsAg and *C. rugosa* lipase

Cell localization options where HBsAg (black quadrangle) and C. rugosa lipase (V5-CRL lip3) (red cut circles) can be expressed in the P. pastoris is shown in the above diagram. (A) Shows the surface display of lip3 containing the PIR transmembrane domain and HBsAg in the inner circle which is the endoplasmic reticulum (ER). This strategy localizes the lipase to the cell surface with the PIR sequence as described earlier. (B) Shows the cytosol retained V5-CRL Lip3 which does not consist of any signal sequence at the N-terminal end of the gene sequence. (C) shows the endoplasmic reticulum retained V5-CRL lip3 containing KDEL sequence along with HBsAg.

4.4 Design and construction of *P. pastoris* strain expressing HBsAg under P_{AOX1}

In *P. pastoris,* the genes involved in the methanol utilization (MUT) pathway are completely repressed under carbon sources like glucose but are strongly induced in the presence of methanol. The repression properties of *P. pastoris*, which contains, strong promoters that can be directly controlled by changing carbon sources, gives a unique control mechanism for the co-expression of multiple proteins. Previous studies show that HBsAg can be expressed under P_{AOX1} (Lünsdorf *et al.* , 2011) forming virus like particles in the endoplasmic reticulum (ER) and the ER extended as the production of HBsAg increases as elucidated in chapter 1 section 1.2.1. It is considered that HBsAg is expressed in the ER and localized in the ER, in this study HBsAg is not tagged with any signal sequence. For the easy detection of *C. rugosa* lip3 lipase expressed under P_{ENO1} and P_{THI11}, lipase gene is cloned along with a simian virus V5 epitope tag sequence.

Among the various therapeutic proteins and vaccines produced in *P. pastoris*, HBsAg has a well-known production process produced under P_{AOX1}. One such example of a study is done by Gurramkonda *et al.* using P_{AOX1} for expression of HBsAg in *P. pastoris* (Bardiya, 2006; Gurramkonda *et al.* , 2009). HBsAg has also been produced under other promoter systems like the GAP promoter, as shown in the study done by Vassileva *et al.* , 2001. In this study, the construction of the HBsAg producing strain was done using the same strategy as described by Gurramkonda *et al.* in their study. HBsAg is expressed under the influence of P_{AOX1}, hence the expression of HBsAg was induced by addition of methanol. The construction of HBsAg expressing strain was done as shown in the following method.

HBsAg gene sequence was synthesised and cloned in pJ902-15 (4252bp) shuttle vector using BsaI sites by Eurogentec. The resulting pJ902-HBsAg (pJ9Sag (4428bp)) was then linearized inhouse using the SacI restriction site in the P_{AOX1} sequence. This pJ9Sag shuttle vector was then used for transformation of the gene into the parent strain PPS9010

wild type. The steps for restriction digestion and transformation is details in chapter 2 section 2.3.8. This gave rise to two P_{AOX1} promoters and two AOX1 terminator sequences along with the zeocin resistant gene. The summary of this strain development is described in Figure 4.5.

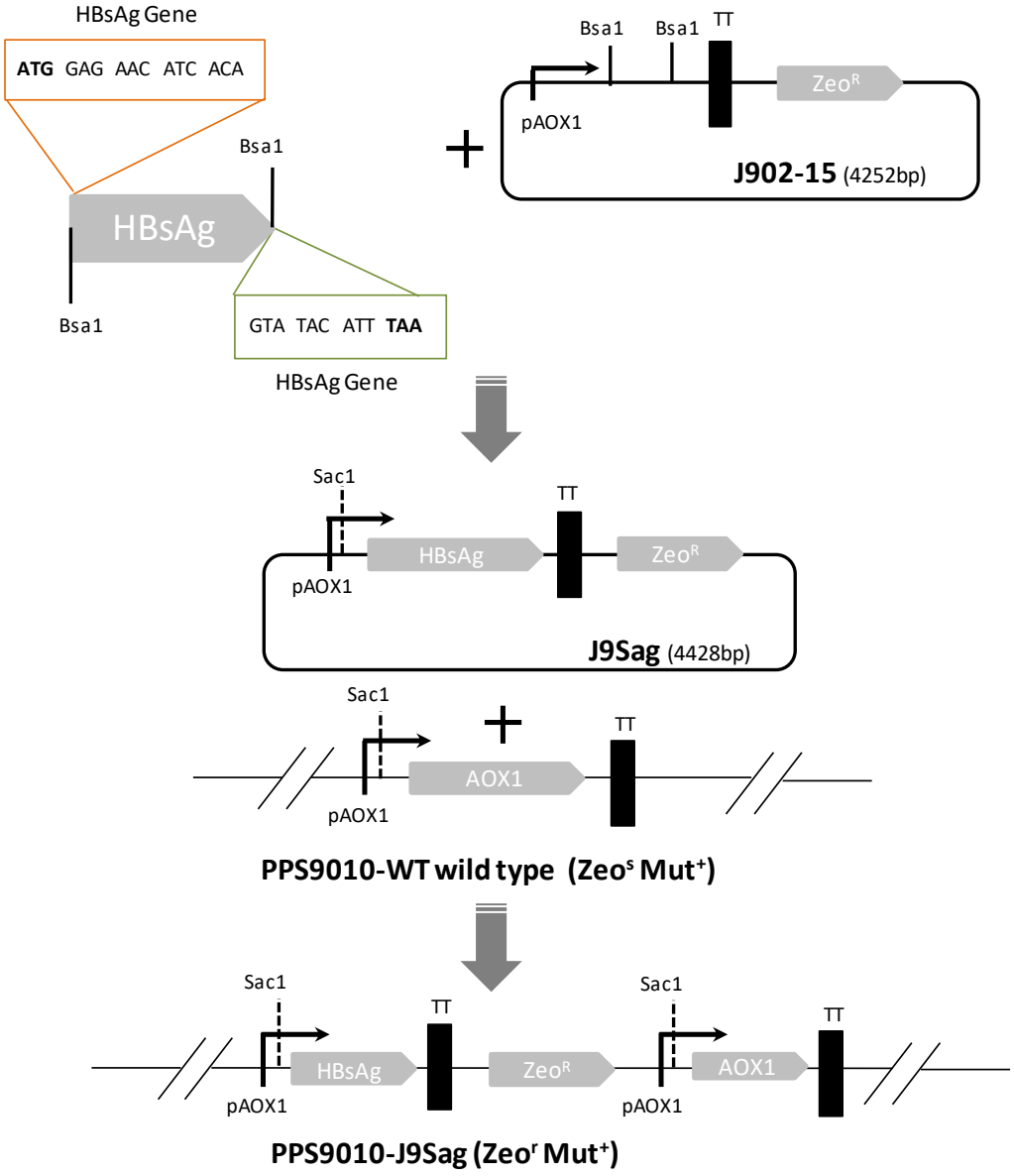


Figure 4.5: HBsAg gene integration in PPS9010 wild type *P. pastoris*

The HBsAg sequence synthesised by Eurogenetec as shown above consisted of Bsa1 restriction site on the 5' end and another Bsa1 restriction site is added at the 3' end of the gene sequence. With restriction digestion this gene sequence was added to the shuttle vector pJ902-15 (4252bp) to obtain the vector construct pJ9Sag (4428bp). The vector was linearized at Sac1 site located inside of P_{AOX1} promoter region and integrated into the

genome of P. pastoris PPS9010 wild type strain. The arrangement of P_{AOX1} and HBsAg gene along with the terminator sequence (TT) and the zeocin resistant gene in pJ9Sag (4428bp) construct resulted in a zeocin resistant Mut^+ strain PPS9010-J9Sag consisted of two P_{AOX1} regions with two P_{AOX1} terminator sequences.

The transformants were further confirmed with by antibiotic resistant yeast peptone dextrose (YPD) plates and the colonies were picked for confirmation of gene integration using polymerase chain reaction. The resulting PPS9010-J9Sag strain was Mut^+ consisting of two AOX1 promoter regions and two AOX1 terminator regions. This strain was used for testing of HBsAg VLP formation as well as a control strain in all further experiments.

4.4.1 Antibiotic selection of *P. pastoris* PPS9010-J9Sag strain expressing HBsAg

The transformed cells were immediately plated on to YPD plates containing zeocin at final concentration of 1mg/mL. The transformed cells were plated along with untransformed *P. pastoris* PPS9010 wild type cells. These untransformed cells were plated in both zeocin containing as well as only YPD plates and treated as positive and negative control of the experiment. All the plates were incubated at 30°C for 48-72 hours for growth of colonies. As shown in the Figure 4.6, the transformed cells gave isolated colonies after the incubation period which were then picked ascetically and grown in zeocin containing YPD broth for construction of master cell banks (MCB) and working cell banks (WCB).

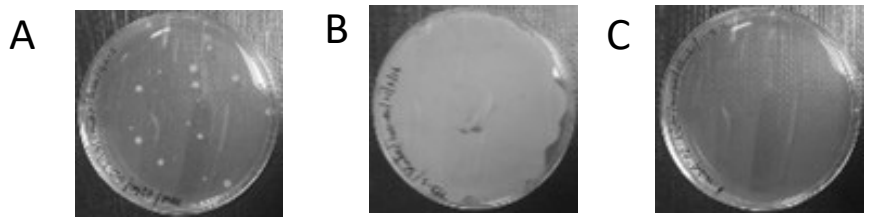


Figure 4.6: Antibiotic resistant colonies obtained from PPS9010-J9Sag strain
The transformed cells were plated in antibiotic resistant (zeocin 1mg/mL) YPD plates. (A) The 700µL of transformed cells were plated on the zoeocin containing plates along with (C) wild type, untransformed cells as negative control. (B) A YPD plate without the zoecin antibiotic was also plated with the wild type, untransformed cells as positive control. These

plates were then incubated in 30°C for 48-72 hours for growth of colonies. After 72 hours isolated colonies were obtained in the YPD plate containing the transformed cells, a lawn of colonies obtained in the positive control and no colonies were observed in the negative control.

4.4.2 Confirmation of HBsAg PPS9010-J9Sag transformants

For the strain producing only HBsAg the transformed cells were selected by growth on zeocin YPD agar plates. After the growth of colonies, they were picked aseptically and streaked on to fresh plates for storage and further experimentation. At the same time the colonies were boiled in 10µL water for 10 mins at 90°C. These were then mixed with the PCR master mix for the PCR reaction, as described in chapter 2, section 2.3.8.3. The primers were designed considering the HBsAg gene as the template. For positive control plasmid pJ9Sag consisting of HBsAg gene was taken as one of the samples. The size of the HBsAg gene was 0.7kb and a band is seen amplified in the same position corresponding to the gene of interest as shown in Figure 4.7. This shows that the gene of interest (HBsAg) has been integrated into the genome of *P. pastoris* following its transformation.

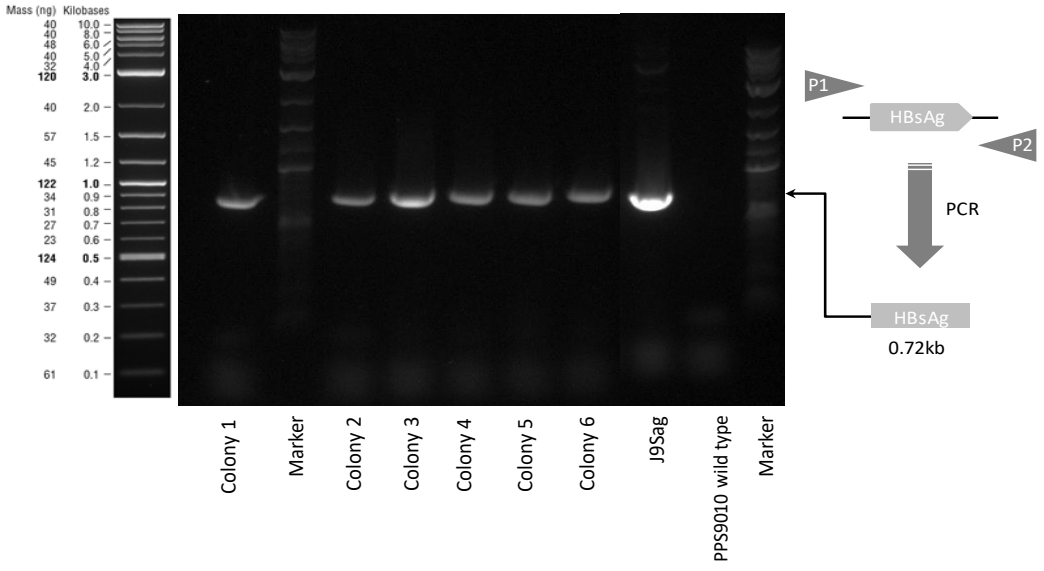


Figure 4.7: PCR confirmation of HBsAg gene integration from PPS9010-J9Sag strains *Polymerase chain reaction product having amplification corresponding to HBsAg (0.7kb). After the PCR reaction samples were run on an 8% agarose gel in 1x TBE buffer and viewed under UV illumination. The samples were run with NEB Quick-Load® 2-Log DNA Ladder and the amplified gene of interest is seen to run in the similar place as the positive*

control plasmid (pJ9Sag) which contains the HBsAg gene. There are no bands observed in the negative control consisting of the PPS9010 wild type strain without transformation. The schematic diagram shows the forward (P1- ATG GAG AAC ATC ACA TCA) and the reverse (P2- AAA TGT ATA CCC AGA GAC) primers used in the PCR reaction. These primers were chosen with HBsAg as template and are found to be unique to HBsAg sequence.

4.5 Design and construction of *P. pastoris* strain expressing HBsAg and V5-CRL lip3 under P_{ENO1} and P_{THI11}

P. pastoris (*Komagataella. phaffii*) (parent strain PPS9010 wild type obtained from ATUM^{SM} (formerly DNA2.0$^{®}$) (Menlo Park, CA, USA), was used to express recombinant HBsAg under the control of P_{AOX1} promoter and recombinant *C. rugosa* lip3 lipase under the control of P_{ENO1}. pJ9Sag construct was made as described in the previous section 0. The design of the vectors is described in the method section 2.1.2 briefly, the P_{ENO1} and P_{THI11} sequences were cloned into the pUC57 vector along with AOX1 terminator sequence (TT) giving rise to pUC57-EAT and pUC57-TAT vector, where EAT stands for the plasmid containing P_{ENO1} and TAT stands for plasmid containing P_{THI11}. The pUC57-EAT and pUC57-TAT were then integrated with the pJ9Sag (4428 bp) with directionless ligation using the BamHI site giving rise to pJ9SEAT (5527bp) and pJ9STAT (5550bp) constructs. The signal sequence from *C. rugosa* lip3 gene was removed rendering the protein to be retained by the cell. In place of signal sequence, a V5 tag sequence was added at the N-terminal region of the lip3 gene was synthesised and sub-cloned in pUC57 vector forming pUC57-LC vector. The pUC57-LC was ligated with pJ9SEAT, pJ9STAT and the final construct, pJ9SEATLC (7123bp) and pJ9STATLC (7142 bp) contained HBsAg gene and the V5-CRL lip3 gene under P_{AOX1} and the P_{ENO1} promoters respectively. The HBsAg gene and the V5-CRL lip3 gene under P_{AOX1} and P_{THI11} in another set of constructs were made. Other strategies were also considered in which the V5-CRL lip3 was tagged with signal peptide that would direct it to the cell surface (Moura *et al.* , 2015) or keep it localised in the ER of the cell. The PIR signal sequence was added along with the signal sequence from *C. rugosa* lip3 gene.

This helped in directing and attaching the protein to the cell surface. After the PIR signal sequence, a V5 tag sequence was added at the N-terminal region of the lip3 gene. It was then synthesised and sub-cloned in pUC57. The final construct, pJ9SEATLS (7399bp) and pJ9STATLS (7454 bp) contained HBsAg gene and the V5-CRL lip3 gene under P_{AOX1} and the P_{ENO1} promoters and the HBsAg gene and the V5-CRL lip3 gene under P_{AOX1} and P_{THI11} in another set of constructs. In the case of endoplasmic retention of the protein, the sequence was complemented with KDEL sequence in place of the signal sequence. This in turn kept the protein of interest to the endoplasmic reticulum of the cell. The final construct, pJ9SEATLkdel (7231bp) and pJ9STATLkdel (7286 bp) contained HBsAg gene and the V5-CRL lip3 gene under P_{AOX1} and the P_{ENO1} promoters in one set and the HBsAg gene and the V5-CRL lip3 gene under P_{AOX1} and P_{THI11} in another set of constructs. These constructs are shown in the following Figure 4.8 and Figure 4.9.

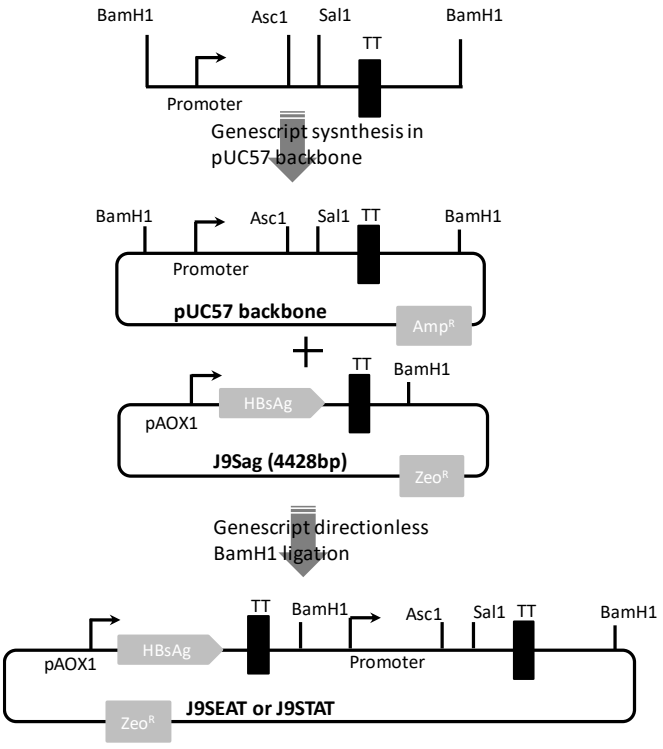


Figure 4.8: Assembly of cloning intermediate for the construction of dual gene plasmid *Above Figure shows the P_{ENO1} along with the AOX1 terminator (TT) sequence is synthesised by GenScript consisting BamH1 restriction site at 5' and the 3' end of the synthesised DNA. In between the P_{ENO1} sequence and the AOX1 terminator sequence Asc1 and Sal1 restriction site were added for integration of the C. rugosa lipase gene.*

With restriction digestion this gene sequence was added to the shuttle vector pJ9Sag (4428bp) to obtain the shuttle vector pJ9SEAT (5527bp) and pJ9STAT (5550bp) construct. This construct consisted of the HBsAg gene under P_{AOX1} and P_{ENO1} in once set and HBsAg gene under P_{AOX1} and P_{THI11} in another set along with two AOX1 terminator sequence and the zeocin resistant gene.

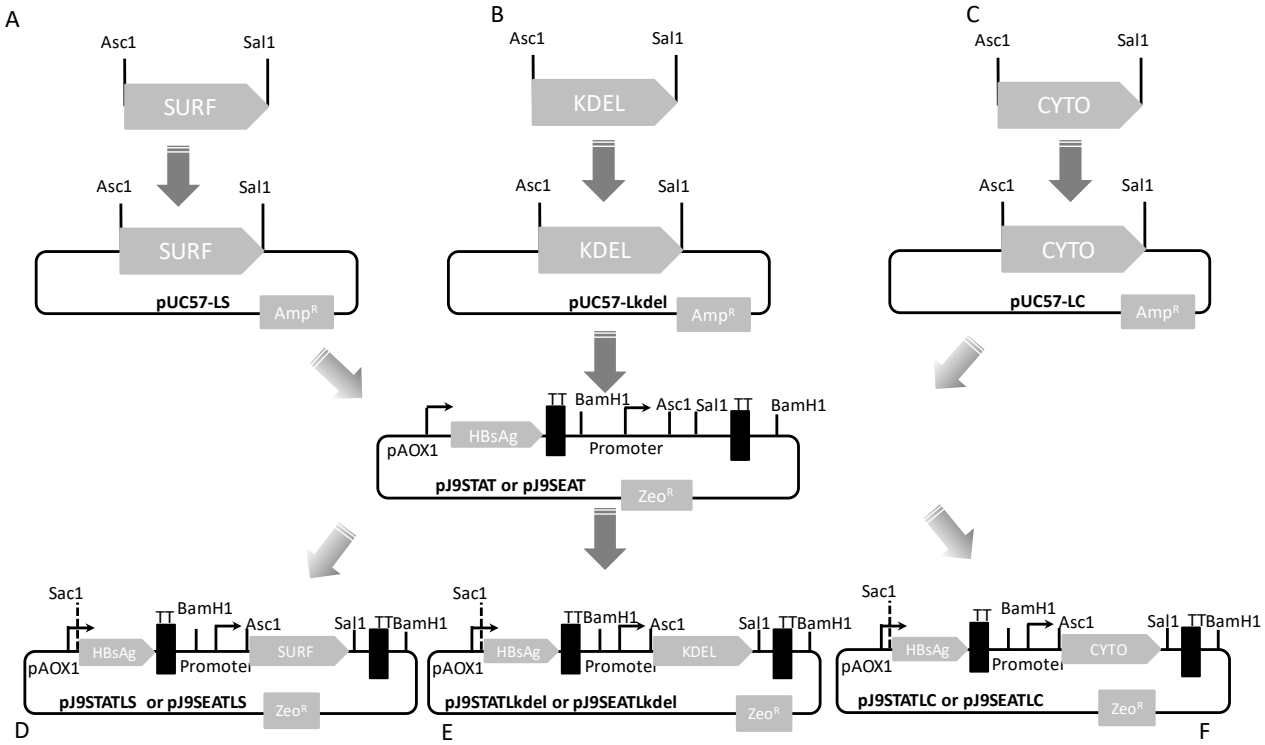


Figure 4.9: *C. rugosa* lipase sub cloning step for vector construction with P_{ENO1} and P_{AOX1}

C. rugosa lip3 lipase containing simian virus V5 tag at the 5' end of the gene sequence was synthesised by GenScript consisting Asc1 and at the 3' end had Sal1 restriction site. (A) The C. rugosa lip3 lipase gene sequence was added with PIR1 sequence for localization of the protein to the cell surface of P. pastoris. This was sub-cloned into the pJ9SEAT (5527bp) and pJ9STAT (5550bp) plasmid using directional Asc1-Sal1 ligation. After ligation the obtained vector had HBsAg gene under P_{AOX1} terminating with AOX1 terminator (TT) sequence followed by surface display tagged and V5 tagged C. rugosa lip3 lipase under P_{ENO1} and P_{THI11} followed by AOX1 terminator sequence in separate constructs. (D) The complete construct is named pJ9SEATLS (7399 bp) and pJ9STATLS (7454 bp) consisting of zeocin resistant marker. (B) The C. rugosa lip3 lipase gene sequence was added with KDEL sequence for localization of the protein to endoplasmic reticulum of P. pastoris. This was sub-cloned into the pJ9SEAT and pJ9STAT plasmid using directional Asc1-Sal1 ligation. After ligation the obtained vector had HBsAg gene under P_{AOX1} terminating with AOX1 terminator sequence followed by KDEL tagged and V5 tagged C. rugosa lip3 lipase under P_{ENO1} and P_{THI11} followed by AOX1 terminator sequence in two different constructs. (E) The complete construct is named pJ9SEATLkdel (7231 bp) and pJ9STATLkdel (7286 bp) consisting of zeocin resistant marker. (C) The C. rugosa lip3 lipase gene sequence did not have any signal sequence for localization of the protein to cytoplasmic lumen of P. pastoris. This was sub-cloned into the pJ9SEAT and pJ9STAT plasmid using directional Asc1-Sal1 ligation. After ligation the obtained vector had HBsAg gene under P_{AOX1} terminating with AOX1 terminator sequence followed by KDEL tagged and V5 tagged C. rugosa lip3 lipase under P_{ENO1} followed by AOX1 terminator sequence. (E) The complete construct is named pJ9SEATLC (7123 bp) and pJ9STATLC (7142 bp) constructs consisting of zeocin resistant markers.

After the constructs have been made, they were linearized and integration into the genome of PPS9010 wildtype *P. pastoris*. For linearization, the constructs were cut with SacI-HF restriction enzyme and transformed into freshly prepared electro-competent cells (Lin-Cereghino *et al.* , 2005) as described in chapter 2, section 2.3.8.1. The genome integrated strains consisted of two P_{AOX1} promoter and two P_{AOX1} terminator sequences, one from the construct and another was the native yeast promoter. Creating six different strains under P_{ENO1} and P_{THI11} promoters shown in Figure 4.10.

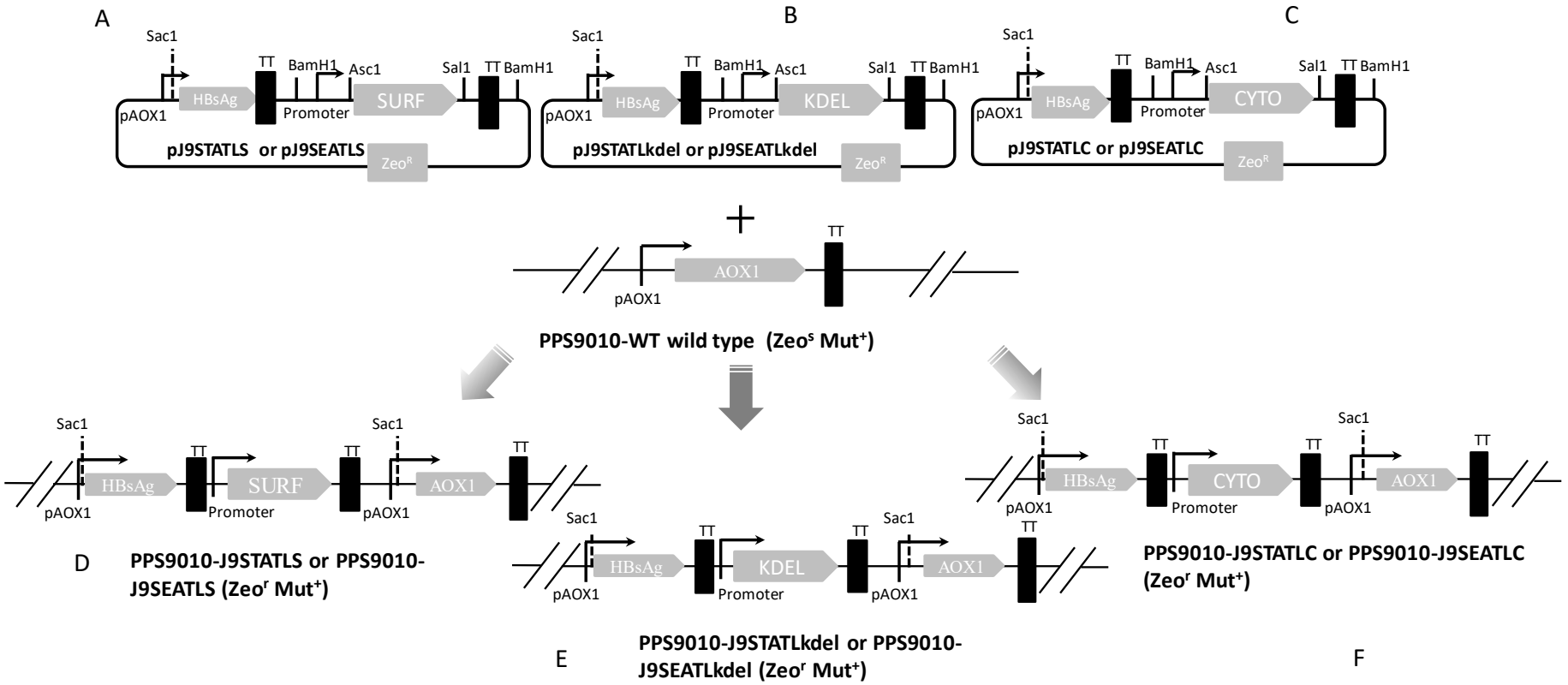


Figure 4.10: HBsAg and *C. rugosa* lipase gene integration under P_{AOX1} and P_{ENO1} promoter in PPS9010 wild type *P. pastoris*

The Figure shows that arrangement of the P_{AOX1} promoter along with P_{ENO1} and P_{THI11} sequences denoted by "Promoter" having HBsAg gene under P_{AOX1} and C. rugosa V5 tagged lip3 under P_{ENO1} or P_{THI11}. (A) The pJ9SEATLS (7399bp) and pJ9STATLS (7454 bp) construct was linearized using the Sac1 site in the P_{AOX1} promoter sequence. The linearized vector was integrated with the PPS9010 wild type P. pastoris Mut$^+$ strain giving rise to the

PPS9010-J9SEATLS (SURF strain) strain. (D) This strain consisted of two P_{AOX1} and two AOX1 terminator sites along with zeocin resistant marker creating a zeocin resistant Mut^+ strain. (B) The pJ9SEATLkdel (7231bp) and pJ9STATLkdel (7286 bp) constructs were linearized using the Sac1 site in the P_{AOX1} promoter sequence. The linearized vector was integrated with the PPS9010 wild type P. pastoris Mut^+ strain giving rise to the PPS9010-J9SEATLkdel strain and PPS9010-J9STATLkdel. (E) This strain consisted of two P_{AOX1} and two AOX1 terminator sites along with zeocin resistant marker creating a zeocin resistant Mut^+ strain. (C) The pJ9SEATLC (7123bp) and pJ9STATLC (7142 bp) constructs were linearized using the Sac1 site in the P_{AOX1} promoter sequence. The linearized vector was integrated with the PPS9010 wild type P. pastoris Mut^+ strain giving rise to the PPS9010-J9SEATLC and PPS9010-J9STATLC strains. (F) This strain consisted of two P_{AOX1} and two AOX1 terminator sites along with zeocin resistant marker creating a zeocin resistant Mut^+ strain.

4.5.1 Antibiotic selection of *P. pastoris* strain expressing lipase under P_{ENO1}, P_{THI11} and HBsAg under P_{AOX1}

The transformed cells were immediately plated on to YPD plates containing zeocin at final concentration of 1000μg/mL. The transformed cells were plated along with untransformed *P. pastoris* PPS9010 wild type cells. PPS9010-J9Sag strain was used as a positive transformation control. All the transformants included the *C. rugosa* lip3 lipase having, CYTO, KDEL and SURF ORFs under P_{ENO1} as well as the HBsAg gene under P_{AOX1} creating J9SEAT strains. These untransformed cells were plated in both zeocin containing as well as only YPD plates and treated as positive and negative control of the experiment. All the plates were incubated at 30°C for 48-72 hours for growth of colonies. As shown in the Figure 4.11, the transformed cells gave isolated colonies after the incubation period which were then picked ascetically and grown in zeocin containing YPD broth for construction of master cell banks (MCB) and working cell banks (WCB).

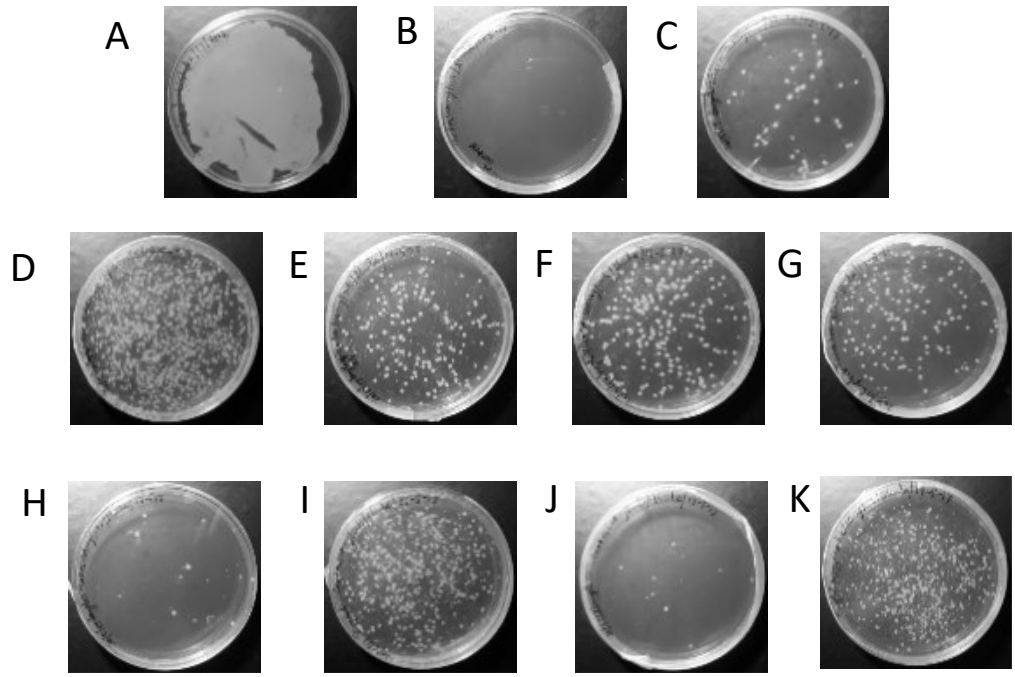
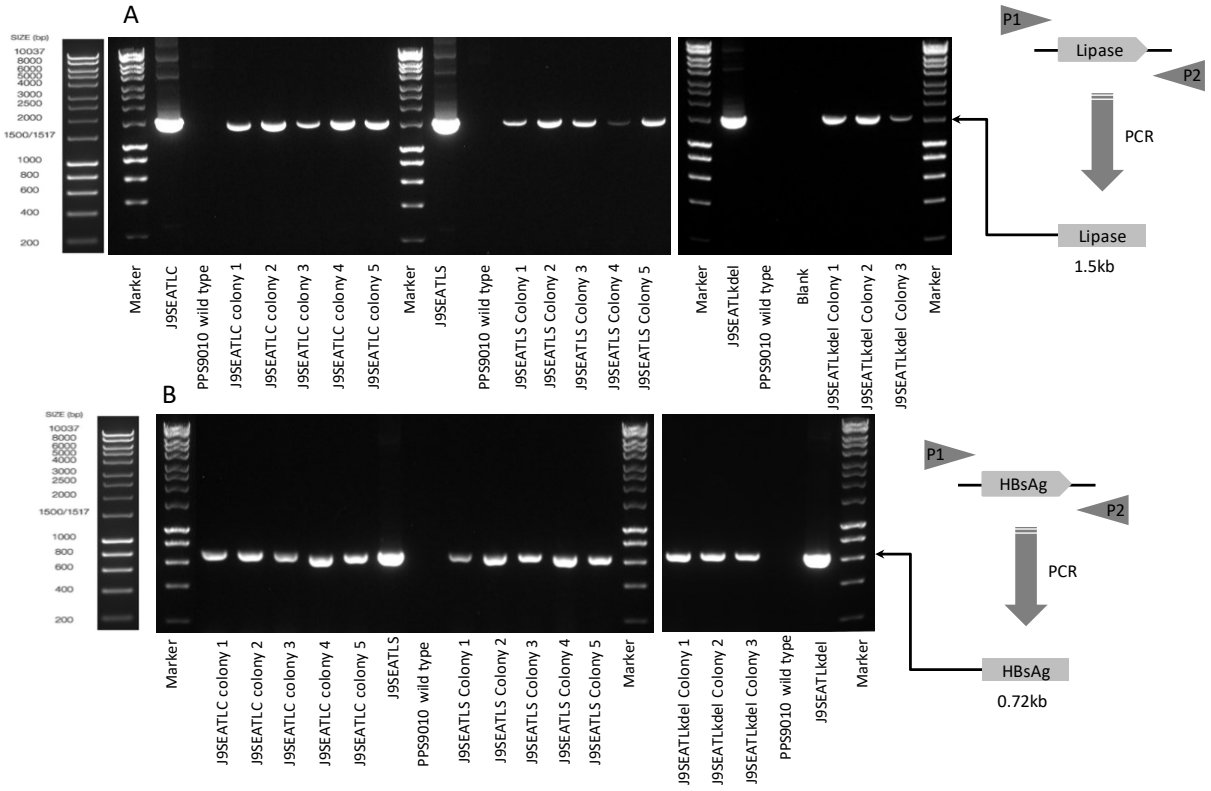


Figure 4.11: Antibiotic resistant colonies obtained from PPS9010-J9SEAT and PPS9010-J9STAT strains

The transformed cells were plated in antibiotic resistant (zeocin 1mg/mL) YPD plates. The 700µL of transformed cells were plated on the plates along with (B) wild type, untransformed cells as negative control. (A) A YPD plate without the zoecin antibiotic was also plated with the wild type, untransformed cells as positive control. (C) For transformation control PPS9010-J9Sag strain was taken, were the linearized pJ9Sag was transformed into the wild type strain together with all the dual expression strains. (D) PPS9010-J9STATLS (E) PPS9010-J9STATLkdel (F) PPS9010-J9STATLC (G) PPS9010-J9STAT (H) PPS9010-J9SEATLS (I) PPS9010-J9SEATLkdel (J) PPS9010-J9SEATLC (K) PPS9010-J9SEAT were plated in individual plates and to obtain isolated colonies. The PPS9010-J9SEAT and PPS9010-J9STAT also grew in the antibiotic resistant YPD plate as these strains had the HBsAg gene along with the zeocin resistant gene. These plates were then incubated in 30°C for 48-72 hours for growth of colonies. After 72 hours two to five isolated colonies were obtained in the YPD plate containing the transformed cells and named as PPS9010-J9SEATLS1, 2, 3 etc, respectively from each plate. A lawn of colonies obtained in the positive control and no colonies were observed in the negative control.

4.5.2 Confirmation of HBsAg and *C. rugosa* lipase from PPS9010-J9SEAT and PPS9010-J9STAT transformants

For the confirmation of the integration of gene into the genome of the *P. pastoris* the colonies were picked at random for the PCR reaction. The colonies were picked using a sterile tip and mixed in 10μL of water. They were then boiled for 10 mins at 90°C. These samples were then mixed with the PCR master mix for the PCR reaction, as described in chapter 2, section 2.3.8.3. The primers were selected based on the HBsAg and *C. rugosa* lipase gene sequence. After the PCR reaction amplification of a DNA band is seen corresponding to the positive control as shown in the following Figure 4.12.



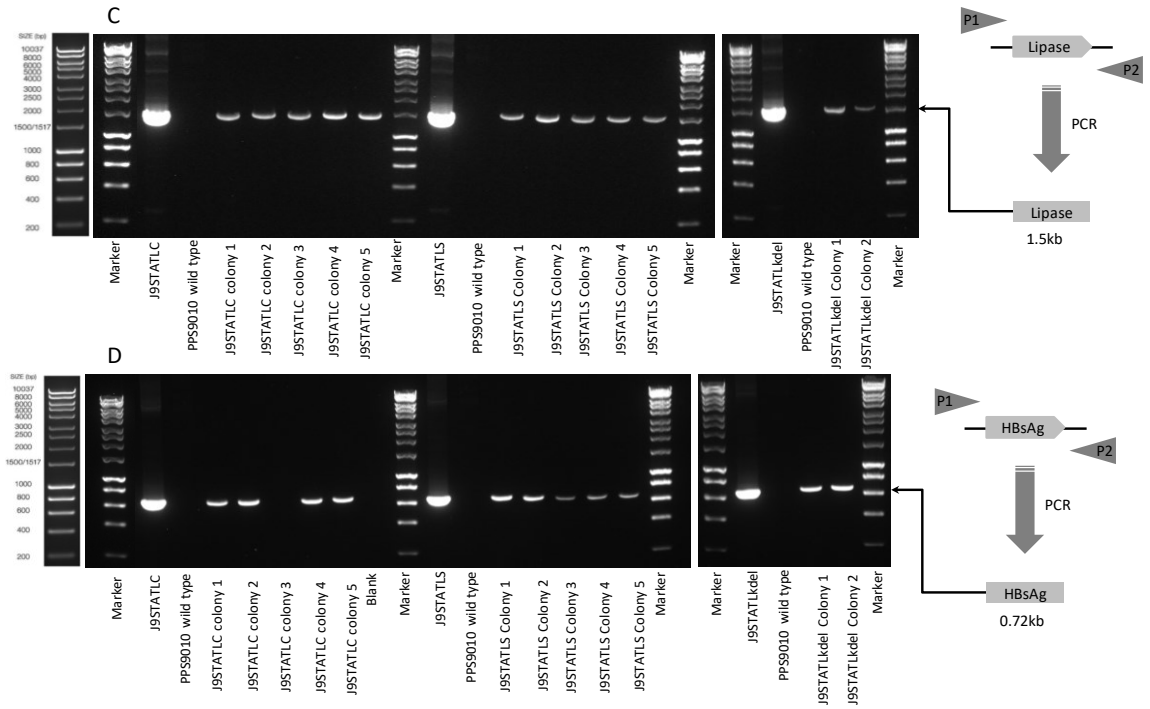


Figure 4.12: *P. pastoris* colony PCR confirmation of HBsAg and lipase gene integration from PPS9010-J9SEAT and PPS9010-J9STAT strains

Polymerase chain reaction product with amplification of the HBsAg (0.7kb) and the C. rugosa lipase (V5-CRL lip3) lipase (1.5kb). After the PCR reaction samples were run on an 8% agarose gel in 1x TBE buffer and viewed under UV illumination. (A), (B), (C) and (D) The gel is run with Bioline HyperLadder™ 1kb DNA Ladder and the amplified genes of interest are shown to run in the similar place as the positive control. The plasmid containing the C. rugosa lipase was used as a positive control. The negative control was the untransformed PPS9010 wild type strains from each set of reactions corresponding to CYTO, SURF and KDEL variants of PPS9010-J9SEAT and PPS9010-J9STAT strains. The schematic diagram shows the forward (P1- GGT AAG CCT ATC CCT AAC) and the reverse (P2- TTA CAC AAA GAA CAG CAG) primers used in the PCR reaction. The primers chosen for the PCR reaction was unique to the lipase gene and did not amplify any other region from the genome of transformed strains. The plasmid containing the C. rugosa lipase was used as a positive control. The negative control was the untransformed PPS9010 strains. The schematic diagram shows the forward (P1- ATG GAG AAC ATC ACA TCA) and the reverse (P2- AAA TGT ATA CCC AGA GAC) primers used in the PCR reaction

Un-transformed cells were taken as negative control and plasmids containing the gene of interest was taken as positive control for the PCR reaction. For the reaction control a reaction blank without PCR primers was also taken along with the colony samples. As shown in the

Figure 4.9 the *C. rugosa* lipase gene (V5-CRL lip3) (1.5kb) and the HBsAg gene (0.7kb) was amplified from the transformants. This confirmed the genome integration of HBsAg and *C. rugosa* lipase into PPS9010-J9SEAT strains.

4.6 Cultivation of *P. pastoris* transformed strains

The growth of strains expressing *C. rugosa* lipase (V5-CRL lip3) under P_{ENO1} (PPS9010-J9SEAT) and the P_{THI11} (PPS9010-J9STAT) was tested with the comparison *P. pastoris* wild type strains to find if the expressed heterologous protein was toxic to the host cell under cell culture conditions. Randomly selected clones of CYTO, KDEL, and SURF from both the sets were grown along with the HBsAg production *P. pastoris* strain (PPS9010-J9Sag) and *P. pastoris* wild type (PPS9010 wild type) as shown in the below Figure 4.13. All the strains namely, CYTO, KDEL, and SURF from both the sets showed similar growth to the wild type strain. All the strains achieved high cell density with an OD_{600nm} of 33 around 96 hours when grown YPD having glucose as the carbon source, in baffled shake flask at 30°C with agitation of 250 rpm suggesting no toxicity due to the production of foreign heterologous protein. The growth condition that was selected to produce lipase was done in presence of glucose. Methanol was not added to the cell culture medium hence the HBsAg production was not induced. After the confirmation of growth of transformed cells in YPD medium, glycerol stocks of the strains were prepared for further experiments. Briefly, colonies were randomly picked from zeocin resistant YPD plates and used for preparation of glycerol stocks as described in chapter 2 section 2.2.1, the details of the seven strains are given in table 4.1. During this experiment's induction to produce HBsAg was not done. The cells were grown with medium containing glucose hence inducing only lipase production under P_{ENO1} promoter. The strains were randomly picked for testing its growth in YPD medium. All the variants, namely CYTO, KDEL, and SURF for both the strains where tested for this experiment.

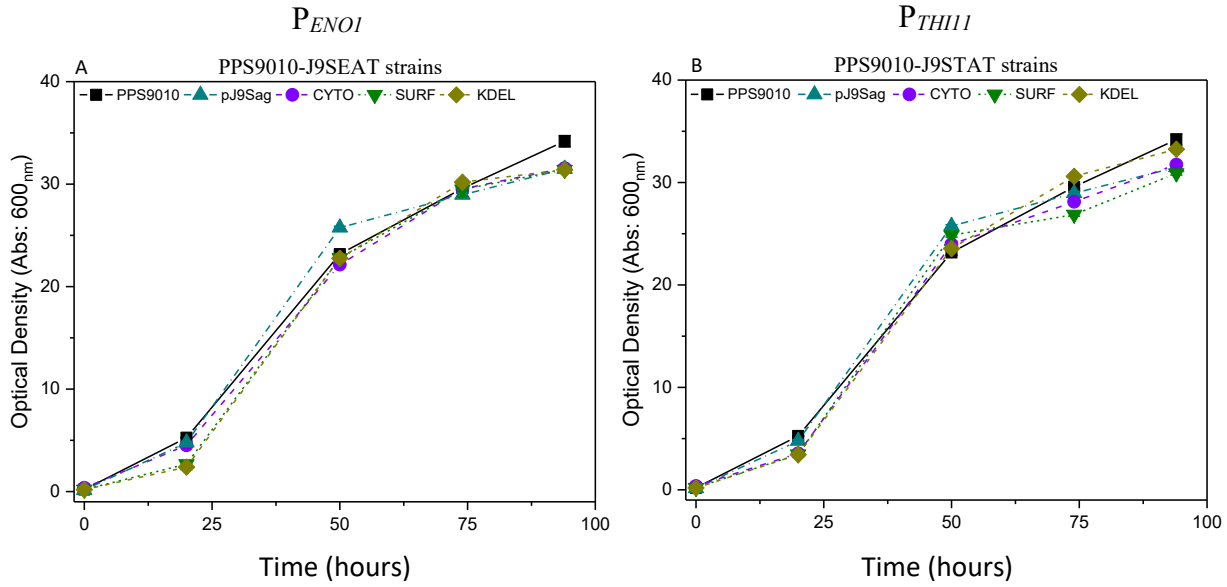


Figure 4.13: Growth curve comparison between wild type and developed strains

Growth comparison of dual expression strains expressing HBsAg under P_{AOX1} and V5-CRL lip3 under P_{ENO1} (A) and P_{THI11} (B) with PPS9010-Wild type strain (solid black squares) and PPS9010-J9Sag P. pastoris strains containing only HBsAg green triangle. Here randomly, 5 positive zeocin resistant colonies containing P_{ENO1} (PPS9010-J9SEATLC4, PPS9010-J9SEATLS2, PPS9010-J9SEATkdel3) and 5 positive zeocin resistant colonies containing P_{THI11} (PPS9010-J9STATLC2, PPS9010-J9STATLS1, PPS9010-J9STATkdel4) (as shown in Figure 4.7 and 4.12) were selected and were grown in YPD (using glucose as the carbon source) along with P. pastoris wild type strain. This experiment was done to compare the growth conditions and not for expression of HBsAg. All the strains were inoculated from glycerol stocks for an overnight culture in YPD. They were then inoculated into fresh media to an OD_{600nm} of 0.2. The growth of the cells occurs similarly over the period of 96 hours.

4.7 Conclusion

From this chapter it the following conclusions can be made.

1. Two genes were transfected into the *P. pastoris* wild type and these transfected strains were then grown. The normal growth of transfected strains in comparison to wild type strains and production of lipase protein suggested that the transfection of these heterologous proteins was not toxic to the organism. The results of expression are shown in the following chapter.
2. The *P. pastoris* strain containing the P_{ENO1} and P_{THI11} promoters did not show toxicity and grew under antibiotic selection on YPD plates.
3. Seven different strains were successfully produced by transfection of PPS9010 wild type *P. pastoris* with HBsAg under P_{AOX1} and *C. rugosa* lip3 lipase genes under P_{ENO1} and P_{THI11} promoters.

The PCR showing the amplification of the lipase and HBsAg genes along with the colonies obtained from the transformed cells proved that the genes of interest were successfully integrated into the *P. pastoris* PPS9010 wild type genome giving rise to seven different strain. These strains grew in YPD media and had comparable growth to wild type *P. pastoris*.

The use of dual promoter system can be used for expression of heterologous proteins such as *C. rugosa* lipase (V5-CRL lip3) which in turn can be used for process improvement for process related impurities. For doing so there is a need to express these proteins sequentially or in phases. Doing so will reduce the load on the cell to produce both the protein in trophophase of the cell culture. This can be achieved by two different promoters which are either induced or produces the protein of interest at different stages of the cell culture. Use of inducible promoter like P_{AOX1} along with unique promoter like P_{ENO1} gives this advantage. As P_{AOX1} is induced with only methanol and P_{ENO1} is supressed by methanol, the combination of their use for dual expression is advantageous. The expression of these proteins needs to be checked before the sequential expression strategies can be tested.

5 Characterisation of *P. pastoris* strains designed to establish independent transcriptional control of transgenes and the effect of intracellular localisation on the function of an exogenous lipase

5.1 Introduction

The design and construction of *Pichia pastoris (P. pastoris)* strains containing V5 tagged *Candida rugosa* lip3 lipase (V5-CRL lip3) and HBsAg, which were positively confirmed with PCR and antibiotic resistant colonies as discussed in chapter 4. In this chapter an attempt is made to find the expression of HBsAg and formation of VLPs along with V5-CRL lip3 positive strains. Further these strains were used for expression and purification of HBsAg followed by confirmation of VLP formation by transmission electron microscopy. The strains were also used to test the activity of the cloned lip3 lipase. The characterization of the cloned strains was done to address the following questions.

1. Can there be a functional HBsAg and V5-CRL lip3 expressed by the dual expression strains?
2. Do signals targeting different intracellular locations impact the expression of a recombinant lipase?
3. Can independent transcriptional control of HBsAg, be achieved along with potentially toxic lipase, intracellularly?
4. Does the expressed HBsAg protein form VLPs after purification?

To answer these questions the expression of HBsAg and V5-CRL lip3 were tested separately. The same dual expression strain was grown in multiple feed media to test the expression of both the heterologous proteins. After the confirmation of the expression, large scale fermentation for purification and preliminary characterization of the heterologous proteins was done. Analytical techniques like transmission electron microscopy was used for detection and identification of VLPs formed after expression of HBsAg. Lipase activity in whole cell, homogenized supernatant as well as purified lipase was tested with zymogram and colorimetry assays. Zymogram used for activity analysis was found to be best suited for crude samples such as yeast homogenate.

5.2 *P. pastoris* strain selection for dual expression of HBsAg and Lipase

5.2.1 Small scale expression of HBsAg in *P. pastoris*

For the expression of HBsAg in *P. pastoris* in shake flask conditions, PPS9010-J9Sag strain was grown in YPD media as described in chapter 2, section 2.2.2. Briefly, the PPS9010-J9Sag strain was cultured in a 10mL culture overnight from the glycerol stock. When the OD_{600nm}~20 was achieved in the overnight culture, it was inoculated to a 2L sterile YPD media aseptically. The culture was grown and transferred into the induction media containing 0.7% (v/v) methanol, the growth curve of which is shown in Figure 5.1. The cells were grown with a daily addition of methanol as shown in Figure 5.5. After the induction of the HBsAg the cells were pelleted aseptically and chilled on ice for cell disruption. The obtained cell culture resuspended in disruption media containing EDTA and ABESF as described in the chapter 2 section 2.3.1 and homogenized for further analysis of HBsAg. The HBsAg produced after induction was confirmed with the help of western blot analysis.

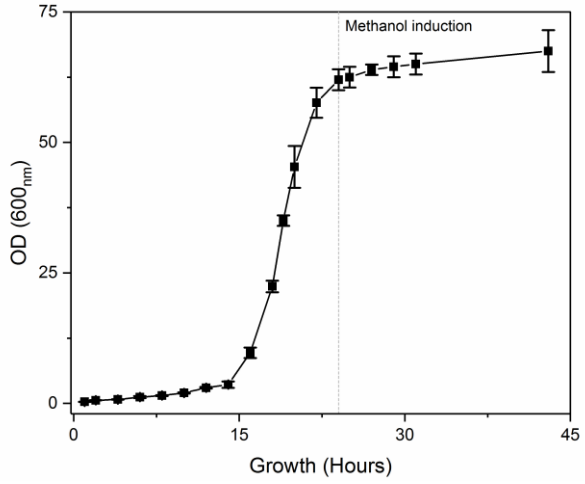


Figure 5.1: Growth curve during HBsAg production under shake flask condition in PPS9010-j9Sag strain

The above graph shows the optical density (OD) at 600nm obtained during the cell culture of P. pastoris producing HBsAg (PPS9010-j9Sag) strain. The highest density of about OD 60 was obtained in YPD media before the cell culture was aseptically centrifuged

and cells were resuspended in the succinate buffer-based induction media (20mM sodium succinate containing 0.7% methanol (v/v)). As described in the chapter 2 the production of HBsAg was done in the presence of 0.7% methanol (v/v). After the growth of P. pastoris for another 48 hours, the cell culture was then harvested. Followed by the cultivation the cells were homogenized and analysed using western blot for presence of HBsAg using anti-HBsAg antibody.

5.2.2 Confirmation of HBsAg expression in recombinant strain PPS9010-J9Sag

It was necessary to check for expression of the HBsAg independently before the test for dual expression system could be characterized. The PPS9010-J9Sag strain, containing on the HBsAg gene under P_{AOX1} was used to check for HBsAg VLPs before testing dual expressing system. For the confirmation of the HBsAg production randomly picked colonies after transformation were selected. As described in chapter 2 section 2.2.2 the colonies were grown under shake flask conditions in YPD media. The culture was grown for 48 hours to obtain high cell density of about OD_{600nm} ~30 before the YPD media was removed aseptically for induction with methanol media. After induction with 0.7% methanol in succinate buffer media for another 48 hours the cells were harvested.

The harvested cells were disrupted using homogenization followed by SDS-PAGE analysis as described earlier in chapter 2 section 2.3.6.2. The SDS-PAGE was stained with coomassie blue for protein bands as shown in the following Figure 5.2. In the SDS-PAGE analysis of the supernatant as well as the pellet of the yeast homogenate no major band corresponding to the HBsAg was observed. The samples were then analysed using western blot of the gel obtained from the SDS-PAGE analysis. Anti-HBsAg antibody was used for detection of the bands using chemiluminescent. There is a band seen corresponding to the HBsAg protein near 16kDa when compared with commercial HBsAg protein. This confirmed

the expression of the HBsAg protein using methanol induction from PPS9010-J9Sag strain. It was also seen that substantial amount of HBsAg is present in the pellet as well as supernatant. This could be due to loss of HBsAg VLPs with the cell debris during the clarification of cell homogenate using centrifugation.

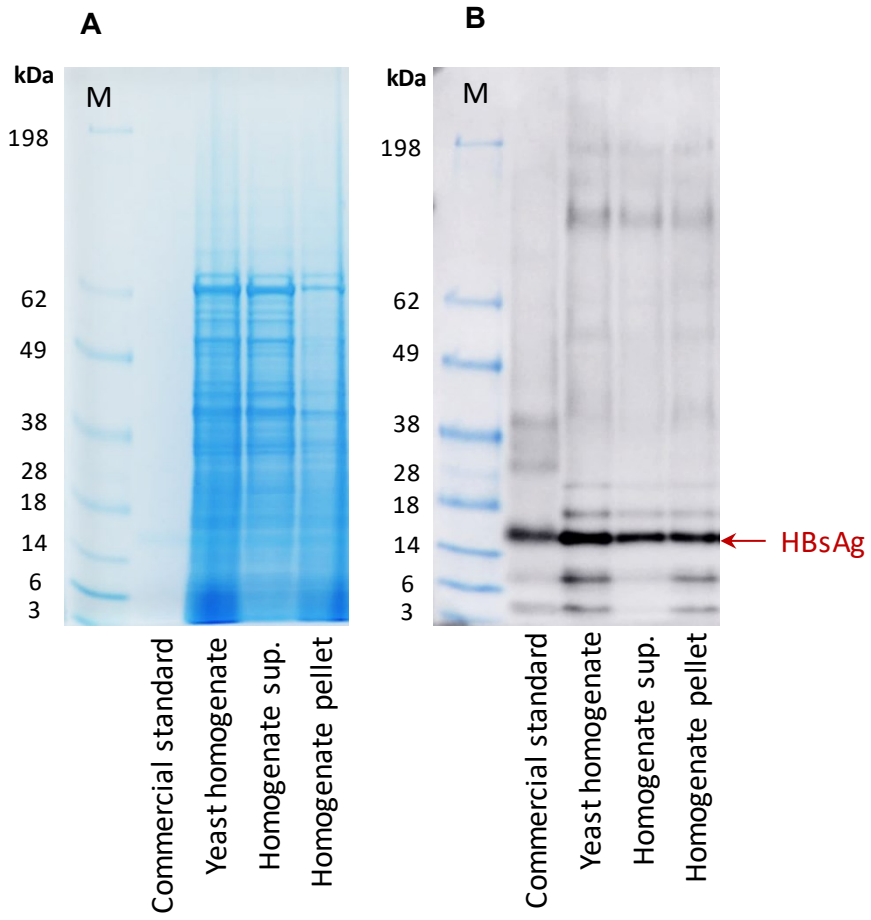


Figure 5.2: Western blot analysis of HBsAg expression from PPS9010-J9Sag

(A) The samples obtained after the cultivation of PPS9010-J9Sag strain for the expression of HBsAg was run in an SDS-PAGE gel. There was a band seen corresponding to the HBsAg protein near 16kDa. This band was found to be present in both pellet as well as supernatant of the yeast homogenate sample. This was confirmed with the western blot analysis. (B) A HRP conjugated primary anti-HBsAg antibody was used for detection of the bands in the western blot analysis and it was developed using ECL reagent as described in chapter 2 section 2.3.6.2. In the obtained western blot, the major band corresponds to the HBsAg commercial standard (Aldeveron). It is seen to be present in both the supernatant as well as the pellet. The SDS-PAGE was run along with molecular weight marker shown as 'M' in the Figure.

5.2.3 Characterising recombinant lipase expression via V5 epitope detection in recombinant strains cultivated with glycerol, glucose or thiamine as carbon source

Two major sets of strains were produced namely strains containing *C. rugosa* lipase (V5-CRL lip3) under P_{ENO1} and P_{THI11}. The strains containing P_{THI11} had the same set of tags in comparison to strains containing P_{ENO1} for cell localization. Randomly selected transformed colonies were chosen to test the expression of V5-CRL lip3 under P_{THI11} and P_{ENO1} promoters. The strains were grown in presence and absence of thiamine in YPD media and chemically defined media respectively using the cell culture method described in chapter 2 section 2.2.2. V5-CRL lip3 expression was clearly observed in YPD medium containing glucose. When the strains containing P_{THI11} are grown in YPD medium containing thiamine, they did not show any expression of V5-CRL lip3 as evident in the western blot analysis shown in Figure 5.3. The grown cell was pelleted after 48 hours of growth and homogenized as explained in the chapter 2 section 2.2.2 and 2.3.1.1 respectively.

The obtained cell homogenate was directly tested for expression of V5-CRL lip3. For this experiment random clones were selected containing P_{ENO1} and P_{THI11} promoters and grown in YPD medium. KDEL strains containing P_{ENO1} shows little to no expression of V5-CRL lip3 in comparison to the CYTO and SURF strains. Majority of the V5-CRL lip3 is found to be present in pellet. The SURF strain contained the signal sequence for attaching the V5-CRL lip3 protein to the cell surface which is the possible reason of having very faint band observed in the supernatant fraction as seen after the western blot analysis. Considering the expression of CYTO strains is found more in comparison to other strains producing V5-CRL lip3 under same conditions this strain is taken up for further experimentation. The expression of V5-CRL lip3 was seen in both glycerol and glucose containing feed.

Further, experiments were done where strain containing P_{ENO1} were grown in thiamine containing media and P_{THI11} strain was grown in glycerol media without any thiamine for

expression of V5-CRL lip3. The strains with P_{THI11} should not show any expression of V5-CRL lip3 in presence of thiamine. To express V5-CRL lip3 in absence of thiamine the cells were cultured in chemically defined medium without any thiamine as yeast peptone broth without thiamine was not available. P_{THI11} containing strains had very low to no growth in chemically defined, thiamine negative media over a period of 48 hours and strain containing P_{THI11} were not considered in further experiments.

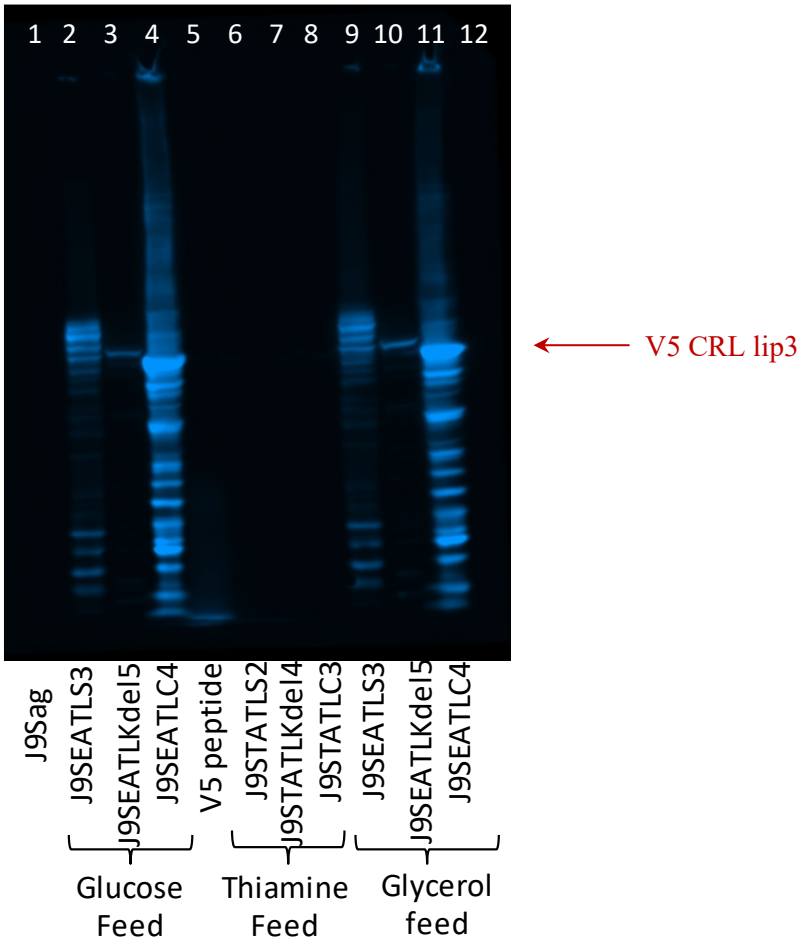


Figure 5.3: V5-tagged *C. rugosa* lipase expression detected by western blot analysis

Fluorescent western blot for the expression of C. rugosa lipase (V5-CRL lip3) using anti-V5 primary antibody and fluorescent tagged secondary antibody was performed after growing the transformed cells in yeast peptone media containing glucose, thiamine, and glycerol. The western blot was run with whole cell homogenate. PPS9010-J9Sag was used as a negative control which did not show any bands related to the V5-CRL lip3 (lane 1). The strains containing P_{THI11} promoter did not show any expression of V5-CRL lip3 (lane 6, 7, 8). PPS9010-J9SEATLkdel (KDEL) (lane 3 and 10) strain containing P_{ENO1} shows little expression of V5-CRL lip3 in comparison to the PPS9010-J9SEATLC (CYTO) (lane

4 and 11) and PPS9010-J9SEATLS (SURF) strains (lane 2 and 9). The expression of V5-CRL lip3 is seen in both glycerol and glucose containing feeds. For glucose and glycerol feed PPS9010-J9SEATLC 4, PPS9010-J9SEATLS 3, PPS9010-J9SEATLCkdel 5 were used and to test for expression in presence of thiamine PPS9010-J9STATLC 3, PPS9010-J9STATLS 2, PPS9010-J9STATLCkdel 4 were randomly selected and tested in this experiment.

5.2.4 Characterising extracellular *C. rugosa* lipase under control of P_{ENO1}

There was no expression seen in the presence or absence of thiamine when the P_{THI11} containing strains were grown in YPD media. These clones were not taken further for this part of the experiment. Strains containing P_{ENO1} were further analysed for the expression and grown as described in chapter 2, section 2.1.2. The strains were grown in presence of glucose by using YPD medium and standard protocol for cell culture. The cells were harvested after 48 hours and were homogenized followed by clarification using centrifugation. Obtained supernatant and the cell pellet were analysed separately with western blot analysis to characterize the strains expressing V5-CRL lip3 under P_{ENO1}. In this experiment a faint to almost no band was seen from KDEL strains producing V5-CRL lip3 in the endoplasmic reticulum region.

The pellet showed multiple bands in both the strains expression V5-CRL lip3 in the cytoplasm (CYTO strain) and the strains localizing V5-CRL lip3 on the cell surface (SURF strain). It is evident from the western blot that the V5-CRL lip3 localised on the cell surface is present in the cell debris as brighter bands are seen in the cell homogenate pellet samples. Single band corresponding to the 58-60kDa band was seen in the soluble supernatant fraction as shown in below Figure 5.4. The strain expressing V5-CRL lip3 with KDEL tag was seen to have reduced to no detectable expression in subsequent experiments. To test the validity of the results, the experiment was repeated, the resultant western blot is shown in Figure 5.3 (C). Similar results were obtained in the repeat experiment, KDEL strains did not show any expression of V5-CRL lip3 and faint bands were obtained from the SURF strains. Maximum expression was seen with CYTO strains. Considering these results, the strains expressing V5-

CRL lip3 in the cytoplasm (CYTO) and PPS9010-J9SEATLS (SURF) strains was taken for further experiments and selection of the strain producing both HBsAg and V5-CRL lip3.

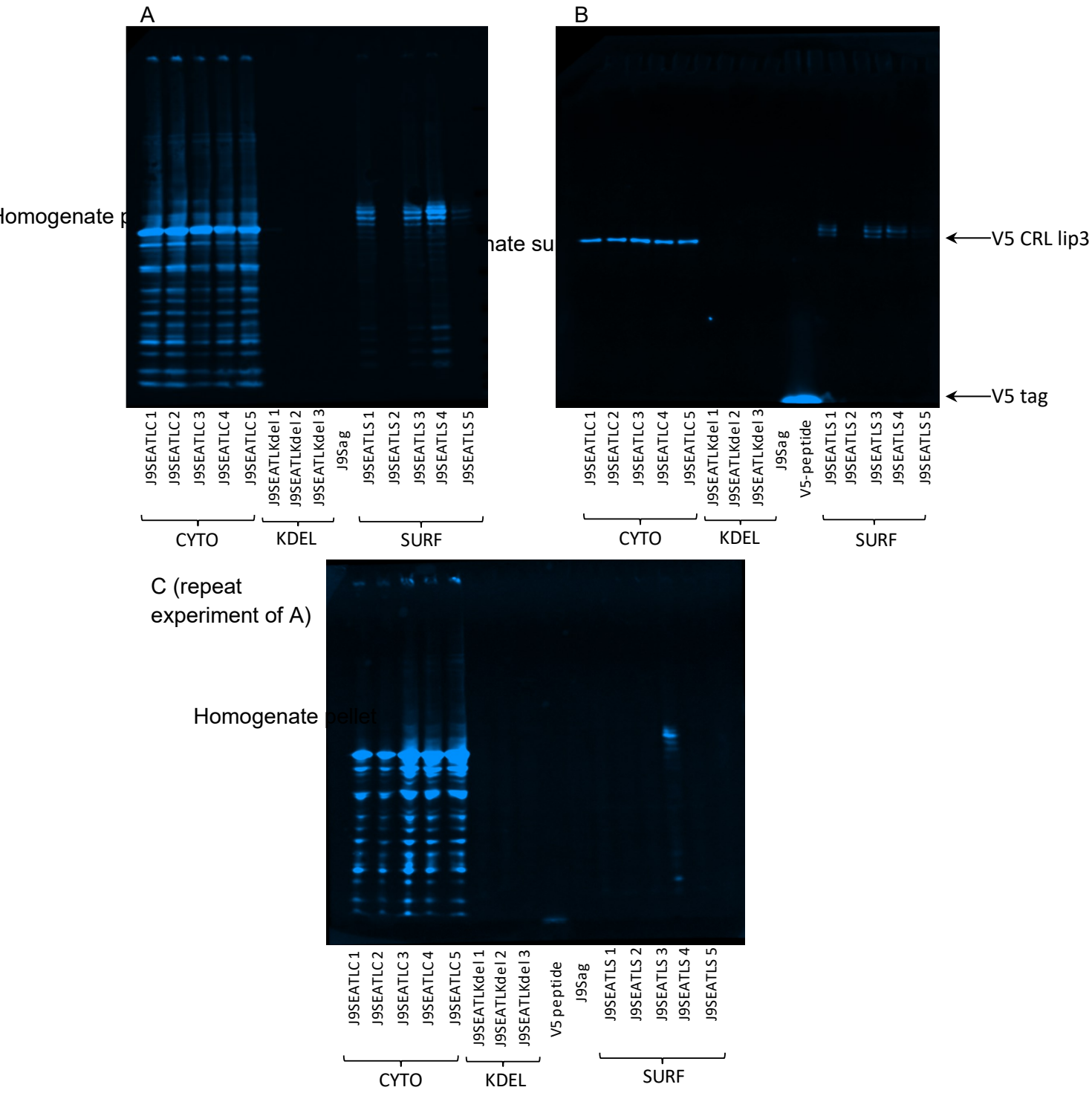


Figure 5.4: Western blot of P_{ENO1} strains expressing *C. rugosa* lipase in recombinant strains *The expression of C. rugosa lipase (V5-CRL lip3) in YPD media. The yeast homogenate pellet (A) is compared with its supernatant (B). The comparison is made to identify the soluble fraction of V5-CRL lip3. KDEL strains containing P_{ENO1} shows little to no expression of V5-CRL lip3 in comparison to the CYTO and SURF strains. Majority of the*

V5-CRL lip3 protein is found to be present in pellet. (C) A repeat biological experiment where the yeast homogenate pellet shows similar results to the previous attempts with no expression of KDEL strain and little or no expression in SURF strains.

5.2.5 Detecting co-expression of HBsAg and *C. rugosa* lipase variants directed for cytosolic and surface-attached expression

The expression of both HBsAg and V5-CRL lip3 in the engineered strain is of pivotal importance. The expression of both HBsAg and V5-CRL lip3 was done after selection of strains, they were then chosen for next step experiments. These cells were grown as previously described chapter 2, section 2.2.2 for the expression of both V5-CRL lip3 under P_{ENO1} and HBsAg under P_{AOX1} with the help of glucose and methanol respectively. After expression the cells were homogenized, and the supernatant were analysed using western blot analysis as shown in the following Figure 5.5.

PPS9010-J9Sag was used as a positive control for the expression of HBsAg. The expressed proteins were compared with simian virus V5 tag and HBsAg commercial standard. It was seen that some of the strains did not show production of both HBsAg and V5-CRL lip3. Also, lower intensity band of V5-CRL lip3 was detected from the supernatant of the cell homogenate from PPS9010-J9SEATLS (SURF) strains. Thus, the remaining strains expressing V5-CRL lip3 in the cell cytoplasm were chosen and were taken further for expression in control conditions. From this experiment the PPS9010-J9SEATLC 4 strain was chosen for further analysis. This strain was chosen as it produced low amount of HBsAg and large amount of V5-CRL lip3. This was done to reduce stress on the host system and as the PPS9010-J9SEATLS (SURF) strains did not produce large amount of V5-CRL lip3 they were not analysed in further experiments.

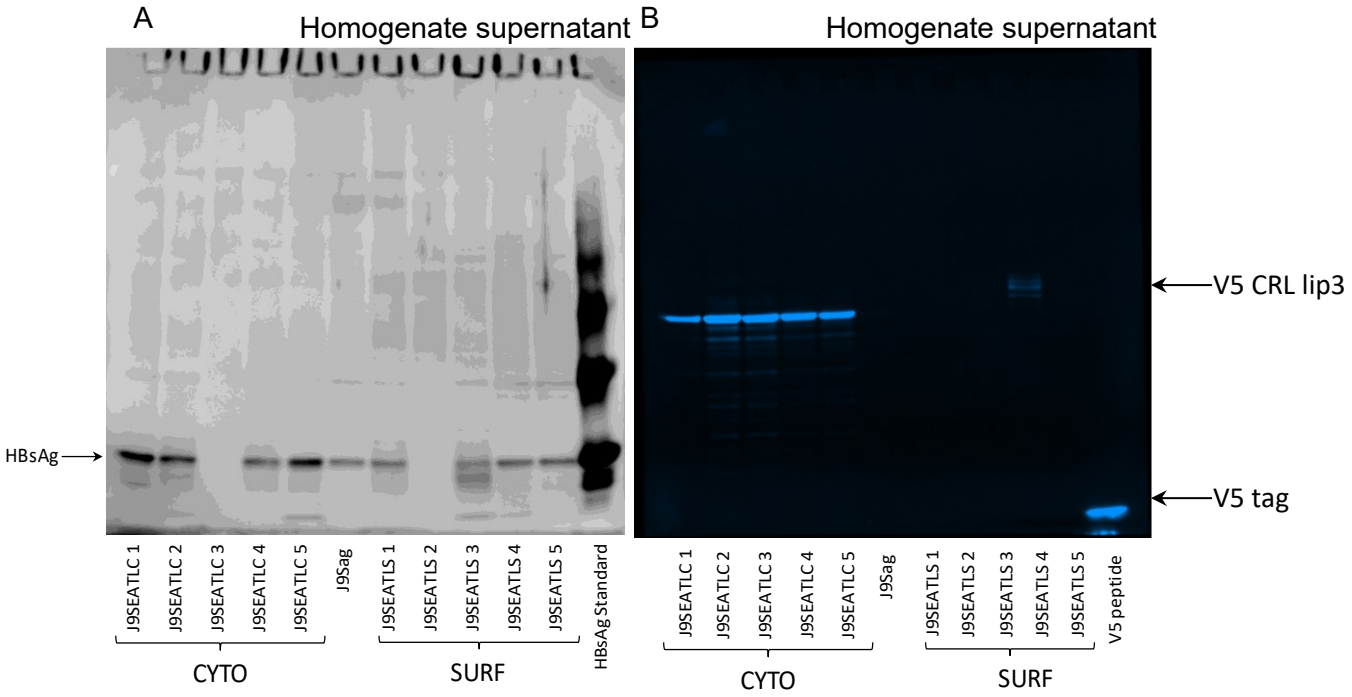


Figure 5.5: Detection of HBsAg and *C. rugosa* lipase V5-CRL lip3 expressed under P_{ENO1}

The samples are collected after the expression of C. rugosa lipase (V5-CRL lip3) in presence of glucose and before the induction of P_{AOX1} with methanol containing feed. The yeast homogenate supernatant shows strains expressing HBsAg and C. rugosa lipase (V5-CRL lip3). (A) chemiluminescence western blot with anti-HBsAg primary antibody having HRP conjugated for the expression of HBsAg and (B) a fluorescent western blot with anti-V5 epitope primary antibody and fluorescent secondary antibody for the expression of C. rugosa lipase (V5-CRL lip3) after growing the transformed cells in yeast peptone media containing glucose. Here PPS9010-J9Sag is used as a positive control for the induction of P_{AOX1} promoter. V5 epitope peptide and HBsAg were used as standards. The experiment was done with separate SDS-PAGE gel as different probes were used for the analysis of VLP and V5 tagged lip3 lipase.

5.3 Large scale expression and characterisation of HBsAg and V5-CRL lip3 in *P. pastoris*

As described in the previous chapter *P. pastoris* strain PPS9010-J9Sag consisting of only HBsAg gene under P_{AOX1} was grown in shake flask conditions. This is to check the expression

of HBsAg and develop the purification steps. The expression was done using the following method.

5.3.1 Large scale production of HBsAg in *P. pastoris*

HBsAg producing strain PPS9010-J9Sag was taken further and expressed in a 30L fermentation vessel. The expressed HBsAg from this fermentation was tested for VLP formation using TEM as previously mentioned. Fermentation of PPS9010-J9Sag strain was as described in the Invitrogen fermentation protocol as described below (Invitrogen Corporation, 2002). PPS9010-J9Sag strain containing HBsAg gene under P_{AOX1} was grown overnight in YPD media to an OD_{600nm} of 20. The bioreactor cultivation was done using Invitrogen's fermentation protocol for *P. pastoris* (Kazaks *et al.* , 2017). Briefly, 0.25L of the culture was inoculated into a 30 L BIOSTAT C plus bioreactor (Sartorius) fermenter with an initial working volume of 13L as described in chapter 2, section 2.2.3.1. The offline and online data obtained from the bioreactor producing HBsAg is showing in Figure 5.6.

The dry cell weight of the cell culture reached more than 100 g/L after 72 hours with OD_{600nm} of about 1000. This high-density cell culture is found to be similar to industrial scale cell culture processes. After 24 hours the bioreactor was found to have loss of air supply due to which the dissolved oxygen and the $\%CO_2$ content had reduced. This error was rectified, and the rest of the fermentation process was completed as per the protocol. There was no effect found in the quality of HBsAg VLP produced at the end of the fermentation process which was later confirmed with the help of transmission electron microscopy. The harvest cells were then clarified from the cell culture broth using fixed angle centrifugation and the cell paste was frozen at -20°C for further analysis.

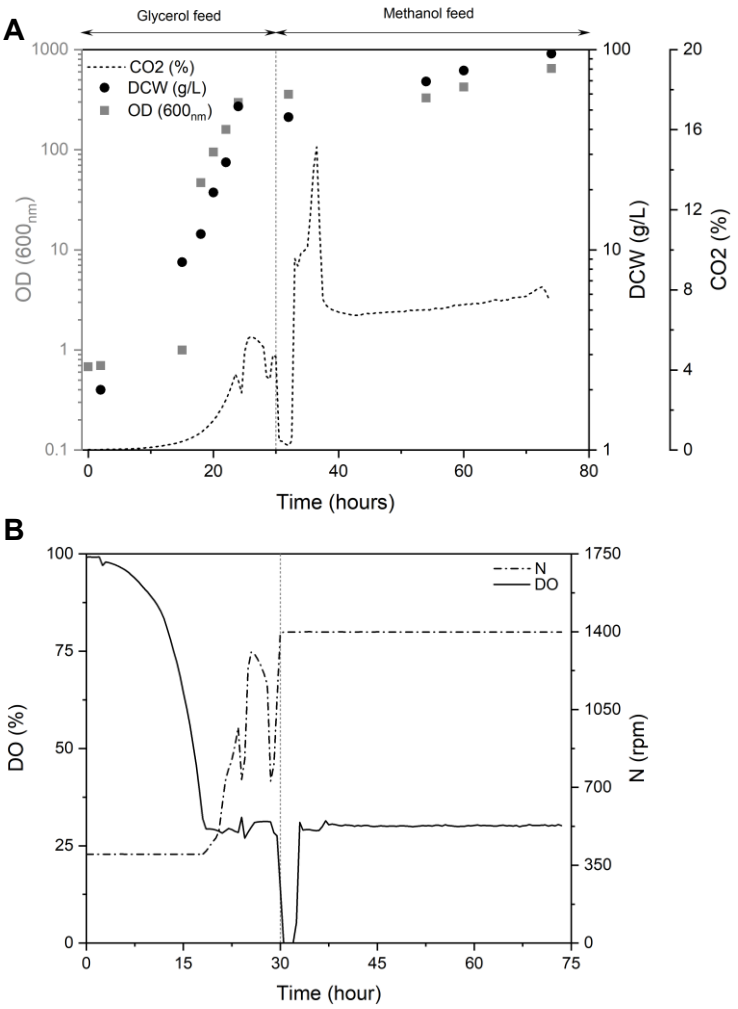


Figure 5.6: Bioreactor batch of PPS9010-J9Sag using BIOSTAT C system

PPS9010-J9Sag strain was grown overnight in YPD media up to an OD of 16. The obtained culture was inoculated into the 13L BioStat C bioreactor containing 40g/L glycerol in Basal Salts Medium with pH 6.0 and supplemented with PTM trace metal salts. The culture was grown in batch mode for 30 hours till there was signs of starvation. Then 50% glycerol containing PTM salts were fed at 18.15mL/hr/L. This was continued for 4 hours before the feed was changed to 100% methanol containing PTM salts. (A) offline data where grey solid squares corresponding to the optical density and black solid circles corresponding to the dry cell weight. This is correlated with % CO_2 (short dash) content in the bioreactor. After 24 hours the bioreactor was found to have loss of air supply due to which the dissolved oxygen and the % CO_2 content had reduced. This error was rectified, and the rest of the fermentation process was completed as per the protocol. There was no effect found in the quality of HBsAg VLP produced at the end of the fermentation process. (B) Online data where straight line corresponds to the dissolved oxygen, dots corresponding to the impeller speed and short dash corresponds to the % CO_2 content in the bioreactor.

5.3.2 Purification of HBsAg from PPS9010-J9Sag strain

The purification of HBsAg was done using multiple steps as shown in Figure 5.7. Briefly, the cells obtained from the 20L fermentation as described earlier in chapter 2, section 2.2.3.1 and batch was washed and re-suspended at (10% wcw/mL) in 1x PBS for homogenization as described in section 2.3.1.1. The obtained homogenate was clarified and the VLPs were extracted using tween 20. The purification of HBsAg from *P. pastoris* is well known in the literature and is done after its extraction using tween (Gurramkonda *et al.* , 2013; Jin *et al.* , 2010). The extraction is typically used for releasing the VLP particles formed of HBsAg from the cell debris. In the present study the homogenized cells were incubated with tween 20 for 30 mins then centrifuged to clarify the cell debris and supernatant is used for the purification of HBsAg using hydrophobic interaction chromatography. The chromatography steps were under taken as described earlier in chapter 2, section 2.3.4.1. This method was used for all the purification trials for detection of HBsAg as well as for transmission electron microscopy (chapter 2, section 2.3.5.1) material generation. The obtained HBsAg was confirmed with the help of western blot analysis. Western blot analysis and SDS-PAGE analysis (chapter 2, section 2.3.6) showed faint but detectable bands of HBsAg in flow-through and strip fractions of the HIC step, shown in Figure 5.8.

Major amount of the protein was eluted in the step gradient and consisted of the major peak. This material generated from fermentation of *P. pastoris* as explained in chapter 2 section 2.2.3 was tested for the transmission electronic microscopy (TEM), the cell culture condition used here was only to produce HBsAg particles and did not consist of lipase gene in the cell. After the TEM analysis it was observed that the sample consisted of structures resembling the cell vesicles. To remove these cell vesicles further purification with the help of anion exchanger was done as described in chapter 2, section 2.3.4.1.2

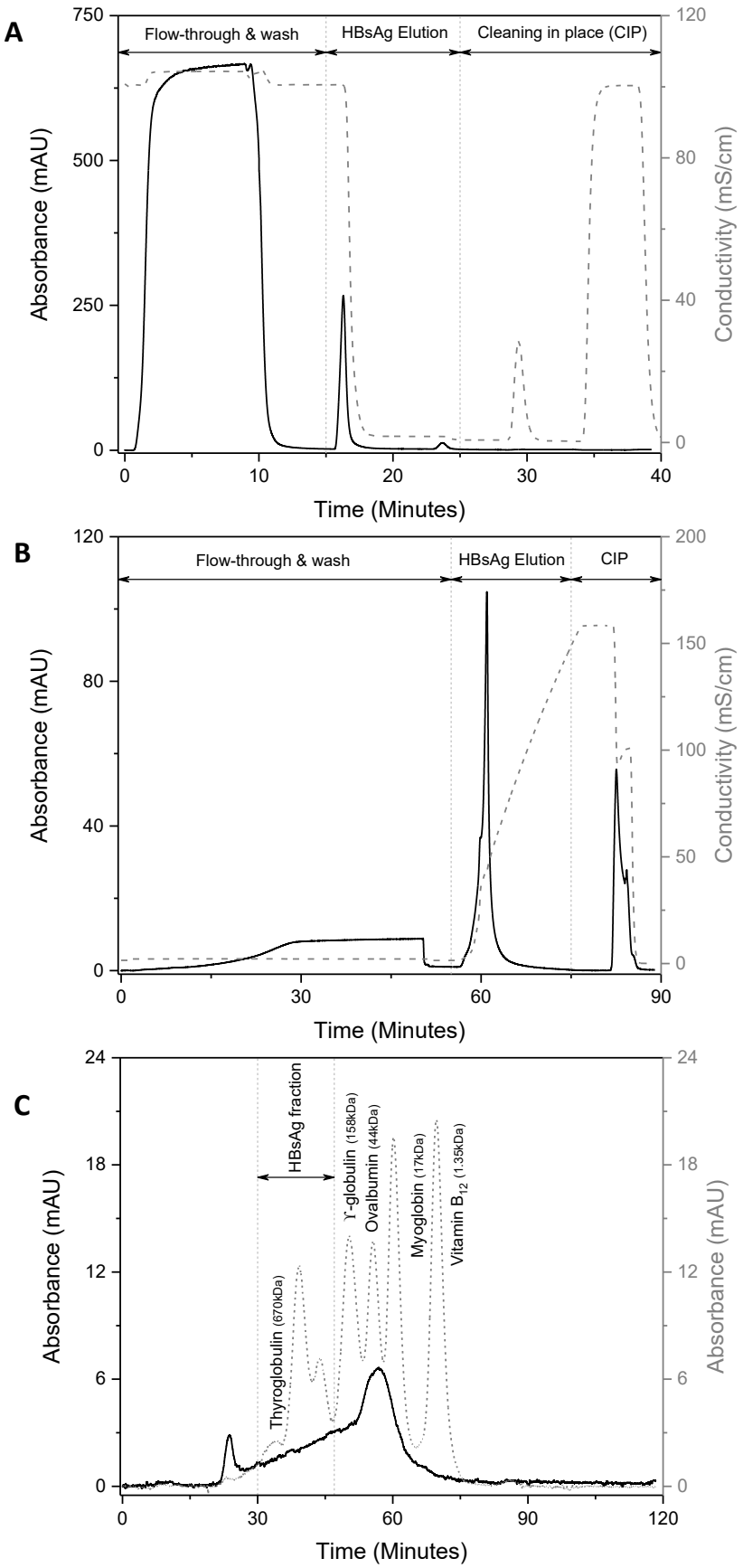


Figure 5.7: Chromatography steps taken for purification of HBsAg from *P. pastoris*

The sequence of the purification step above shows the chromatograms from the purification of the HBsAg from the harvested P. pastoris cells. (A) The purification of HBsAg virus like

particles using hydrophobic interaction chromatography (HIC). 5mL HiTrap Butyl-S 6 FF from GE was used for the purification of HBsAg. The column was saturated with 20mM sodium phosphate equilibration buffer at pH 7.0 containing 0.6M ammonium sulphate. HBsAg was eluted with the help of a decreasing gradient of buffer B of 20mM sodium phosphate without ammonium sulphate. The column then underwent cleaning with 0.5M sodium hydroxide and storage in 20% ethanol. The eluent was then dialysed and purified using an anion exchange column. (B) The purification of HBsAg virus like particles was further done using anion exchanger (AEX) 5mL HiTrap Q Sepharose FF column. The reconditioned HIC eluted fraction obtained from the HIC step was then loaded on to the 25mM Tris-HCl equilibrated Q Sepharose FF column at pH 8.0. HBsAg was eluted with the linear gradient of (0-1M) sodium chloride. The column then underwent cleaning with 0.5M sodium hydroxide and storage in 20% ethanol. (C) Eluted fraction from the anion exchanger chromatography column was then dialysed in 1x PBS before loading on to a Superose GL 300 size exclusion column. The obtained peak was fractionated for analysis of HBsAg. The fraction corresponding to thyroglobulin elution retention time had maximum amount of HBsAg. The column showed separation of different proteins with large size range. The other fraction of the eluted peak consisted of host cell proteins.

The HIC eluted fraction as shown in Figure 5.7 reconditioned with the help of equilibration buffer (20mM Tris-HCl pH 8.0) before loading on to the anion exchanger column. The reconditioned material was then loaded on to the column and a linear gradient was employed for the purification of HBsAg VLPs from the HIC eluate. The major peak was collected in fractions and concentrated using 10kDa MWCO ultrafiltration membrane. This material was then further purified with the help of size exclusion chromatography with the help of a gel filtration column.

Eluted fraction from the anion exchanger chromatography column was loading on to a Superpose GL 10/300 size exclusion column as described in chapter 2, section 2.3.4.1.3 . The obtained peak was fractionated for analysis of HBsAg. To identify the fractions for further analysis of HBsAg VLPs, a protein mixture with known molecular weight was loaded on to the same column with identical run conditions. The HBsAg VLPs eluted from the anion exchanger when analysed by TEM, it was found to have single HBsAg VLPs and aggregated

HBsAg VLPs along with other half formed VLP debris. To isolate the single HBsAg VLPs the peak eluted from size exclusion column was fractioned.

The size of the monomeric HBsAg VLPs is reported to be about 20nm and was found to be eluted in the same retention time as thyroglobulin which was identified as the peak number with reference to the known elution profile of the commercial standards. The protein mixture consisted of thyroglobulin (670kDa), γ-globulin (158kDa), ovalbumin (44kDa), myoglobin (17kDa), and Vitamin B_{12} (1.35kDa). The peak fraction corresponding to the thyroglobulin elution retention time was concentrated with the help of 100kDa MWCO and analysed with western blot and TEM for detection of HBsAg and HBsAg VLPs respectively.

5.3.3 Functional characterization of HBsAg VLPs produced by dual expression strains using transmission electron microscopy

Transmission electron microscopy (TEM) as described in chapter 2, section 2.3.5.1 was used for the detection and identification of purified VLPs. As shown in Figure 5.8 both the PPS9010-J9Sag as well as the dual expression strain PPS9010-J9SEATLC4 was purified using the above method and analysed using TEM. The flowchart shows the steps undertaken for the purification and intermittent reconditioning steps. The strains which are producing V5-CRL lip3 lipase was compared with the PPS9010 wild type strain. Both the strains were grown under identical conditions. In shake flask in the first instance and then the purified VLPs were compared from bioreactor batches.

There was no VLP like structures found from the PPS9010 wild type strain with TEM analysis and no band was observed when the SDS-PAGE gel as shown in Figure 5.8. Purified HBsAg protein analysed with commercial standard and the *P. pastoris* PPS9010 wild type. The following gel and western blot proved that with the protocol mentioned in this study for purification of HBsAg protein can be achieved. Also, *P. pastoris* PPS9010 wild type did not express any HBsAg protein as shown by the western blot analysis.

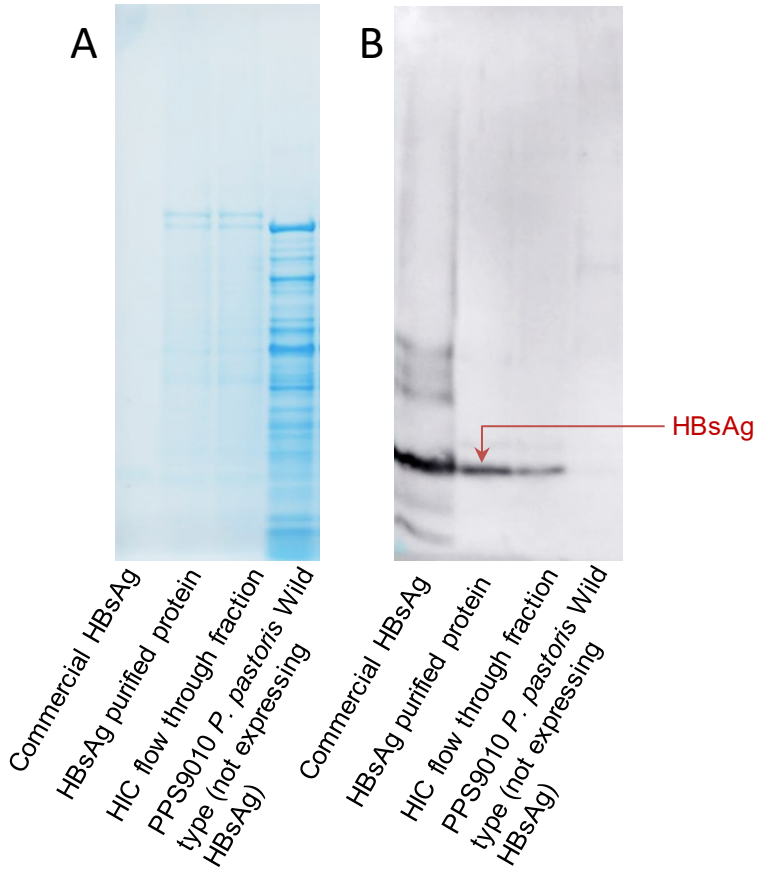


Figure 5.8: Purified HBsAg comparison with *P. pastoris* PPS9010 wild type

The purified protein obtained after a sequence of purification steps described in the previous section was analysed in comparison with the P. pastoris PPS9010 wild type strain. (A) SDS PAGE showing the purified protein with reduced impurities bands compared to the P. pastoris PPS9010 wild type. HIC flow through fraction was also analysed along with the purified protein as this fraction showed loss of HBsAg from the column during purification. (B) Western blot analysis showed the purified HBsAg and HBsAg was not detected in the P. pastoris PPS9010 wild type.

The VLP structures obtained from the dual expression strains were visually identical to the control strain**.** Multiple purification steps were employed to find the best results with transmission electron microscopy as shown in Figure 5.9 and Figure 5.10. As described above the HBsAg VLPs were purified from the yeast homogenate using three chromatography steps. Multiple TEM images were taken before the last polishing step with gel-filtration column. It is found that using the last polishing step multiple large structures resembling the organelles were purified from the VLPs whereas by dialyzing the sample overnight before gel-filtration gave

rise to hollow VLP structures. These were then considered to find the best method for purification of the HBsAg VLPs from the yeast homogenate before TEM analysis as shown below.

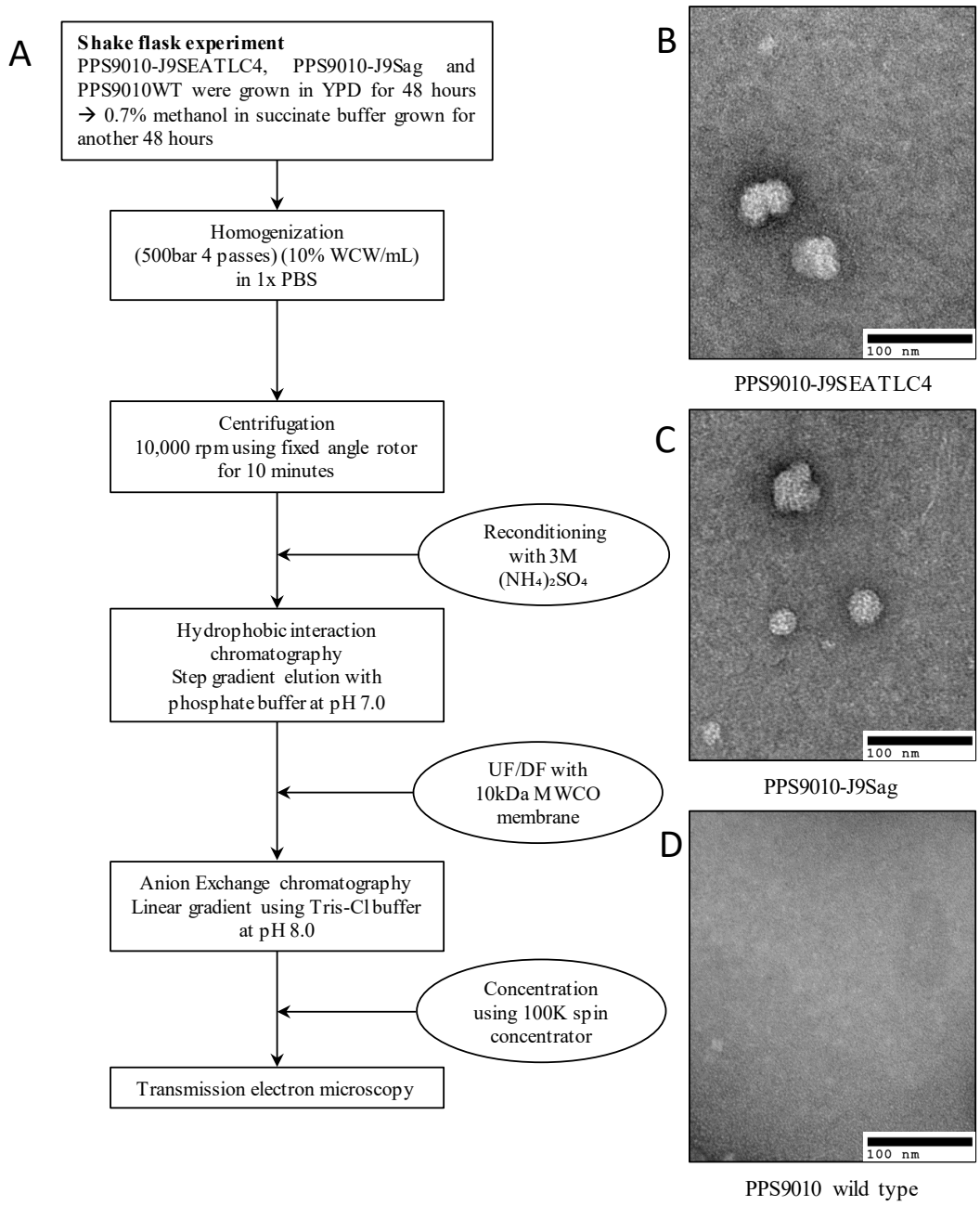


Figure 5.9: Flow diagram for purification of HBsAg VLPs from dual expression strain from shake flask cultivation

(A) Purification of HBsAg virus like particles. PPS9010-J9SEATLC4 and PPS010-J9Sag strains were grown in YPD media followed by methanol induction and harvests were homogenized and clarified using centrifugation. The homogenate supernatant was purified using hydrophobic interaction chromatography followed by anion exchange chromatography. The eluted protein was then analysed using TEM. (B) TEM image obtained from PPS9010-J9SEATLC4 strain expressing both HBsAg and V5-CRL lip3

enzyme and (C) TEM image obtained from PPS9010-J9Sag strain expressing only HBsAg, whereas (D) TEM image obtained from the wild type strain PPS9010.

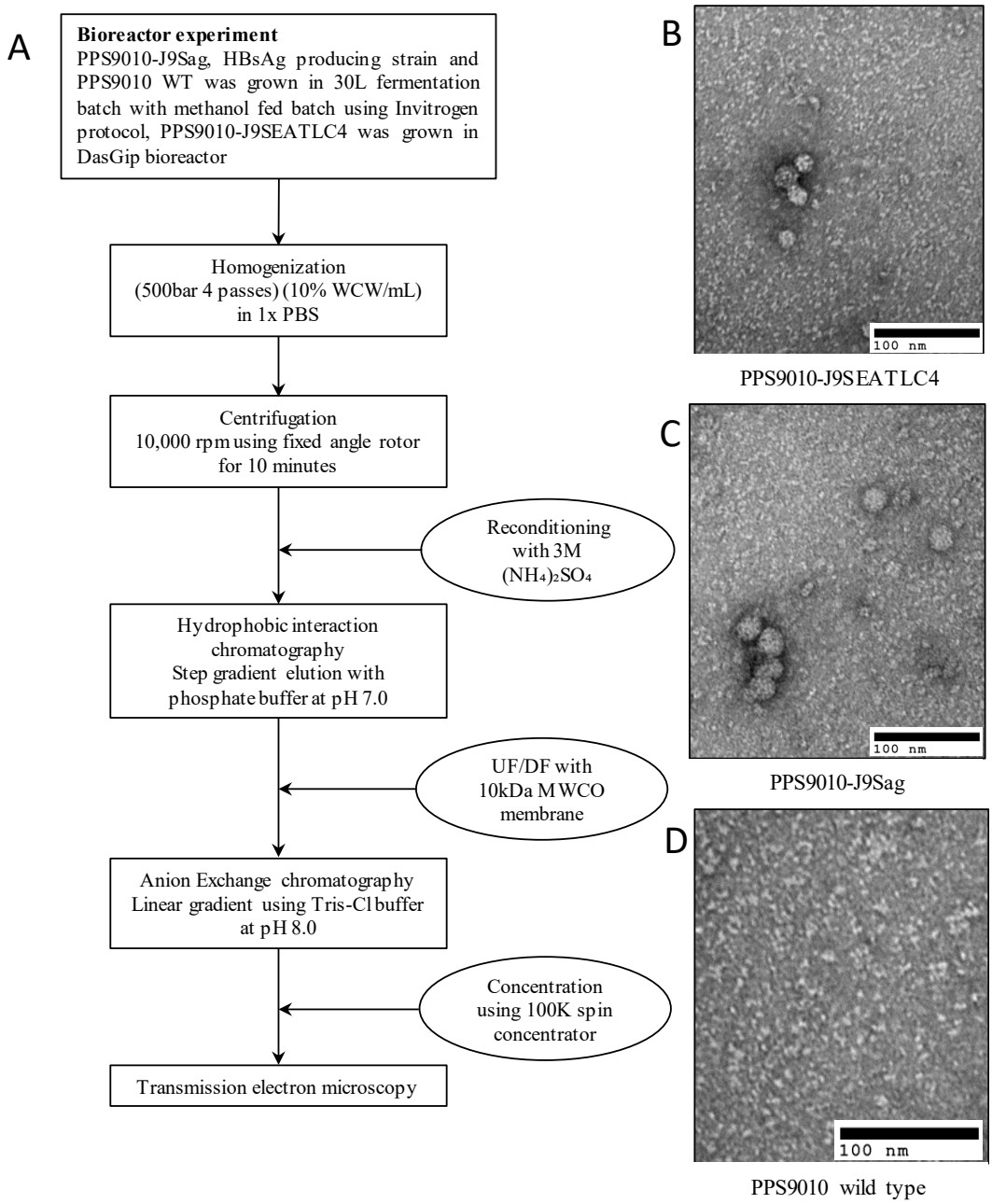


Figure 5.10: Flow diagram for purification of HBsAg VLPs from dual expression strain in a bioreactor fermentation

(A) purification of HBsAg virus like particles from PPS9010-J9SEATLC4 and PPS010-J9Sag strains. The PPS9010-J9Sag strain was grown using the Invitrogen protocol in a 30L sartorius bioreactor and the PPS9010-J9SEATLC4 strain was grown in 0.25L DasGip bioreactor. Both the cell harvests were homogenized and clarified using centrifugation. The homogenate supernatant was purified using HIC followed by AEX chromatography. The eluted protein was then analysed using TEM. (B) TEM image obtained from PPS9010-J9SEATLC strain expressing both HBsAg and V5-CRL lip3 enzyme and (C) TEM image

obtained from PPS9010-J9Sag strain expressing only HBsAg, whereas (D) TEM image obtained from the wild type strain PPS9010.

5.3.4 Purification of V5 tagged *C. rugosa* lip3 lipase from PPS9010-J9SEATLC4 strain

Lipase from PPS9010-J9SEATLC4 strain was purified to measure its in-vitro activity. The expression of *C. rugosa* lip3 lipase (V5-CRL lip3) was done in shake flask (lab scale) cell culture. PPS9010-J9SEATLC4 strain was grown for the expression of V5-CRL lip3 with the initial experiments as described in chapter 2, section 2.2.2; it was seen from the shake flask experiments described in chapter 4 that the expression of V5-CRL lip3 can be done with the help of glucose or glycerol feed. To obtain higher cell density the V5-CRL lip3 containing *P. pastoris* PPS9010-J9SEATLC4 strain was grown in YPD media and purified with the help of hydrophobic interaction chromatography. The resulted purity of 95% as observed from Western blot analysis shown in Figure 5.8.

The purification of V5-CRL lip3 from *P. pastoris* has been reported in literature. In the present study the *P. pastoris* PPS9010-J9SEATLC4 strain was grown for about 50 hours. As it was not known if there is any degradation of the V5-CRL lip3 when produced in large quantities in the cell cytoplasm, the *P. pastoris* were harvested after 50 hours. The harvested cells were then homogenized using Gaulin lab 40 homogenizer at 500bar pressure with 5 passes. The obtained homogenate was clarified using centrifugation at 10,000xg for 20 minutes at room temperature. The supernatant was reconditioned using ammonium sulphate ($(NH_4)_2SO_4$). This supernatant was then used for purification using hydrophobic interaction chromatography as described in chapter 2, section 2.3.4.2. The purification of V5-CRL lip3 was done using multiple steps as shown in Figure 5.8. The obtained fractions were then analysed using western blot analysis as described in chapter 2, section 2.3.6.3.

It was seen that the flow through and wash fraction had considerable about of the V5-CRL lip3 protein. This suggests that there can be improvement in the purification step. Increasing the concentration of ammonium sulphate in the equilibration buffer can help in

retaining most of the protein of interest in the column. In the linear gradient fractions, small amount of the protein was found to be eluted as represented by the faint band shown in the Figure 5.8. Majority of the single band protein was eluted with the help of 10mM CHAPS buffer. The obtained chromatograph and the western-blot are shown in Figure 5.8 (B).

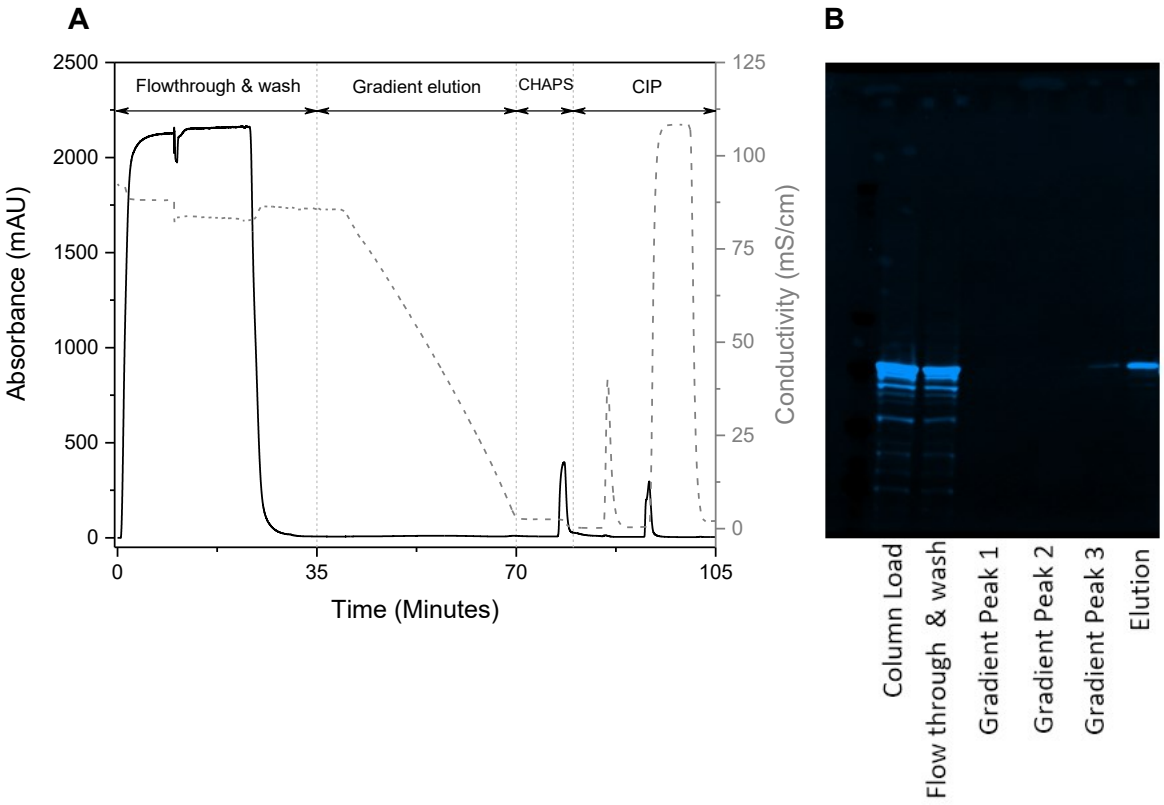


Figure 5.11: Hydrophobic interaction chromatography purification of *C. rugosa* lip3 lipase

The flow above Figures shows the hydrophobic interaction chromatogram for the purification of the C. rugosa lip3 lipase (V5-CRL lip3) from the harvested P. pastoris PPS9010-J9SEATLC strain. (A) The purification of V5-CRL lip3 was done using 5mL HiTrap Butyl-S 6 FF from GE. The column was saturated with 25mM Tris-Cl pH 7.0 equilibration buffers at containing 0.5M ammonium sulphate. V5-CRL lip3 was eluted with the help of 10mM CHAPS without ammonium sulphate. The column then underwent cleaning with 0.5M sodium hydroxide and storage in 20% ethanol. The eluted fraction was then analysed using western-blot analysis as described in chapter 2. (B) Purification samples were loaded onto an SDS-PAGE and after the gel was run under electrophoretic conditions, the protein bands were transferred on a nitrocellulose membrane. This membrane was strained with anti-V5 tag fluorescent antibody for the visualization of the V5-CRL lip3 protein.

5.4 Lipase assay development

5.4.1 Development of colorimetry assay if lipase activity

The activity of *C. rugosa* lipase (V5-CRl lip3) in comparison with commercial lipase from *C. rugosa* was tested with 4-nitrophenyl laurate as a substrate. As described earlier in chapter 2, section 2.2.2 the dual expression strains were grown in YPD media. The standard curve was created with 4-Nitrophenol. Activity was measured over time at 460nm using a TECAN plate reader. The experiment was done as per previously published (Ruiz *et al.* , 2004) using microwell plate as described in chapter 2, section 2.3.7.1. Briefly, the lipase assay buffer was used to dilute the standards and substrates from the stock solutions. After 48 hours of growth the cells were harvested and homogenised (chapter 2, section 2.3.1.1) in the lipase assay buffer. Both the pellet of the homogenate as well as the supernatant were analysed using this assay. This was done to have an idea as to which fraction of the homogenate consisted of maximum amount of the active lipase.

The samples and the substrate were mixed using 2sec orbital shaking before taking the reading. Specific activity of the enzyme was calculated as activity is equal to number of enzyme units per mL of reaction mixture per milligram of protein. Commercial enzyme was used as control along with non-expressing *P. pastoris* wild type strain which were grown together with the dual expression strain. This was also spiked with the commercial enzyme and taken as a system control as shown in Figure 5.11. It was seen from this experiment as this assay is not best suited for the analysis of the lipase enzyme from yeast homogenate. The spiked sample showed less activity in comparison to the commercial enzyme and indicated the presence of inhibitory elements in the yeast homogenate. The homogenate pellet fraction showed maximum activity of the expressed V5-CRL lip3 although this activity was much lower in comparison to the commercially available purified *C. rugosa* lipase VIII.

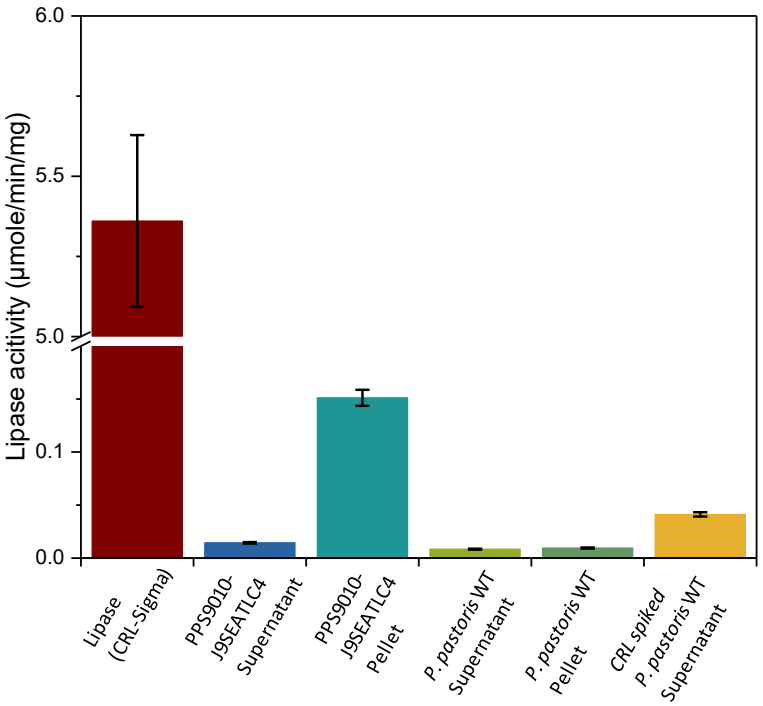


Figure 5.12: Lipase activity obtained from colorimetry assay

The above graph shows the comparison between commercial lipase and the expression of lipase obtained from the dual expression strain. As described in chapter 2 section 2.3.7.1 the formation of 4-nitrophenol from 4-nitrophyl laurate was done spectrophotometrically at 460nm. The commercial lipase was re-suspended lipase activity assay buffer (55.56mM phosphate buffer pH 8.0 with 1.33% Triton X 100). The expressed yeast cells were clarified using centrifugation and re-suspended followed by homogenization in the assay buffer media. P. pastoris wild type (PPS9010-WT) was used as a control that did not express any recombinant lipase. The supernatant of the control sample was also spiked with commercial Candida rugosa lipase VIII from Sigma with the same concentration (0.1mg/mL). Both the supernatants from the lipase expressing (PPS9010-J9SEATLC4) (CYTO) strain and the P. pastoris wild type did not show high activity. When the supernatant of the P. pastoris wild type was spiked with the commercial Candida rugosa lipase VIII it failed to give activity as obtained while analysing the commercial lipase in the aqueous media.

5.4.2 Zymogram for *C. rugosa* lip3 lipase activity

The activity of the lipase was studied using zymogram analysis of *C. rugosa* lipase (V5-CRL lip3). The method used here is similar to commonly used protocols (Diaz *et al.* , 1999; Prim *et al.* , 2003; Singh *et al.* , 2006). The strains were grown overnight in 10mL YPD using 50mL falcon tubes. They were then centrifuged and re-suspended in Tris-HCl pH 7.5 buffer

and sonicated using SoniPrep as described in chapter 2, section 2.3.1.2. The Zymogram was developed after running the samples in a 4-16% BisTris SDS PAGE (Laemmli, 1970) gel under non-reducing conditions as described in chapter 2, section 2.3.6.1. The imaging using Amersham gel imager was done after soaking the gel in 2.5% Triton X 100 in 25mM TrisCl pH 7.5 buffer containing 50% MeOH as described in chapter 2, section 2.3.7.2. It was observed that during the method development the commercial standard and the samples were boiled following the standard sample preparation steps during SDS-PAGE analysis. Doing so the protein activity bands were found to be lower than the known molecular weight bands. This can be due to the denaturation occurring due to boiling of the sample during sample preparation.

Due to this observation the protein samples were not boiled or reduced during sample preparation. An SDS-PAGE gel was preferred in this analysis in case of native PAGE gel as SDS-PAGE produced much sharper distinguishable protein activity bands. Such compact and sharp bands are not obtained when the analysis was done using native PAGE gel. The multiple wash done containing 2.5% Triton X 100 helped in removal of the SDS from poly acrylamide gel. The samples that were obtained from PPS9010-J9Sag strain producing only HBsAg under P_{AOX1} did not show any difference from the strain PPS9010-J9SEATLC co-expressing both the HBsAg as well as the V5-CRL lip3. The lipase was expressed using above mentioned culture conditions. The samples from the batch culture were analysed using SDS-PAGE analysis. When the gel was incubated with MUB (4-Methylumbelliferyl butyrate) a band of activity was seen over time. The presence of methanol in the washing media helps in visualization of the product formed due to the catalytic activity of the lipase enzyme. PPS9010-J9SETALC 4 showed faint band for activity of the enzyme and was taken forward for the cell culture experiments under bioreactor conditions as shown in Figure 5.13. The reference standard showed clear bands of degradation of laurate and release of the florescent product which was visualised using UV light. The samples expressing V5-CLR lip3 did not show prominent bands which might be an indication less active protein or production of protein in

very low concentration. Further optimization of this technique is needed for better results indicating clear activity bands.

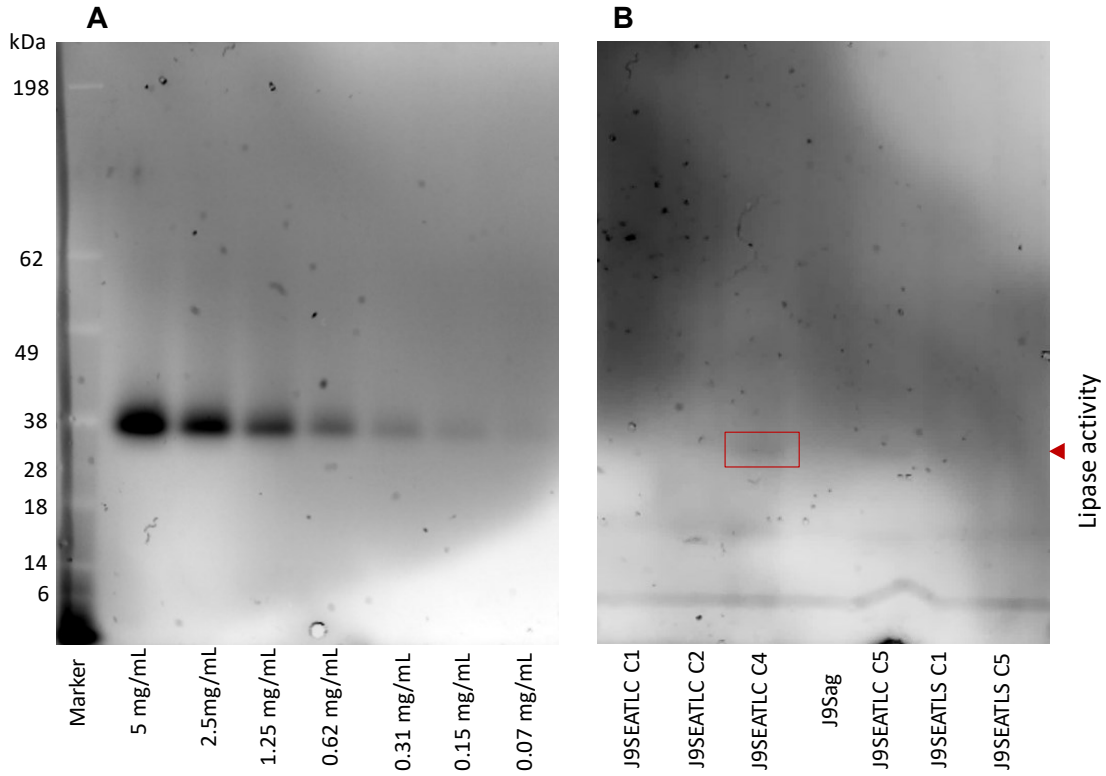


Figure 5.13: Lipase activity zymogram of strains producing both lipase and HBsAg

The gel shows the lipase activity from the dual expression strain with 4-Methylumbelliferyl butyrate (MUB) substrate. (A) Commercial lipase from C. rugosa VIII (Sigma), used as a reference standard was prepared in PBS buffer with a serial dilution from 5 to 0.07mg/mL. (B) The dual expression strain expressing C. rugosa lipase (V5-CRL lip3) were grown in YPD overnight culture and selected based on V5 detection as seen from the western blot analysis shown in Figure 5.5. The reference standard as well as the samples were denatured by boiling followed by running them in an SDS-PAGE gel electrophoresis under non-reducing conditions. After the completion of the electrophoresis the gel was washed with buffer containing triton X 100 for 45 mins and layered with buffer containing MUB substrate for 1 hour. After incubation distinct bands appeared corresponding to the product from the lipase activity reaction under UV light. The bands obtained were faint and did not show strong activity bands as seen in case of the reference standard against MUB laurate.

Table 5.1: Recombinant strain selected producing V5-CRL lip3 and HBsAg

The strains were tested for the expression of V5-CRL lip3 and HBsAg under P_{ENO1} and P_{AOX1} respectively. The strain PPS9010-J9SEATLC4 was chosen on the based on confirmed production of V5-CRL lip3 in glycerol containing media and HBsAg in methanol containing media. In the table the strains with P_{THI11} was not tested (NT) for HBsAg as these strains did not produce any detectable V5-CRL lip3. Here the detection is classified from no expression (✕) to expression which is visible (✓), clearly visible (✓✓) and intense band (✓✓✓) as concluded from western blot results shown in Figure 5.2 and 5.3.

	Strains	V5-CRL lip3	HBsAg
1	J9SEATLS	✓	✓
2	J9SEATLC	✓✓✓	✓
3	J9SEATLkdel	✕	✓
4	J9STATLS	✕	NT
5	J9STATLC	✕	NT
6	J9STATLkdel	✕	NT
7	J9Sag	-	✓

5.5 Conclusion

From this chapter it can be concluded that *P. pastoris* can be successfully used as a system to express two different heterologous recombinant proteins. It can also be concluded that

1. The dual expression of HBsAg can be achieved along with potentially toxic lipase intracellularly.
2. The potentially cytotoxic V5-CRL lip3 can be expressed with detectable amount of lipase activity along with formation of VLPs. To have higher lipase activity the cell culture conditions needs to be optimized so that larger quantity of lipase is expressed. It can be assumed that the purified lipase will show higher activity in aqueous solution as crude cell culture solution gives interference to the detection of lipase.

The use of dual promoter system can be used for expression of heterologous proteins such as V5-CRL lip3 which in turn can be used for process improvement for process related impurities. For such improvement there is a need to express these proteins sequentially or in phases. Doing so can reduce the load on the cell to produce both the protein in trophophase and can be achieved by two different promoters which are either induced or to produce the protein of interest at different stages of the cell culture. Use of inducible promoter like P_{AOX1} along with unique promoter like P_{ENO1} gives this advantage. As P_{AOX1} is induced with only methanol and P_{ENO1} is supressed by methanol, the combination of their use for dual expression is advantageous. The following table 5.1 shows the tested six strains with dual expression and HBsAg producing strain. The strain PPS9010-J9SEATLC was chosen on the based on confirmed production of V5-CRL lip3 in glycerol containing media and HBsAg in methanol containing media.

6 Using carbon source to direct independent transcriptional control of lipase and VLP in *P. pastoris*

6.1 Introduction

Based on the consistency of observations, the strain (PPS9010-J9SEATLC4) with positive detection of V5 tagged *C. rugosa* lipase lip3 (V5-CRL lip3) and HBsAg under P_{ENO1} and P_{AOX1} respectively was selected. In this strain the V5-CRL lip3 was produced in the cytoplasm of the *P. pastoris* cell and HBsAg was localized in the endoplasmic reticulum. These strains were further taken for analysis and following questions were addressed.

1. Can different carbon feed like sorbitol, methanol and glycerol be used for controlled independent expression of *C. rugosa* lipase and HBsAg in *P. pastoris* trophophase?
2. Can this independent control strategy be used to maximize VLP expression through fermentation while accumulating lipase only at the terminal period of the cultivation?

To answer these questions the expression of HBsAg and V5-CRL lip3 were tested separately. After the confirmation of both the heterologous proteins expression, induction of multiple proteins by alternative carbon source (IMPACS) were tested. Multiple combinations of sequences of carbon feed during these cell culture runs were tested to find the best combination where both the heterologous proteins are expressed and are accumulated at the end of the fermentation process. After the selection of a feed strategy the dual expression strain was then grown under controlled conditions of a bioreactor with feed consumption monitoring.

6.2 Intended bioprocess benefits of dual expression system

Most cell culture strategies involve production of a single protein under a single promoter. The details of the heterologous expression system are given in chapter 1 section 1.4. Briefly, *P. pastoris* is grown in the presence of glucose or glycerol and methanol is used for the induction of heterologous proteins under P_{AOX1}. Alternative carbon and energy sources are also used such as, alanine, sorbitol, mannitol and trehalose, although the doubling time on are notably very long when such carbon sources are used as shown by Inan and Meagher, 2001.

Novel promoters described earlier gives an innovative option for the expression of more than one protein under single cultivation process. It is known that the repression of P_{AOX1} occurs in presence of glycerol. It is also known that P_{ENO1} is classified as a constitutive promoter (Vogl and Glieder, 2013). There is some evidence that the P_{ENO1} can be repressed in the presence of methanol, ethanol and glucose (Stadlmayr *et al.* , 2010).

In the present study *P. pastoris* PPS9010-J9SEATLC4 strain did produce V5-CRL lip3 protein under P_{ENO1} in presence of glucose, when grown in YPD medium, it was evident that glucose does not completely repress the action of P_{ENO1}. It was confirmed that there can be an increased production of V5-CRL lip3 in presence of glycerol and more repressed in presence of methanol. The experiments were designed keeping in mind the induction and repression of both the promoters. The objective here was to obtain the expression of both the products, namely HBsAg as well as V5-CRL lip3 sequentially also, to have maximum accumulation of HBsAg and V5-CRL lip3 at the end of the process. Expression of heterologous protein was done sequentially in presence of methanol for expression of HBsAg under P_{AOX1}. It was seen in small scale experiments that when P_{ENO1} was activated in the beginning of the cultivation then over time the total amount of V5-CRL lip3 gets reduced. It suggested that to obtain maximum expression of V5-CRL lip3 enzyme the induction of the P_{ENO1} should be done at the end of the cultivation process as shown in Figure 6.1.

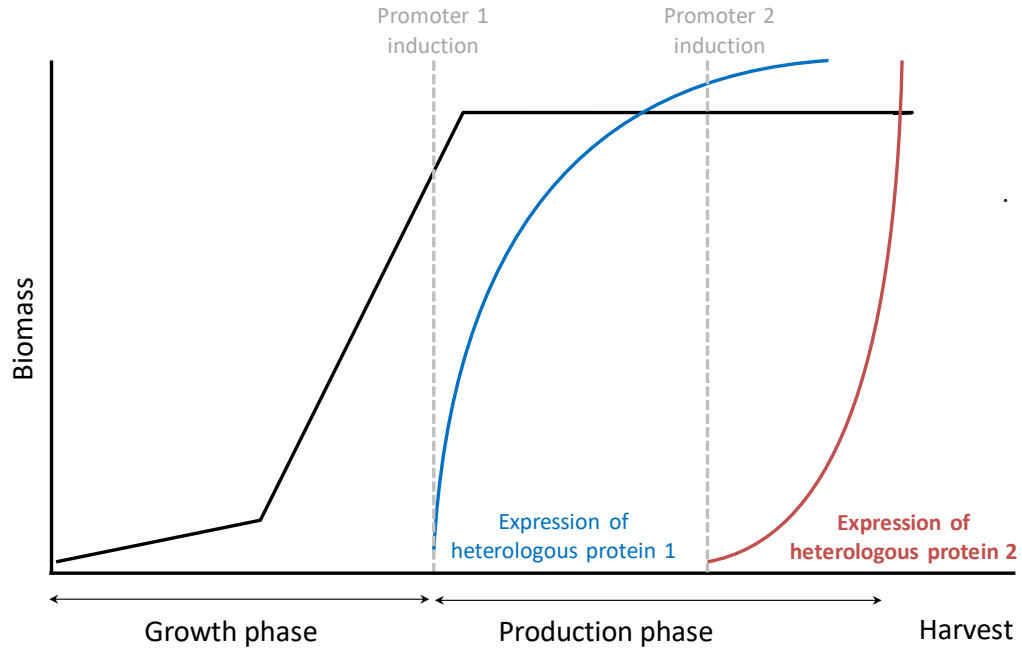


Figure 6.1: Model for dual promoter expression using different sequential feed containing sorbitol, methanol and glycerol under shake flask conditions

The Figure shows ideal expression of two heterologous proteins expressed under two different promoters namely P_{AOX1} and P_{ENO1}. The expression of lipase is predicted to initiate at the start of the growth phase it is under constitutive promoter P_{ENO1} and the expression of HBsAg is initiated under P_{AOX1} with addition of methanol. At the end, during harvest both the heterologous protein products are ideally with maximum production.

6.2.1 Establishing an IMPACS framework with P_{ENO1} and P_{AOX1}

Keeping in mind the objective of maximal expression for both the proteins at the end of the cultivation period, a strategy of sequential expression of HBsAg and V5-CRL lip3 lipase was done as shown in Figure 6.2. *P. pastoris* cells were cultivated in 250mL shake flask at 30°C under 250rpm shaking. Culture media was prepared from EasySelect™ *Pichia* Expression Kit. YNB containing trace elements, vitamins, nitrogen sources and salts without amino acid was used for this experiment. 0.02% Biotin per litre was added to each culture media. Final media concentrations of 1% Methanol (M), 1% Glycerol (G), 2M sorbitol (S) and 1 M potassium phosphate buffer, pH 6.0 was prepared for the cultivation experiments. Phosphate buffered saline (PBS) tablets from Gibco were used for washing the *P. pastoris* cells. Culture media was changed after every 24 hours sequentially with fresh media by centrifugation, washing and resuspension. Washing with 1x PBS was employed in between the steps to remove residual glycerol, sorbitol or methanol.

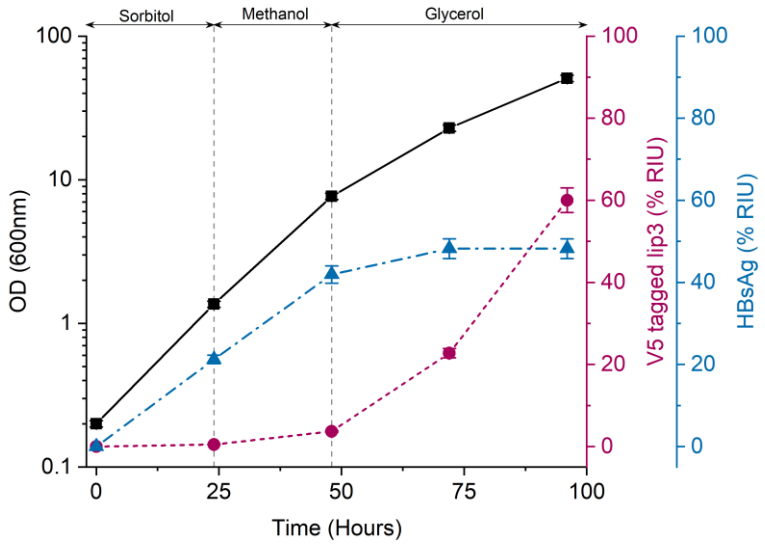
The resuspension on the cells after wash was done in fresh media. In the case of the 'SMG' strategy, the cultivation was started with 1M Sorbitol as the sole carbon source in potassium phosphate, pH 6.0 buffered minimal media containing YNB and biotin. The next growth phase used the same medium, containing 1% Methanol and the final step used the same medium containing 1% Glycerol. The notation is retained for the different cultivation strategies namely, MSG, MGS, and GMS where the sequence of sorbitol (S), methanol (M) and glycerol (G) were interchanged. As described earlier the expression of HBsAg and V5-

CRL lip3 lipase was done in under shake flask conditions along with the study of substrate consumption.

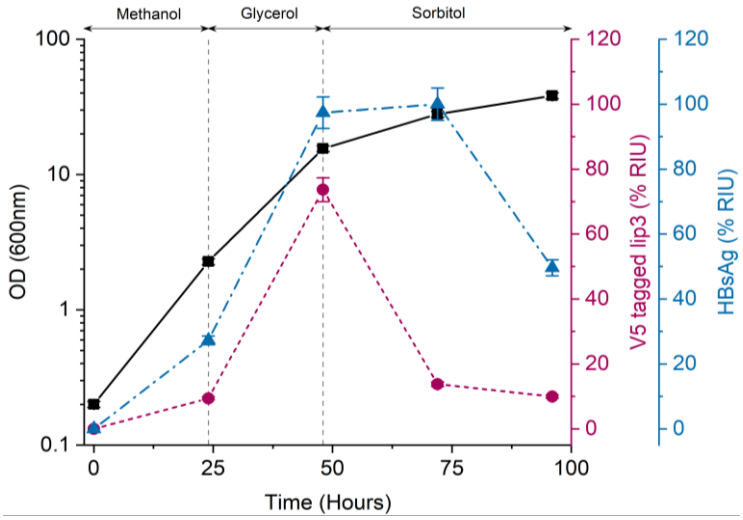
During the sorbitol growth phase (S) it was seen that the growth of the cells was slower than in other carbon sources such as glucose and glycerol. During the sorbitol growth phase there was expression of HBsAg under P_{AOX1} found with little to no expression of V5-CRL lip3 found which is under P_{ENO1}.In the methanol phase (M) of the cultivation of HBsAg under P_{AOX1} was expressed. The buffered minimal media containing glycerol did not show any the production of HBsAg expressed under P_{AOX1} and showed the production of V5-CRL lip3 under P_{ENO1}. In the glycerol phase (G) there was accelerated growth. This phase was continued for another 48 hours and samples for OD_{600nm} analysis and immunoblotting were taken at regular intervals. The expression of V5-CRL lip3 increased with cell growth which indicated that the P_{ENO1} expression did not affect the cell growth in presence of glycerol.

The expression of the proteins was analysed using dot blot analysis. Dot blot analysis for quantification of HBsAg and V5-CRL lip3 expressed protein; same steps were as described in chapter 2 section 2.3.6.4. Briefly, the samples were reduced using the Invitrogen reducing agent (Cat No. NP0004) and boiled at 70°C for 10 mins before spotting the dots on to the nitrocellulose membrane. The membranes were then spotted with 1µL of reduced samples. The blocking of the membrane followed by use of primary and secondary antibody was done in the same manner as described in chapter 2 section 2.3.6. For quantification the intensity of the dots was then measured using Amersham 600 gel imager and analysed using four parameters logistic (4PL) fitting algorithm.

A


Sorbitol
Methanol
Glycerol
OD (600nm)
V5 tagged lip3 (% RIU)
HBsAg (% RIU)
Time (Hours)

B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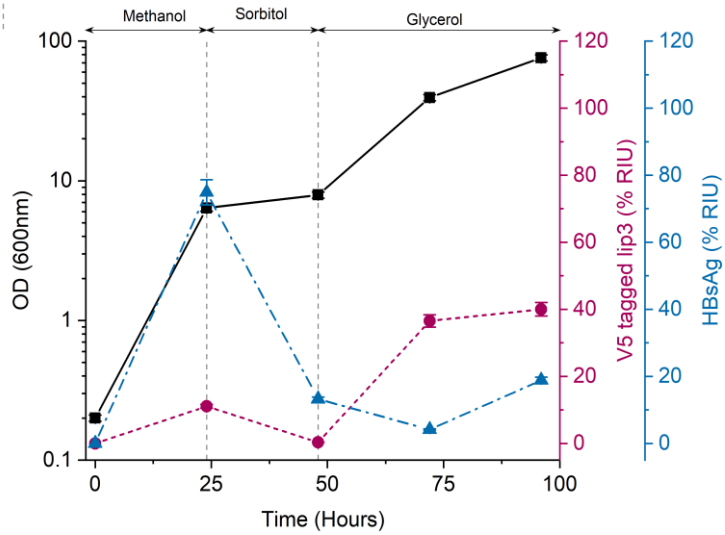
Glycerol
Sorbitol
OD (600nm)
V5 tagged lip3 (% RIU)
HBsAg (% RIU)
Time (Hours)

C


Methanol
Sorbitol
Glycerol
OD (600nm)
V5 tagged lip3 (% RIU)
HBsAg (% RIU)
Time (Hours)

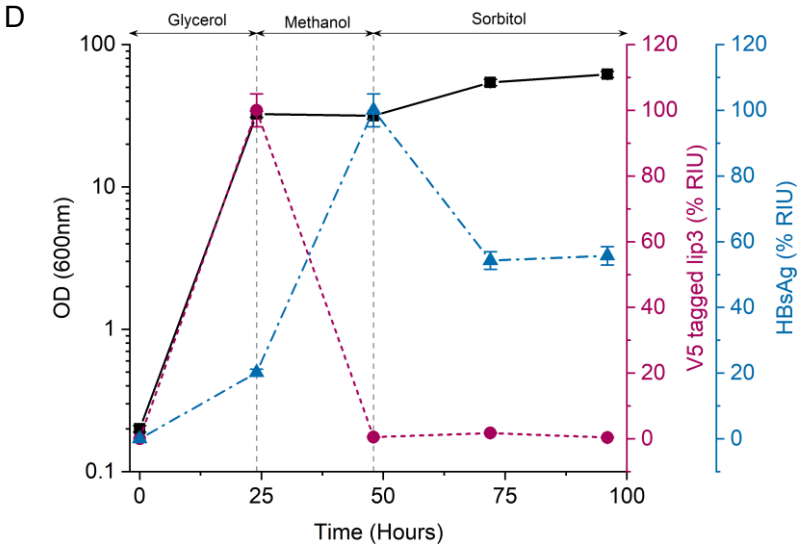


Figure 6.2: Strategies for dual promoter expression using different sequential feed containing sorbitol, methanol and glycerol under shake flask conditions

As described earlier in methods section (A), (B), (C), and (D) corresponds to different feed strategies shown in the above Figure. PPS9010-J9SEATLC strain was grown in 250mL baffled shake flask at 30°C under 250rpm shaking containing buffered minimal media. Samples were taken at every 24 hours interval until 96 hours and measured for expression of HBsAg (inverted triangles) and V5tagged CRL lip3 (solid circles). The samples were then analysed using western blot analysis and the intensity obtained from the band is represented as % relative intensity units (%RIU). Each sample was run at least two times to have 5% standard deviation with n=2 error on each time point. The graphs were plotted with normalized values from the complete set.

The expression of HBsAg and V5-CRL lip3 was done under a fixed feed strategy. PPS9010-J9SEATLC strain containing HBsAg under P_{AOX1} and cytoplasm retained V5-CRL lip3 under P_{ENO1} was grown in 250mL baffled shake flask at 30°C under 250rpm shaking containing buffered minimal media. Samples were taken at every 24 hours interval till 96 hours and measured for expression of HBsAg (blue) and V5-CRL lip3 (red). The samples were then analysed using dot blot analysis. This analysis was done to see the expression of HBsAg and V5-CRL lip3 in comparison to the consumption of feed. As seen in the Figure 6.3, the methanol containing feed was used after 24 hours of cell growth in sorbitol. The samples obtained from after 24 hours shows the expression of HBsAg which is controlled under P_{AOX1}

and is induced with methanol. The methanol bolus which was added after 24 hours is consumed but HBsAg is found in later stages of the cell culture. Similar production of V5-CRL lip3 is found with the addition of glycerol feed. It is also seen that the V5-CRL lip3 is reduced as glycerol starts reducing in the cell culture media. This correlated with the expression of V5-CRL lip3 under P_{ENO1}. In this study the cell culture was not washed with PBS in between the feed changes and the production of V5-CRL lip3 as well as HBsAg was still obtained. Considering this the feeding strategy of sorbitol, methanol, and glycerol was selected for further analysis of the developed dual expression strain under controlled conditions of a bioreactor.

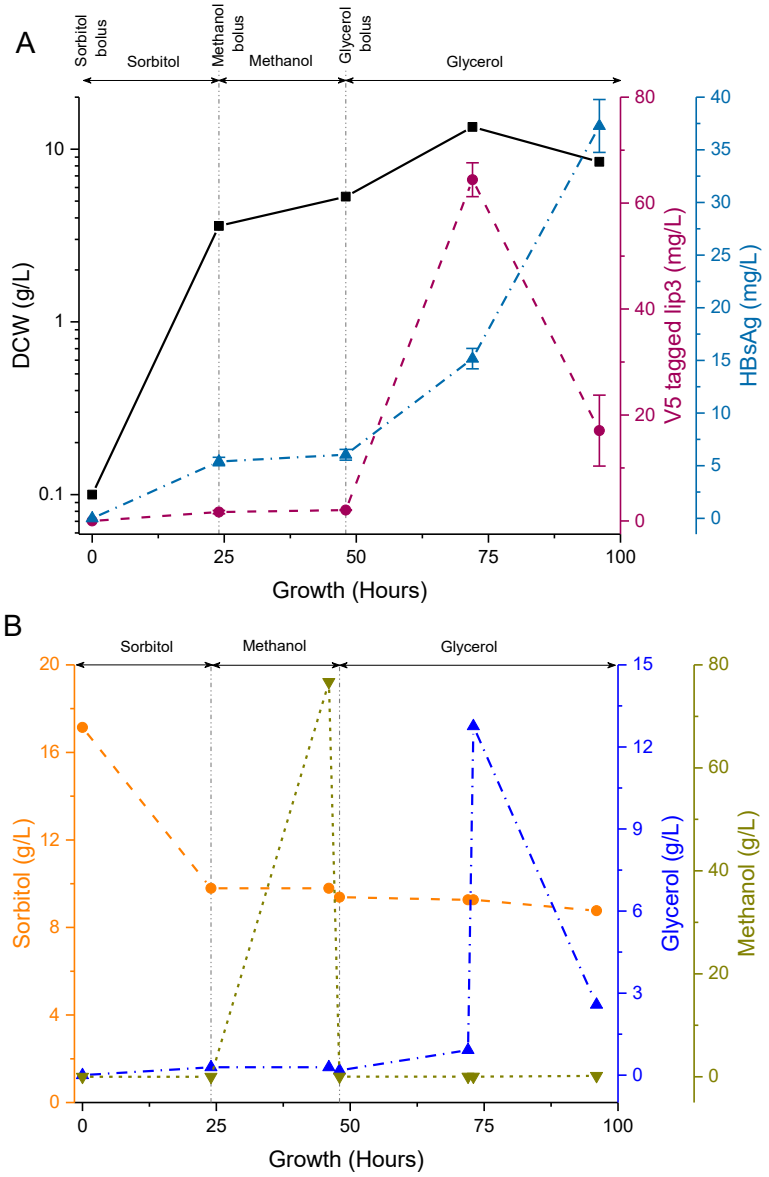


Figure 6.3: Product accumulation and expression of lipase along with HBsAg in shake flask cultivation

As described earlier in methods section (A) corresponds to expression of HBsAg and V5-CRL lip3 lipase under a fixed feed strategy shown in the above Figure. PPS9010-J9SEATLC strain was grown in 250mL baffled shake flask at 30°C under 250rpm shaking containing buffered minimal media. Samples were taken at every 24 hours interval till 96 hours and measured for expression of HBsAg (blue) and V5tagged CRL lip3 (red). The samples were then analysed using dot blot analysis. Each sample was run at least two times to have 5% standard deviation with n=3 error on each time point. The graphs were plotted with normalized values from the complete set. (B) Corresponds to the graph showing the presence of sorbitol throughout the process in comparison with methanol and glycerol feed at 24 hours and 48 hours respectively, where the respective feed is added and consumed over time. The graphs are plotted in comparison with expression of HBsAg and V5-CRL lip3.

6.2.2 Performance of the IMPACS framework for cells cultivated in a fermentation system

The PPS9010-J9SEATLC4 strain expressed V5-CRL lip3 and showed lipase activity evident from the zymogram described in chapter 5 section 5.4.2. The same strain in separate experiment showed expression of HBsAg forming VLPs that were confirmed from the TEM analysis given in chapter 5 section 5.3.3. The PPS9010-J9SEATLC4 strain was taken further for expression under bioreactor conditions.

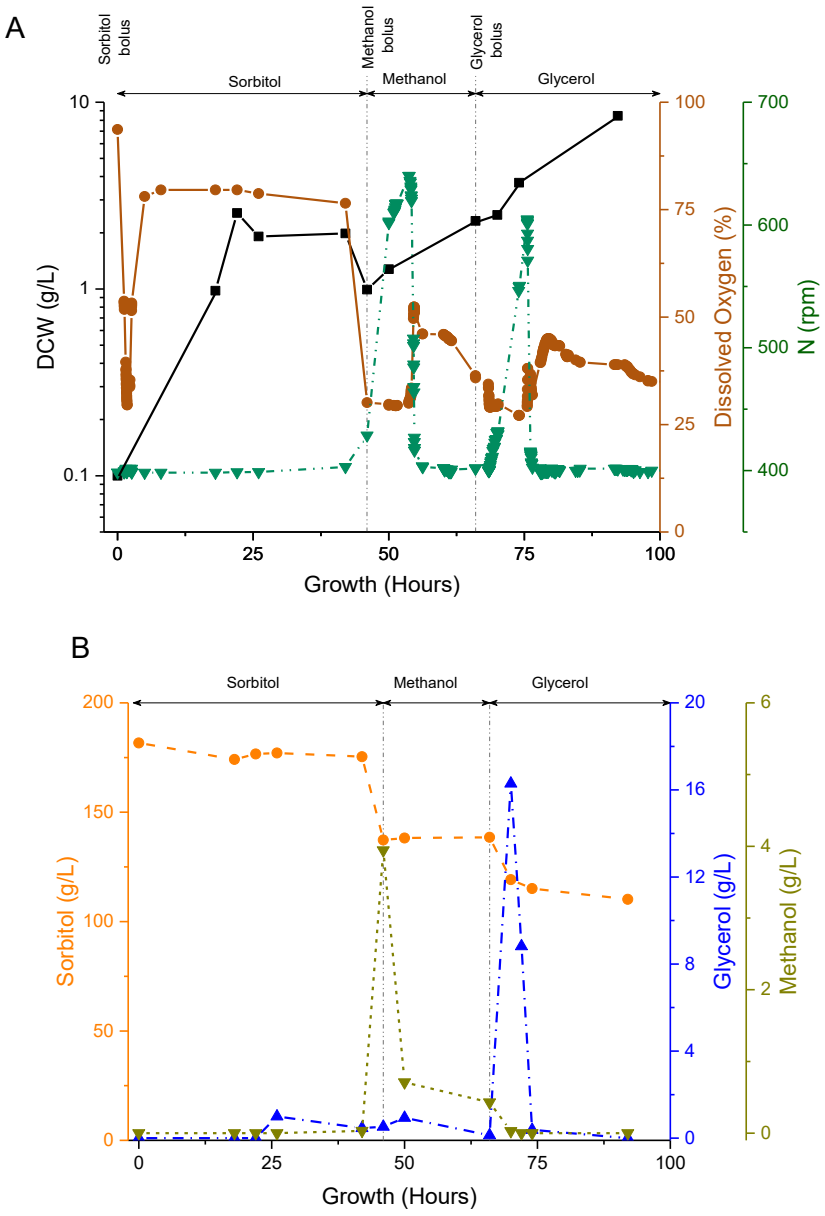
As the Co-expressing strain showed expression of both heterologous proteins in shake flask conditions, the media and bolus dose of carbon sources were added to mimic the small-scale conditions. Bioreactor cultivation was carried out to mirror the sequential nature of the shake flask experiments; however, changes were made to allow for a more industrially relatable process. The main difference between the shake flask and bioreactor experiments was the removal of the washing and resuspension step, which led to the presence of sorbitol throughout, due to which the strain had low specific growth rate μ (0.69). This meant that it did not allow carbon source to be depleted, and the typical signs thereof (Dissolved oxygen spike, pH shift, agitation decrease) to be used as a trigger to start the second phase of growth

(utilising glycerol). However, these changes notwithstanding, there is a clear sequential induction of HBsAg and V5-CRL lip3 lipase, which serves to reinforce the applicability of a sequential induction system within *P. pastoris*.

6.2.3 Migrating experiment design from shake flask to closed system using a DasGip multi-bioreactor system

The sequential expression of both heterologous proteins where achieved under closed bioreactor system. For the small-scale bioreactor studies, 7 ml of defined media culture from an overnight YPD shake flask cultivation described earlier was used to inoculate 0.15 L of the same modified defined media (YNB supplemented with sorbitol, as above) in an Eppendorf DasGip 0.25L bioreactor, employing the 'SMG' cultivation strategy. A dissolved oxygen cascade was employed to maintain a minimum 30%, the bioreactor utilised an increase in agitation rate (400-700rpm) along with the ability to blend oxygen if required. 17.5% w/v ammonia solution and 14% v/v orthophosphoric acid were used to maintain pH at 6.0. The culture was maintained at a temperature of 30 °C. After 46hrs the culture had an additional 50mL of YNB Medium supplemented with methanol to start the production of HBsAg. The culture was grown until 66 hrs and then had another 50mL of YNB medium supplemented with glycerol added. This induced the production of V5-CRL lip3 enzyme under P_{ENO1}. The obtained expression of V5-CRL lip3 and HBsAg from DasGip bioreactors are shown in Figure 6.4. *P. pastoris* was grown in minimal media with bolus addition of sorbitol for 46 hours. During this phase the cell growth was slow and achieved a plateau at 10g/L DCW shown in Figure 6.4 (A). The addition of methanol and glycerol and their consumption was tracked using HPLC technique described in chapter 2 section 2.3.3.3, where total amount of methanol, glycerol and sorbitol present in the culture supernatant was measured shown in Figure 6.4 (B). During the cell culture the expression of HBsAg and V5 tagged CRL lip3 was also measured with DOTBLOT analysis.

The growth of *P. pastoris* was seen to be lower than that is obtained when the same strain is grown under glycerol or glucose carbon source. This observation is consistent with other studies done by Inan and Meagher, 2001 with sorbitol as the sole feed source. The present study is designed to test the dual expression of HBsAg and V5 tagged CRL lip3 heterologous proteins. For the higher biomass and improved production of both the heterologous proteins, further optimization of the feed strategies and fermentation process is required.



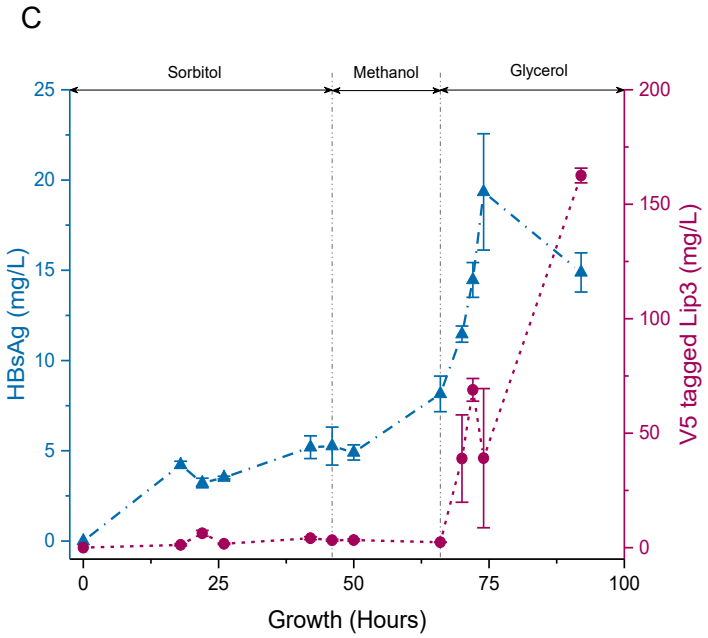


Figure 6.4: Closed system of PPS9010-J9SEATLC using DasBox system

PPS9010-J9SEATLC strain was grown overnight in YPD media up to an OD600nm of 23. The overnight culture was then inoculated into the 0.25L DasGip bioreactor containing 150ml of 1M sorbitol in buffered minimal media at pH 6.0. The culture was grown in batch mode for 46 hours and OD600nm was observed to have decreased. 50mL of methanol 1% was added to the bioreactor and the culture was grown in this phase for another 20 hours. The presence of methanol induced the production of HBsAg. In the final phase, the production of V5-CRL lip3 was induced by addition of 50mL of 1% glycerol to the bioreactor. (A) The graph shows the % dissolved oxygen (DO) (brown line) and rpm (N) (green dash-dot line) of the bioreactor at each phase of the fermentation process. (B) Graph showing the presence of sorbitol throughout the process in comparison with methanol and glycerol spike obtained during after the addition of feed at 46 hours and 66 hours respectively, where the respective feed is added and consumed over time. (C) Graph showing increased accumulation of HBsAg (blue triangles) and V5-CRL lip3 (solid circles) production at different phases of the culture. The amount of HBsAg and V5-CRL lip3 lipase was measured using Dot Blot analysis at the end of the cultivation. The error bar represents standard deviation with n=2 error obtained from the dot blot band intensity.

6.3 Conclusions

From this chapter the following can be concluded

1. *P. pastoris* can be successfully used as a system to express two different heterologous recombinant proteins under controlled conditions.
2. The production of *C. rugosa* lipase (V5-CRL lip3) and HBsAg can be controlled using different feed strategies containing glycerol and methanol along with sorbitol during the trophophase of *P. pastoris*.

There are certain limitations seen in terms of biomass accumulation due to carbon source and inducer constraints during the fermentation experiments. The use of different carbon sources in the cell culture feed can be monitored using modern HPLC techniques. This gives us a better understanding of consumption of carbon and energy metabolism along with production of heterologous proteins. As seen in this chapter, the growth of *P. pastoris* is not sufficient with sorbitol as a sole carbon source. Even though the expression of HBsAg and *C. rugosa* lipase (V5-CRL lip3) can be achieved similar results as seen from shake flask experiments. In the controlled bioreactor conditions, the cell densities do not reach up to industrial standards.

For the downstream process improvement, the expression of *C. rugosa* lipase (V5-CRL lip3) needs to be further studied. In the present chapter it was seen that the expression obtained from the developed PPS9010-J9SEATLC4 (CYTO) strain had much less lipase activity in-comparison to the commercially purified enzyme (*C. rugosa* lipase VIII from sigma). To see similar effect on lipid impurities and its clearance from the process feed large quantities of active lipase needs to be produced. The use of dual promoter system does give a unique advantage for achieving this goal. Further work is needed on production of high-density cultures which will be capable in producing larger quantities of active lipase at the end of fermentation process.

7 Conclusions

In this thesis the importance of lipid impurities in fouling of downstream matrices and membranes was assessed with the goal of using lipase against such impurities which are released while extraction of intracellular therapeutic products from *Pichia pastoris (P. pastoris*).

Objective 1 (chapter 3)

It was observed that exogenous use of commercially available lipase from *Candida rugosa* (*C. rugosa)* helps in reduction of triacylglycerol lipids from downstream process feed, yeast homogenate. Triacylglycerol lipase used in this study is specific for the triacylglycerol found as the major foulant during downstream operations. As the action of lipase needs an optimum temperature of 37°C to reduce lipid content, the crude yeast homogenate was incubated at the same temperature. During this incubation, the cell homogenate showed formation of flocculants which would be easily removed along with cell debris, formed after homogenization with centrifugation. The remaining suspended lipids in the cell supernatant was removed and a total reduction of 50% was seen in overall triacylglycerol content of the yeast homogenate. This removal of the triacylglycerol had a positive effect on filtration, which was as high as 60% improvement in throughput at 800LMH flow rate while using a dead-end filtration setup.

Objective 2 (chapter 4)

Considering the effect of lipase on the lipid impurities, an approach is taken to co-express the lipase along with intracellular therapeutic products like VLPs for further process improvement. To reduce stress on the host cell producing two heterologous proteins, an approach of sequential expression is taken. The intracellular localization of potentially toxic triacylglycerol *C. rugosa* lipase (V5-CRL lip3) is considered such that the product of interest can be produced followed by localization in endoplasmic reticulum while lipase is expressed and localized in the cytoplasm of the host cell. Other strategies involved localization of lipase in the endoplasmic reticulum (KDEL strains) and surface displayed (SURF strains). The KDEL strains having P_{ENO1} promoter stopped expressing V5-CRL lip3 after first few experiments whereas the SURF strains did not produce large quantities of V5-CRL lip3 lipase as seen from

western-blot analysis. Hence, the strains obtained from these strategies did not show promise and were not take further.

This strategy of expression gives a unique advantage for co-expression and bioprocessing of different proteins. The use of dual promoter system can be used for expression of heterologous proteins which in turn can be used for process improvement of process related impurities. This was achieved by two different promoters which are induced to produce the protein of interest at different stages of the cell culture. Use of inducible promoter like P_{AOX1} along with unique promoter like P_{ENO1} gave this advantage. As P_{AOX1} is induced with only methanol and P_{ENO1} is supressed by methanol, the combination of their use for dual expression was advantageous. Seven different strains were created which followed the strategies for dual expression. These strains were selected under antibiotic resistant markers and the gene integration was confirmed using PCR analysis. Further expression and characterisation of the heterologous proteins under two different promoters was necessary.

Objective 3 (chapter5)

P. pastoris strains were grown in standard YPD media and growth protocol used for *P. pastoris* cultivation. It was successfully concluded that the *P. pastoris* strains can be used for expression of two different heterologous recombinant proteins without being toxic to the organism. The expression of the HBsAg antigen was confirmed using western blot analysis with anti-HBsAg antibody. The strains expressing V5 tagged lipase from *C. rugosa* (V5-CRL lip3) were screened with different carbon feed. It was observed that the V5-CRL lip3 was expressed in large quantities in strains where it was expressed in the cytoplasm. The strain PPS9010-J9SEATLC4 (CYTO), expressing both the HBsAg and the V5-CRL lip3 in the soluble fraction (homogenate supernatant) were taken further for the development of dual expression cell culture conditions.

Objective 4 (chapter 6)

It can also be concluded that *P. pastoris* can be successfully used as a system to express two different heterologous recombinant proteins under controlled conditions. The production

of V5-CRL lip3 and HBsAg can be controlled using different feed strategies containing glycerol and methanol along with sorbitol during the trophophase of *P. pastoris*. There are certain limitations seen in terms of biomass accumulation due to carbon source and inducer constrains during the fermentation experiments. The use of different carbon sources in the cell culture feed was monitored using modern HPLC techniques. The growth of *P. pastoris* was not sufficient with sorbitol as a sole carbon source. Even though the expression of HBsAg and V5-CRL lip3 was achieved similar to the results as seen from shake flask experiments.

In the controlled bioreactor conditions, the cell densities do not reach up to industrial standards. For the downstream process improvement, the expression of V5-CRL lip3 needs to be further studied. It is seen that the lipase activity obtained from the developed PPS9010-J9SEATLC4 (CYTO) strain was much lesser than the commercially purified enzyme when added to the yeast homogenate. To see similar effect on process impurities and clearance of lipid from the process feed large quantities of active lipase needs to be produced. The use of dual promoter system can give a unique advantage for achieving this goal. Further work is needed on production of high-density cultures along with large quantities of active lipase at the end of fermentation process.

8 Future work

8.1 Short term studies

The present work is separated into the upstream section where independent expression of lipase along with VLP under the influence of two different promoters and the downstream section were the effects of exogenous lipase on yeast homogenate lipids is studied. These sections have been done with the partial fulfilment of the original scope of work. Given more time, additional areas could be explored for further in-depth analysis of filter membrane fouling due to lipid impurities. These are explained in the below sections with some proof of concept experiments, separated into short term and long-term projects that can be undertaken to enrich this research.

1. Confirmation of cellular localization using confocal microscopy

The strains used in this work consists of the strains producing heterologous proteins which is localized in different cellular compartments. It has been demonstrated in this work that the strains, in this case producing V5 tagged lipase has been produced by all the three variants namely the cytoplasmic retention (CYTO) strain, ER retained (KDEL) strain and the surface displayed (SURF) strain. The confirmation of the cellular localization was not done in this work. To confirm the proper localization of heterologous protein in different cellular compartment confocal microscopy can be used. Previously study done by Liang *et al.* with *Candia antarctica* lipase B was anchored to the cell surface using a flocculation functional domain of FLO1p (Liang *et al.* , 2013). They have used confocal microscopy to show the expression of the green fluorescent protein on the cell surface. Similar technique can be employed to study the localization of the protein expressed with different signal peptide targeting it to cytosol or by use of KDEL sequence the peptide targeting it to the endoplasmic reticulum region of the cell.

2. Optimization of *C. rugosa* lip3 lipase production in cytosol of *P. pastoris*

The strains used in this work consists of the strains producing heterologous proteins in the cytoplasm of *P. pastoris*. During western blot analysis the antibody binds to the protein of interest and allows detection of the protein. In the present study, when the V5 tagged lipase protein was expressed and the expression was qualitatively measured using western blot analysis, large number of higher molecular weight as well as lower molecular weight bands were observed. This experiment is described in detail in chapter 5, section 5.2. It was also seen that when this crude harvest containing V5 tagged lipase was clarified using centrifugation and only the supernatant was analysed, single band representing the V5 tagged lipase was seen. Further optimization experiments are required so that such bands, with lower molecular weight (possible fragmentation) and high molecular weight (possible aggregation) can be avoided. This can be done by use of other tags to detect the expressed lipase. Other possible options for optimization of western-blot analysis can be by not boiling the samples before loading onto the SDS-PAGE gel before the western-blot and running the gel in non-reducing conditions. If by optimization of the sample preparation step the production of high and low molecular weight V5 tagged lipase is not reduced, then other strategies involving optimization of culture conditions needs to be evaluated.

3. Development of dual promoter system for high density *P. pastoris* fermentation

The strains developed in this work where not treated as a process mimic, consisted of the strains producing heterologous proteins under P_{AOX1} and P_{ENO1}. The present work used a strategy of expressing two heterologous proteins sequentially where both the proteins maximally accumulated inside the cell at the end of the fermentation process. This strategy was used as a control system and had not been optimized for process improvement. With the selected strategies the developed strains could only be grown with the help of sorbitol as the

carbon source, as P_{ENO1} is expressed in glucose and glycerol. Doing so the cell culture obtained was not having high cell densities. To obtain high cell density cultures as well as use the strategies studied in this work, new promoters such as P_{PHO89}, a Phosphate-Responsive promoter (Ahn *et al.*, 2009) or P_{ICL1} promoter based on isocitrate lyase gene (Umemura *et al.*, 2004) could be used in place of P_{ENO1} which does not gets induced in the presence of glycerol or glucose in the media.

4. Development of lipase activity method to detect lipase activity in crude cell culture samples.

In the present study the lipase activity was detected using two different methods namely, colorimetry and with the help of lipase activity zymogram. As per the obtained results it was observed that colorimetry method was not suitable for analysis of crude samples containing lipase. A large amount of interference was seen with the crude harvest while using the colorimetric method. And this was confirmed when the yeast homogenate was spiked with commercially available lipase which gave reduced activity measurement in comparison to the same lipase in aqueous medium. In case of the zymogram method used for the detection of lipase activity (Kwon *et al.*, 2011), it was observed that the substrate which gave the maximum response with the commercial lipase could only be achieved using a small chain substrate. It was easily optimized with the commercial lipase. But it was difficult to find a response when the crude harvest was used for detection of lipase activity using the same substrate. Due to these results, for the measurement of lipase activity either of these method needs to be optimized with the use of more sensitive detection system or different method should be developed.

5. Immobilization of lipase on cell surface for degradation of lipid fouling and process improvement using SURF strain.

The strains used in this work consisted of SURF strain which expressed *C. rugosa* lip3 lipase consisting of the PIR1 signal sequence. This strain was not optimized as it did not produce large amount of V5-CRL lip3 lipase in comparison to the CYTO strain. For further studies this strain could be optimized for expression of the lipase in large quantities which is displayed on the cell surface. This strategy gives a particular advantage were the lipase displayed on the cell surface could be used as an immobilized enzyme for the degradation of lipids after homogenization. This in turn helping in process improvement while purifying high value VLP products like HBV. For detection of the immobilized lipase on the surface of the cell, confocal microscopy is used as shown in the study done by Jiang *et al.*, 2008. This technique can be first used for detection of the surface expressed strains and then optimized for detection of lipase activity which can be used for its quantification.

6. Optimization of endoplasmic reticulum retained *C. rugosa* lipase in *P. pastoris*

The strains developed in this work consisted of KDEL strategy which was used to develop strains that can expression lipase and retain them in the endoplasmic reticulum (ER) of *P. pastoris*. The strains did not expression large amount of V5-CRL lip3 lipase as shown with western-blot analysis. These strains could be optimized so that we are expressed and retained in the ER region of the cell. Further re-transformation and screening of more clones is required for such optimization and development of the KDEL strain.

8.2 Long term studies

Effect of the dual expression strain on downstream process improvement

Due to vast financial consequences due to the fouling of membrane and matrices that is incurred during the development of the a biotherapeutics, it is a major concern to develop a reliable manufacturing process that will be as quick and cost effective. For this reason, the present study is an attempt to develop a dual expression system which can be used for the improvement of downstream processing. From the present study, a long-term study can be further undertaken which will help investigate the present knowledge for process improvement. It evident from the present study that by expression of process enhancing protein like lipase and benzonase can be co-expressed along with a protein of interest. This co-expression needs to be optimized such that the production of the process enhancing protein is obtained at the end of the fermentation process, thus reducing metabolic load to the cell, and is accumulated at high concentration which will be sufficient to reduce the lipid load, in this case, in the feed stream. To achieve such production conditions proper selection of promoters is required. As shown in this study the use of sorbitol does not give high density cell mass but is essential to be used for the growth of the *P. pastoris* cells as the selected promoters expressed under glycerol. It is believed that selection of promoter which is only invoked at the end of the fermentation process will be the optimal scenario for production of process enhancing proteins. Use of such process enhancing proteins will in turn help in reduction of the overall process cost incurred due to fouling. Advances in this area of research to produce specifically engineered cell lines for process enhancement along with producing high value proteins can be beneficial in reducing fouling and overall cost from co-expression.

List of publications by author

1. A dual promoter strategy for effective co-production of viral antigen and *Candida rugosa* triacylglycerol lipase during post-trophophase growth of *Komagatella phaffi* (*P. pastoris*) strain PPS9010 by **Sushobhan Bandyopadhyay**, Qing Tee, Behzad Karkaria, Michael Sulu, Chris Barnes, Daniel G. Bracewell, Darren N. Nesbeth (publication in preparation).

2. Exogenous lipase to reduce lipid load in primary recovery operations for yeast bioprocessing by **Sushobhan Bandyopadhyay**, Darren Nesbeth, Daniel G. Bracewell (publication in preparation).

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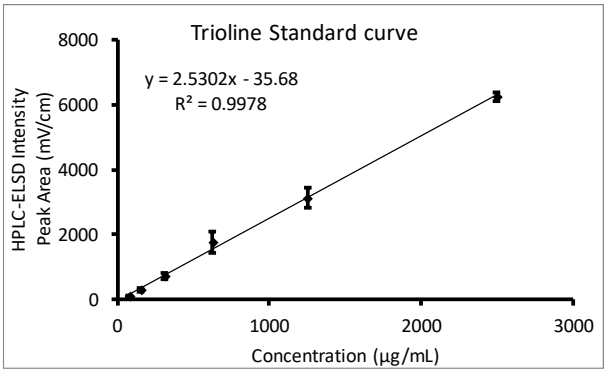
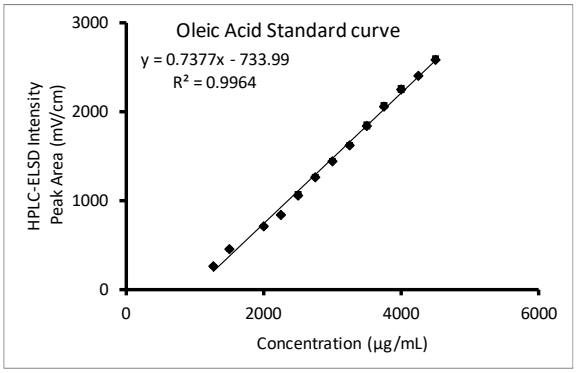
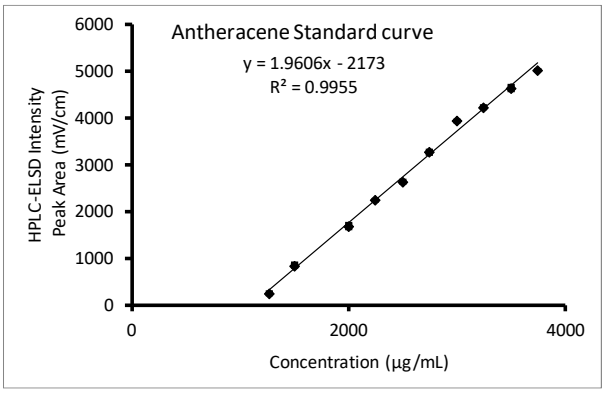
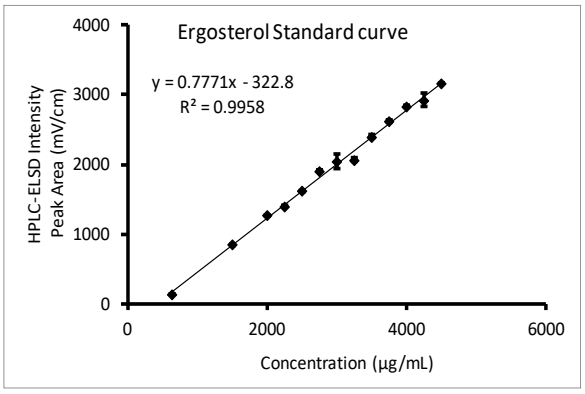
Appendix I

Standard curves for quantification

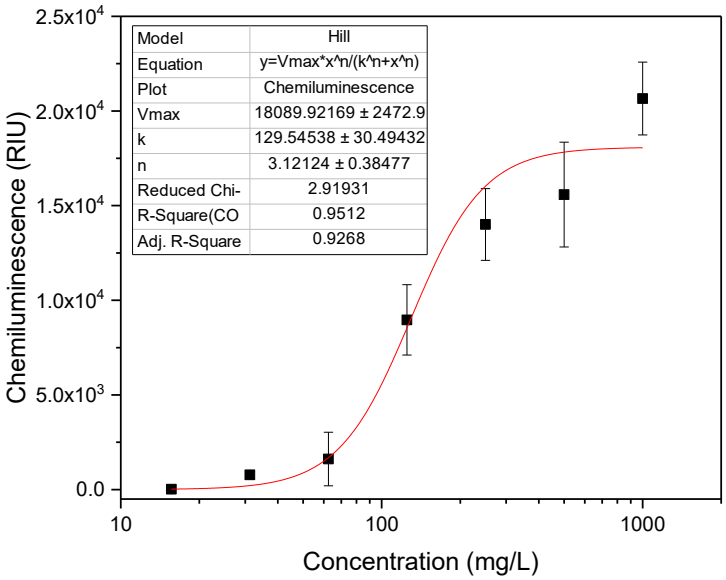








The above calibration curve was used for quantification of triacylglycerol, oleic acid, ergosterol and anthracene. The standards were commercially obtained from sigma and were dissolved in chloroform, methanol mix (2:1). The obtained stock was then used to make serial dilutions for the standard curve. The samples were then analysed on HPLC-ELSD system and the peak area was recorded and plotted as HPLC-ELSD intensity. The obtained curve was then fitted using a liner curve fitting algorithm using an Microsoft EXCEL software. The standard curves were then used for quantification of triacylglycerol and oleic acids from the yeast homogenate samples.



The above calibration curve was used for quantification of HBsAg and V5-CRL lip3 lipase produced by P. pastoris expression both the proteins in DASgip Bioreactor. For the quantification of both HBsAg as well as the V5 tagged CRL lip3 lipase expressed by the engineered strains, DOTBLOT analysis and western blot analysis were used. As described in chapter 2 the samples were spotted on to a nitrocellulose membrane which was then dried and strained with HPR tagged anti-HBsAg primary antibody and anti-V5 antibody for HBsAg and V5-CRL lip3 lipase respectively. The chemiluminescence was measured using the gel documentation system and the image was analysed using image J. The intensity obtained from the image was then plotted using Origin graphing software for curve fitting. The best curve fitting obtained was using the Hill model and further used for quantification of expressed HBsAg and V5-CRL lip3 lipase.

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Appendix II

DNA sequences

Gene sequence

Candida rugosa lip3 lipase DNA sequence

>X66006.1 *C.cylindracea* LIP3 gene

ATGCCGCCGGTGGGCAACCTCCGCTTCAAGGACCCTGTGCCGTACTCTGGCTC
GCTCAACGGCCAGAAGTTTACTCTGTACGGCCCGCTGTGCATGCAGCAGAAC
CCCGAGGGCACGTTTGAAGAGAACCTTGGCAAGACGGCACTCGACTTGGTGA
TGCAGTCCAAGGTGTTCCAGGCGGTGCTTCCCCAGAGTGAGGACTGCCTCAC
CATCAACGTGGTGCGGCCGCCGGGCACCAAGGCGGGCGCCAACCTCCCGGTC
ATGCTCTGGATCTTTGGCGGTGGGTTTGAGATCGGCAGCCCCACCATCTTCCC
TCCCGCCCAGATGGTCACCAAGAGTGTGCTCATGGGCAAGCCCATCATCCAC
GTGGCCGTCAACTACCGTGTTGCCTCGTGGGGGTTCTTGGCTGGTGATGACAT
CAAGGCCGAGGGCAGCGGGAACGCCGGCTTGAAGGACCAGCGTTTGGGCAT
GCAGTGGGTGGCAGACAACATTGCCGGGTTCGGCGGCGACCCGAGCAAGGTG
ACGATCTTTGGCGAGCTGGCGGGCAGCATGTCCGTGTTGTGCCACCTCATCTG
GAACGACGGCGACAACACGTACAAGGGCAAGCCGTTGTTCCGCGCGGGCATC
ATGCAGCTGGGAGCCATGGTGCCGCTGGACCCGGTGGACGGCACGTACGGCA
ACGAGATCTACGACCTCTTTGTCTCGAGTGCTGGCTGTGGCAGCGCCAGCGAC
AAGCTCGCGTGCTTGCGCAGTGCGCTGAGCGACACCTTGCTCGATGCCACCA
ACAACACTCCTGGGTTCTTGGCGTACTCCTCGTTGCGGTTGCTGTACCTTCCCC
GGCCCGACGGCAAGAACATCACCGATGACATGTACAAGTTGGTGCGCGACGG
CAAGTATGCAAGCGTTCCCGTGATCATTGGCGACCAGAACGACGAGGGCACC
ATCTTTGGTCTCCTGCTGTTGAACGTGACCACGAATGCTCAGGCCCGTGCTTA
CTTCAAGCAGCTGTTCATCCACGCCAGCGACGCGGAGATCGACACCTTGATG
GCGGCGTACCCCCAGGACATCACCCAGGGTCTGCCGTTCGACACCGGCATCT
TCAACGCAATCACCCCGCAGTTCAAGAGAATCCTGGCGGTGCTCGGCGACCT
TGCATTCATCCACGCCCGCCGCTACTTCCTCAACCACTTCCAGGGCGGCACCA
AGTACTCGTTCCTCCTGAAGCAGCTCCTGGGGTTGCCAATCATGGGCACCTTC
CATGCCAACGACATTGTGTGGCAGGACTACTTGTTGGGAAGCGGCAGCGTCA
TCTACAACAACGCGTTTATCGCGTTCGCCACCGACTTGGACCCCAACACCGCG
GGGTTGTTGGTGAACTGGCCCAAGTACACCAGCAGCCTGCAGCTGGGCAACA
ACTTGATGATGATCAACGCCTTGGGCTTGTACACCGGCAAGGACAACTTCCG
CACCGCTGGCTACGACGCGTTGATGACCAACCCGCTGCTGTTCTTTGTGTAA

Candida rugosa lip3 lipase amino acid sequence

>sp|P32947|LIP3_DIURU Lipase 3 OS=*Diutina rugosa* OX=5481 GN=LIP3 PE=1 SV=1

MKLALALSLIASVAAAPTAKLANGDTITGLNAIINEAFLGIPFAEPPVGNLRFKDP
VPYSGSLNGQKFTSYGPSCMQQNPEGTFEENLGKTALDLVMQSKVFQAVLPQSE
DCLTINVVRPPGTKAGANLPVMLWIFGGGFEIGSPTIFPPAQMVTKSVLMGKPIIH
VAVNYRVASWGFLAGDDIKAEGSGNAGLKDQRLGMQWVADNIAGFGGDPSKV
TIFGESAGSMSVLCHLIWNDGDNTYKGKPLFRAGIMQSGAMVPSDPVDGTYGNE
IYDLFVSSAGCGSASDKLACLRSASSDTLLDATNNTPGFLAYSSLRLSYLPRPDGK
NITDDMYKLVRDGKYASVPVIIGDQNDEGTIFGLSSLNVTTNAQARAYFKQSFIH
ASDAEIDTLMAAYPQDITQGSPFDTGIFNAITPQFKRISAVLGDLAFIHARRYFLNH
FQGGTKYSFLSKQLSGLPIMGTFHANDIVWQDYLLGSGSVIYNNAFIAFATDLDP
NTAGLLVNWPKYTSSSQSGNNLMMINALGLYTGKDNFRTAGYDALMTNPSSFF
V

HBsAg DNA sequence

>J02205.1 Hepatitis B virus surface antigen gene, complete cds

GGATCCCAGAGTCAGGGGTCTGTATCTTCCTGCTGGTGGCTCCAGTTCAGGAA
CAGTAAACCCTGCTCCGAATATTGCCTCTCACATCTCGTCAATCTCCGCGAGG
ACTGGGGACCCTGTGACGAACATGGAGAACATCACATCAGGATTCCTAGGAC
CCCTGCTCGTGTTACAGGCGGGGTTTTTCTTGTTGACAAGAATCCTCACAATA
CCGCAGAGTCTAGACTCGTGGTGGACTTCTCTCAATTTTCTAGGGGGATCTCC
CGTGTGTCTTGGCCAAAATTCGCAGTCCCCAACCTCCAATCACTCACCAACCT
CCTGTCCTCCAATTTGTCCTGGTTATCGCTGGATGTGTCTGCGGCGTTTTATCA
TATTCCTCTTCATCCTGCTGCTATGCCTCATCTTCTTATTGGTTCTTCTGGATTA
TCAAGGTATGTTGCCCGTTTGTCCTCTAATTCCAGGATCAACAACAACCAGTA
CGGGACCATGCAAAACCTGCACGACTCCTGCTCAAGGCAACTCTATGTTTCCC
TCATGTTGCTGTACAAAACCTACGGATGGAAATTGCACCTGTATTCCCATCCC
ATCGTCCTGGGCTTTCGCAAAATACCTATGGGAGTGGGCCTCAGTCCGTTTCT
CTTGGCTCAGTTTACTAGTGCCATTTGTTCAGTGGTTCGTAGGGCTTTCCCCCA
CTGTTTGGCTTTCAGCTATATGGATGATGTGGTATTGGGGGCCAAGTCTGTAC
AGCATCGTGAGTCCCTTTATACCGCTGTTACCAATTTTCTTTTGTCTCTGGGTA
TACATTTAAACCCTAACAAAACAAAAAGATGGGGTTATTCCCTAAACTTCAT
GGGCTACATAATTGGAAGTTGGGGAACTTTGCCACAGGATC

HBsAg amino acid sequence

>AAA45524.1 surface antigen [Hepatitis B virus]

MENITSGFLGPLLVLQAGFFLLTRILTIPQSLDSWWTSLNFLGGSPVCLGQNSQSP
TSNHSPTSCPPICPGYRWMCLRRFIIFLFILLLCLIFLLVLLDYQGMLPVCPLIPGST
TTSTGPCKTCTTPAQGNSMFPSCCCTKPTDGNCTCIPIPSSWAFAKYLWEWASVR
FSWLSLLVPFVQWFVGLSPTVWLSAIWMMWYWGPSLYSIVSPFIPLLPIFFCLWV
YI

PIR tag DNA sequence

ATGTACAGGAACTTAATAATTGCTACTGCCCTTACTTGCGGTGCATACAGTGC
CTACGTGCCTTCCGAACCATGGAGCACACTGACACCTGATGCTAGCCTTGAA
AGTGCCCTCAAAGATTACTCACAAACTTTTGGAATAGCTATTAAGTCCTTAGA
TGCCGACAAGATTAAGAGAGAG

PIR tag amino acid sequence

MYRNLIIATALTCGAYSAYVPSEPWSTLTPDASLESALKDYSQTFGIAIKSLDADK
IKRE

Simian virus V5 tag DNA sequence

GGTAAGCCTATCCCTAACCCTCTCCTCGGTCTCGATTCTACG

Simian virus V5 tag amino acid sequence

GKPIPNPLLGLDST

Promoter sequences

ENO1 promoter sequence

TGGCAAAAGGCAACCGTTATAAATGTGATCTTTCTTGGCAGTTATCTGTCAAT
TTTTCTAAGGAACAGTGAATTCATCATAGGAGAGATGTTATACGTTACATAAT
CATACATACTGCATGTATCTCACCTACTTTACCTCATCAACTCTAAAACAGTT
CTAGTCCCAACCCCAGATTCCTAGTCATGACACAAGTCCGCACCGGACAGGA

CTCACAACCAGCAAGAGAAGCTAACAAATTTACGCCCCGGTAAAACATTCTT
TAGGGGCCGTTCAATGGTAATTTTCCTCTCACCCGTTTAAACTTACCTCCGGG
CGGTATCTTCAATAACCTCTGTTGTCCCCGGGTATCATTGGAAACAGTGAGGG
ACGTTGAACAGAAGAGAGGATCACCGTAAATTTGCCTTGCAATTGGCCCTAA
CCACGGATGGTTAACTTCAAGCCATCACGACAGCAATTGAGTCGGCGCATAG
CTACCCTCCTCTTCTTGACCCCATGCATAGGACCAACCTTAACCGATGGAACA
GGTTCCTCCGCTCCGTCCCCTGGTAGTGTCTCTGCGCAAGAAATAGTTAAGGT
ATGAAGACTGATCTCTCGCACCCCCCTCACAGTACTGTTATGGTGAATTGACA
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CGAGCTTCGTACAGTCTCAATCTAGACGTCTGATAATGGCGTTTGTGCTCCTA
ATCGATGAGCCATCTCATGTGACGTCTATACGCTTCGATGGCTTCCGTCGCGA
ATATAGAACCACTTGAAATATGCTGCAAACCACGATCCACCCTGGTCCTGAA
AAGATATAAATACAGCACATCTAGCAGGCTTTTGTCTTCTTGGTTGAAACACA
CAATTATAACAATCTACATCTAAAAATGGCTATTTCAAAAGTATTTGCCCGTT
ACGTCTACGACTCCAGAGGAAACCCAACCGTTGAGGTTGACCTCTACACTG

THI11 promoter sequence

TTGCGCAAGTCAAGCAGAGTTTAATCTTTTCAGCTTCATCGTCAGTGATATTT
CTCAGCCCACAGACCAAGTCAACTTTGGAATCTAACAACCTTGTTCTTACAAT
GTTAGAACTCTTAAGTCGCATGCCATGATCTTCAAGCTGAATTTTGTGAAGGA
GGTCAAACCCCACAATGGCATCTAGTTGTTTAGAATACATGCCTTCGACAAGT
GTTTGAGTGTCCAAAATCAAGAGCTCAAAATTATTGAATTTGTCTGCCAATAA
CGCCGTAAATTGATTAGTGTCCAGCCCACCAACAATAGGAGCACCTATAGTT
AATTTTTCAGATAAATTTAAGTTATCAAGGTAAAGGAGCTCTAAGTTTACCCC
TTCCAACAGGGTTATTTGAGAACTCAATAAATTGTTGAATTCAAAACCAATTG
TCTTTGAATTCTCCACTGGAGCTTCCTTGCTGAAATTGATTTTGATACCATTGG
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AGTTCAGAAACTGGAATTGAGCTCAAAAAACTGGAATCGAGCGGATATTTGA
AGATTGATGCCTTACTCATGAATTGATTGATAAGAGCTCCGTGATTCACTCTG
TCAATGATTACCCCTCTCCTACCCGATTTGGGACTTTTTCTTCAGTCTTGGGGA
CTTTTTTTCATATGACTTGACCTTGCTTTCCCAATAGGGAAGGACTCACCCAT
GGATGATTAAGTTTGGATTACTCGTTTAGGAAATAGTAGCCATGAATCAATTT
GAATCATACCATCATGAAATAGGGTTAGGCTGTAAATGCCTCAAAAATGGCT
CTTGAGGCTGGATTTTTGGGTATTGGAATGTTGGTAGCAATTGGTATAAAAGG
CCATTTGTATTTCACTTTTTTGTCCTTCATACTTTACTCTTCTCAACTTTGGAAA
CTTCAATAAATCATC

Genscript-synthesised fragments

>>pTHI1_AOXTT_100217

AAAAGGATCCTTGCGCAAGTCAAGCAGAGTTTAATCTTTTCAGCTTCATCGTC
AGTGATATTTCTCAGCCCACAGACCAAGTCAACTTTGGAATCTAACAACCTTG
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