A SYSTEMATIC BIOPROCESSING APPROACH FOR THE CO-PRODUCTION OF ETHANOL AND SUCCINIC ACID FROM LIGNOCELLULOSIC FEEDSTOCK USING NOVEL YEAST STRAINS

A THESIS

Submitted in partial fulfillment of the
requirements for the award of the degree
of
Master of Science

by

Duddugunta Mohanchaitanya Reddy

Department of Biosciences and Biomedical Engineering
INDIAN INSTITUTE OF TECHNOLOGY INDORE
May 2023

INDIAN INSTITUTE OF TECHNOLOGY INDORE

CANDIDATE'S DECLARATION

I hereby certify that the work which is being presented in the thesis entitled **A SYSTEMATIC BIOPROCESSING APPROACH FOR THE CO-PRODUCTION OF ETHANOL AND SUCCINIC ACID FROM LIGNOCELLULOSIC FEEDSTOCK USING NOVEL YEAST STRAINS** in the partial fulfillment of the requirements for the award of the degree of **MASTER OF SCIENCE** and submitted in the **DEPARTMENT OF BIOSCIENCES AND BIOMEDICAL ENGINEERING, Indian Institute of Technology Indore**, is an authentic record of my own work carried out during the time period from Septempber 2021 to May 2023 under the supervision of Dr. Ganti Suryanarayana Murthy, Professor and Dr. Prashant kodgire, Professor.

The matter presented in this thesis has not been submitted by me for the award of any other degree of this or any other institute.

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Date:

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मातृ देवो भव।
पितृ देवो भव।
आचार्य देवो भव।

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Last, but not least, all I can say is that I will always be grateful to the almighty God for his grace.

Mohanchaitanya Reddy Duddugunta

DEDICATION

Dedicated to my mother, Varalakshmi.

Abstract

Saccharomyces cerevisiae consumes Glucose present in lignocellulosic biomass and converts it into ethanol. However, the utilization of five-carbon sugars, like xylose, would result in a 40% increase in product yield compared to utilizing Glucose alone. Much research has already been done on utilizing xylose by integrating xylose dehydrogenase and xylose reductase genes into the saccharomyces genome. However, product yield was lower compared to when utilizing Glucose alone. Several bottlenecks are identified using genome-scale dynamic flux balance analysis of metabolic pathways limiting ethanol production when grown on xylose and Glucose. Most recent studies showed this is due to the imbalance in reducing equivalents produced in respiratory pathways.

Our project aims at modifying *Saccharomyces cerevisiae* by integrating a set of five genes regulating the respiratory pathways of the organism to produce ethanol as well as succinate in significantly large amounts and to develop a systematic bioprocessing an approach that is technically feasible, economically viable, and environmentally low impact in a biorefinery level, which would result in producing more than 200g/L sugar titers after enzymatic hydrolysis and having more than 90% xylose consumption the efficiency of five- and six-carbon sugars and converting them into succinate and ethanol with an efficiency of more than 90%. This would result in about a 40% decrease in product cost.

Keywords:
Saccharomyces cerevisiae, dynamic Flux balance analysis, ethanol, succinate.

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Acronyms

LB Agar	Luria Bertini Agar
LB broth	Luria Bertini broth
MCT	Micro-centrifuge tube
LAF chamber	Laminar Air Flow chamber
$CaCl_2$	Calcium chloride
NEB	New England Biolabs
PCR	Polymerase chain reaction
YPD media	Yeast extract, peptone, and dextrose media
EDTA	Ethylenediaminetetraacetic acid.
Pfu	*Pyrococcus furiosus*
XDH	Xylitol dehydrogenase
pmoles	pico-moles
XR	Xylose reductase
XK	Xylulose kinase
PPP	Pentose phosphate pathway
FBA	Flux Balance Analysis
DFBA	Dynamic Flux Balance Analysis
CoBRA toolbox	Constraint-Based Reconstruction Analysis tool

Chapter 1

Introduction

1.1 Biofuels

Biofuels are fuels produced from biomass of organic matter derived from living or recently living organisms. Biomass of organic materials with high glucose content like corn, sugarcane, the biomass of algae, lignocellulosic biomass of rice straw, and wheat straw generates biofuels that can produce various types, including bioethanol, biodiesel, biogas, and biohydrogen. Biofuels are considered renewable energy sources because of their property to produce comparatively lower greenhouse gas emissions, having a higher octane number than gasoline, and being produced by organic biomass, making it an alternative renewable fuel source to non-renewable fossil fuels[1].

1.2 Current State of Biofuels in India

India is the third largest energy consumer in the world, and the government is trying to gain energy independence and replace non-renewable petroleum-based transport fuels with biofuels[2]. The Indian government has implemented several policies and initiatives to promote the production and use of biofuels, including the National Biofuels Policy (2020), which aims to promote the use of advanced biofuels and bio-compressed natural gas (CNG). The country has set a target of achieving 20% blending of ethanol with petrol and 5% blending of biodiesel with diesel by 2030.

1.3 Co-production of succinic acid with ethanol for sustainable Biorefineries

Saccharomyces cerevisiae is most the suitable model organism that has been used for fermentation as it can survive a wide range of pH and osmolarity, convenient for performing genetic modifications, and produces large amounts of bioethanol[3]. *Saccharomyces cerevisiae* consumes Glucose present in lignocellulosic biomass and converts it into ethanol. Nevertheless, consuming only Glucose is not enough for higher ethanol production. However, utilizing five-carbon sugars, like xylose, would result in a 40% increase in product yield compared to utilizing Glucose alone[4]. Hemicellulose in lignocellulosic feedstock contains around 30-40% of xylose[5]. Therefore, using lignocellulose as the substrate for the fermentation would increase the product yield. Much research has already been done on utilizing xylose by integrating xylose dehydrogenase and xylose reductase genes into the *Saccharomyces sp.* genome. Several bottlenecks are identified using genome-scale dynamic flux balance analysis of metabolic pathways limiting ethanol production when grown on xylose alone. Most recent studies showed this is due to the imbalance in reducing equivalents produced in respiratory pathways.

Moreover, ethanol production alone will not be economically viable at a biorefinery level because of the low-value commodity market and poor ethanol commercialization. Therefore, an integrated biorefinery approach of producing two or more products of different economic values is needed.

Succinic acid is among the 12 top highly valued chemicals that can potentially improve biorefineries' profitability[6]. Biobased succinic acid can be used as a platform chemical for producing various industrial chemicals, such as 1,4-butanediol, used in bioplastic production. Studies on techno-economic analysis at biorefinery levels show that the co-production of succinic acid leads to significant profitability[7], has a

global market size of USD 110.4 million, and is expected to expand at a 10.6% compound annual growth rate.

Therefore, our project aims at modifying *Saccharomyces cerevisiae* by integrating a set of five genes regulating the respiratory pathways of the organism to produce ethanol, as well as succinate in significantly large amounts and develop a systematic bioprocessing an approach that is technically feasible, economically viable, and environmentally low impact in a biorefinery level, which would result in producing more than 200g/L sugar titers after enzymatic hydrolysis and having more than 90% xylose consumption the efficiency of both five-carbon and six-carbon sugars and converting them into succinate and ethanol with an efficiency of more than 90%. This would result in about a 40% decrease in product cost.

Chapter 2

Background and literature review

In nature, *Scheffersomyces stipitis*, *Candida shehatae*, and *Spathaspora passalidarum* convert xylose to xylulose using two enzymes: xylose reductase and xylulose dehydrogenase. *Saccharomyces cerevisiae* cannot utilize xylose in the external environment but can convert xylulose to ethanol under fermentative conditions[8]. It has an internal metabolic pathway of converting xylulose-5-phosphate to ethanol via a non-oxidative pentose phosphate pathway and glycolysis with Ribulose-5-phosphate, Glyceraldehyde-3-phosphate, and pyruvate as intermediate metabolites in that pathway. However, the challenge was the utilization of xylose and converting it into xylulose-5-phosphate. A wide range of research has been done on engineering *Saccharomyces cerevisiae* to express the genes that encode the enzymes in converting xylose to xylulose-5-phosphate. A metabolic engineering strategy was put forth by Ho et al. to introduce a high copy number of shuttle plasmids called pLNH plasmids which had xylose reductase(*XR*), xylose dehydrogenase gene(*XDH*), and xylulose kinase(*XK*) collectively called as the XYL genes with efficient glycolytic promoters which made xylose non-fermenting *Saccharomyces cerevisiae* to xylose-fermenting *Saccharomyces cerevisiae* both in aerobic and a*nae*robic conditions without being induced by xylose or being repressed by high glucose[9].

However, in recombinant hosts with recombinant plasmids, there is a possibility for copy number variation, leading to different metabolic outputs at different times. These strains must constantly be maintained under selection pressure, which is not economically feasible at a biorefinery level. Therefore, genetic changes at a chromosomal level would increase the stability and longevity of the target gene's expression inside the host and remove the cost of antibiotics in maintaining the recombinant strains.

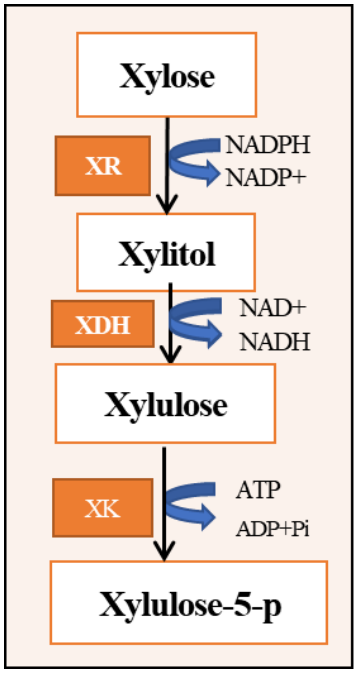


Figure 1 Representation of xylose uptake pathway mediated by XR, XDH, and XK

Several metabolic engineering strategies to address the challenges of xylose uptake, mainly inefficient xylose transport, NADH/NAD+, and NADPH/NADP+ imbalance due to variable co-factor specificity[10], increased xylitol formation, and xylose flux through the pentose phosphate pathway for efficient xylose fermentation to produce significant amounts of ethanol, were studied. Studies on Hxt1, Hxt2/4, Hxt5, Hxt6/7, and Gal3 transporter proteins in *S.cerevisiae*[11] the heterologous expression of xylose transporter from *Arabidopsis thaliana* in *S.cerevisiae*[12], kinetic modeling studies of glucose transporters during batch fermentations[13] and dynamic flux balance analysis studies(DFBA) on xylose fermentations[14] were revealed xylose transporters to be the most important for xylose uptake but are not the determining factor for the rate of xylose uptake. Apart from xylose uptake, converting xylose to xylulose and the downstream reactions are limiting bottlenecks for the growth of xylose[15]. Chromosomal integration and over-expressing XYL1 and XYL2 encoding xylose reductase and xylose dehydrogenase from a naturally xylose-fermenting yeast*: Pichia stipitis* and an endogenous XKS1 gene

encoding xylose kinase enzyme at *HIS3* locus of *S.cerevisiae* with CEN. PK 113-7A background showed a comparable increase in xylose uptake, and a reduction in xylitol formation, which is not present in *P. stipitis* was observed in *Saccharomyces cerevisiae*[16]. These downstream reactions are of the non-oxidative pentose phosphate pathway, which governs the flux of xylose fermentation[17, 18].

The enzymes of non-oxidative PPP were over-expressed in the xylose-fermenting *S. cerevisiae* TMB3001 strain. They showed increased xylulose fermentation rate but not of xylose, which suggested that xylose fermentation rates were controlled by reactions upstream of xylulokinase, whereas xylulose conversion rates by those of non-oxidative PPP[17, 19]. The former statement can be countered by the studies done by integrating a gene in the RWB217 strain of *S. cerevisiae* that encoded the xylose isomerase gene, which could substitute the function converting xylose to xylulose done by XDH and XR. However, the a*Nae*robic growth rates on xylose only reached 0.03 per hour[20]. These studies support that oxidative PPP is essential for regenerating NADPH for XR, which mediates xylose uptake under aerobic conditions. Recent studies show that overexpressing the downstream reactions after xylose uptake to allow xylitol to proceed into PPP instead of constraining them to prevent its formation, there was no significant increase in xylose utilization under a*nae*robic conditions, which shows that there is still an NAD+/NADH imbalance and if we proceed in the direction of aerobic fermentation then the ethanol yield might be negatively impacted[21]. Further studies of genome-scale DFBA modeling on *S.cerevisiae* suggested that NAD+/NADH balance can be increased by decreasing the succinate production inside the mitochondria, which stimulates its production via the glyoxylate pathway in the cytoplasm[22]

Chapter 3

Rationale, Hypothesis, and Objectives

In this project, we are trying to address the significant issues of producing renewable Bio-ethanol and Bio-succinic acid in environmentally sustainable and techno-economically feasible ways from lignocellulosic feedstock. This approach of producing both ethanol and succinic acid in the same bioreactor will reduce production costs and increases profitability compared to producing them individually.

A *Saccharomyces cerevisiae* strain with a xylose uptake pathway of XYL genes already integrated into its genome was taken for this project. To reduce NAD+/ NADPH imbalance during xylose utilization, in this project, we are aiming to integrate five genes with the strong promoter and terminator sequences that overexpress a pathway of reactions acting as a redox sink for the excess NADH, which limits the xylose utilization and at the same time produces succinate in large amounts. Among these five genes, four are native to *Saccharomyces cerevisiae*, and the fifth gene, a malate transporter, will be introduced from *Schizosaccharomyces pombe*, which helps export succinic acid. Additionally, to decrease the succinate production in mitochondria, we are aiming to delete the gene that regulates the conversion of D-Isocitrate to α-ketoglutarate and the intermediates of the citric acid cycle will be replenished by the glyoxylate shunt that takes place in the cytoplasm which can be used for cellular metabolic activities. The first four genes will be expressed in the cytoplasm, except the fifth gene, which gives malate transporter and will be localized on the cell wall of the yeast for succinate export.

A DFBA study is being done to analyze the dynamics of metabolic fluxes of the utilization of Glucose, xylose, and the production of ethanol and succinate.

Therefore, our project has two main objectives,

Objective 1: To perform and analyze the dynamics of metabolic fluxes for utilizing Glucose and xylose and the production of ethanol and succinate, which will help design further experiments.

Objective 2: To create a recombinant vector to perform chromosomal integration in *Saccharomyces cerevisiae* strain, enhancing the xylose uptake capability and co-production of ethanol and succinate in significant amounts.

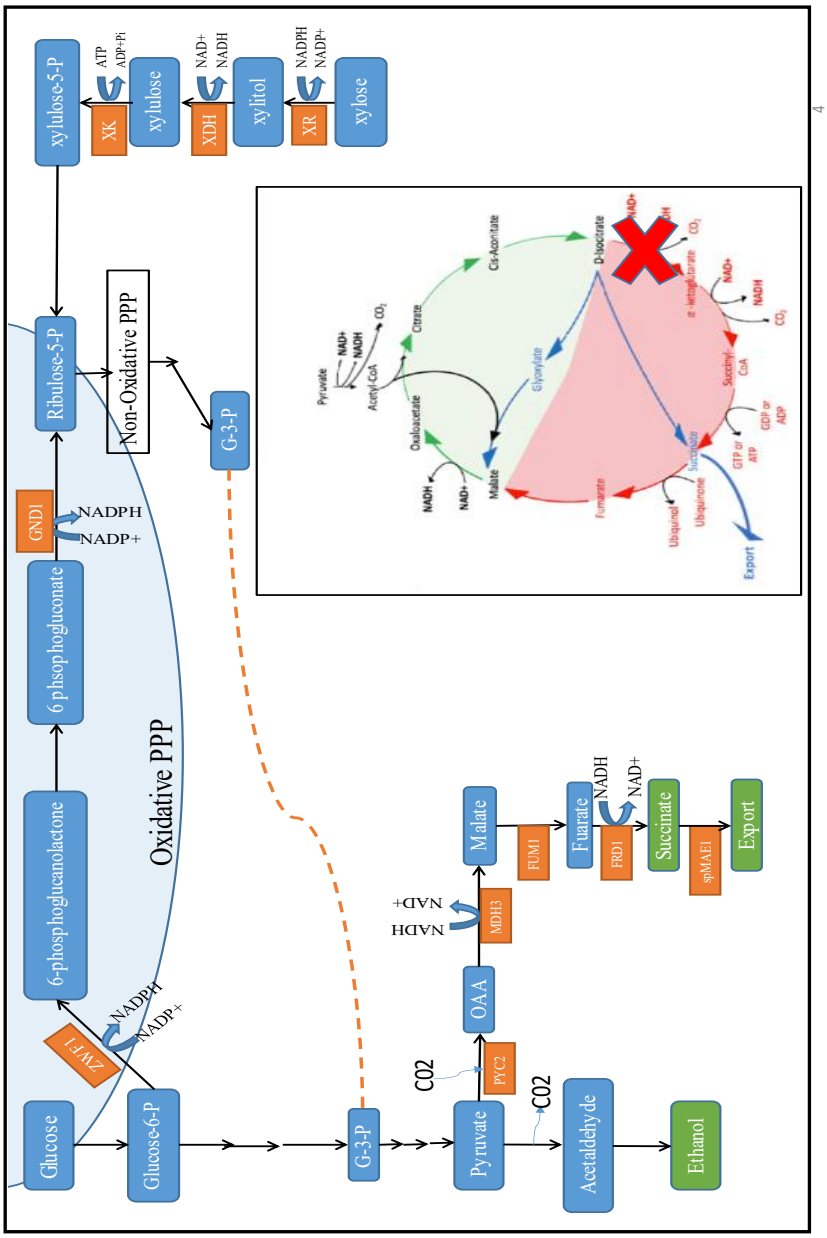


Figure 2 Representation of the proposed hypothesis

Chapter 4

Material and methods

4.1 Materials

4.1.1. Strains

Saccharomyces cerevisiae Strains listed in Table 4.1 were obtained from Dr. Ronald E. Hector's laboratory at USDA. All the strains were received on an aluminum foiled-wrapped filter paper with a small amount of dry media.

DH5α cells were used as host cells for maintaining and cloning pRS414, pRH1018, and recombinant vectors created in our lab.

4.1.2 Chemical Reagents and Kits

Agarose special (HiMedia), Luria Bertani broth (HiMedia), Luria Bertani agar (HiMedia), Ampicillin and kanamycin(HiMedia), G418-geneticin(TCI chemicals), Yeast extract, Peptone, Dextrose, Xylose, Restriction endonucleases – *Nae*I, *Xho*I, *Sca*I, *Hinc*II, *Ssp*I, *Sac*I (NEB), NEBuilder HiFi DNA assembly master mix(New England Biolabs), Taq DNA polymerase, *Pfu* DNA polymerase, dNTPs, Taq Buffer, HiProof Buffer, Ethidium bromide (Sigma Aldrich), Favor Prep plasmid extraction mini kit, Favorgen gel extraction/ PCR purification kit, Tris-Cl(Sigma Aldrich), $CaCl_2$ (HiMedia), Ethidium bromide (EtBr), Luria-Bertani agar (LB agar), Luria-Bertani broth (LB-broth), Glycerol ($C_3H_8O_3$), Methanol (CH_3OH).

4.2 Methods

4.2.1 Chemically competent *E. coli* cell preparation

A single isolated colony from a freshly streaked plate of DH5α cells is inoculated in 5 mL of LB broth and incubated at 37 °C and 200 rpm

overnight. 0.5 mL of this primary culture was added to 50 mL of freshly autoclaved LB broth at 37 °C and 200 rpm for 2.5 to 3 hours till the O.D. at 600nm reached 0.4 to 0.5. This culture was kept on ice, and all further steps were carried on ice strictly. The culture was distributed into two 50 mL centrifuge tubes and centrifuged at 4000 rpm for 10 minutes in a refrigerated centrifuge whose sample temperature was set at 4 °C. The supernatant was discarded, and the cell pellet was kept on ice in the LAF chamber. The cell pellet in each tube was resuspended with 5mL of 0.1M $CaCl_2$(Freshly prepared and autoclaved) and transferred into one single tube. This mixture was incubated on ice for 45 minutes and centrifuged at 2500 rpm for 10 minutes. Discarded the supernatant, gently resuspended the cell pellet with 2 mL of 0.1M CaCl2, and incubated on ice for 4 hours. To make 30% Glycerol stocks, 865 μL of 50% Glycerol(freshly prepared and autoclaved) was added to 2 mL of CaCl2 resuspended cells, 0.2 mL of this mixture was distributed into pre-cooled MCTs, and pipette tips and frozen at -80 °C till further use.

4.2.2 Plasmid recovery

Plasmids were received on filter paper wrapped in aluminum foil. The filter paper was carefully placed into an MCT using sterile forceps, and 40 μL of the freshly prepared, autoclaved 1X TE buffer(pH 8.0) was added. The MCT was then incubated in a 37 °C water bath for 20 minutes. A 2 μL volume was taken from the tube and transformed into DH5α competent cells, plated on the L.B. Agar plates with appropriate selective media for a different plasmid that we received, and one of the colonies was inoculated into a 5 mL LB broth, incubated at 37°C, 220 rpm, and the plasmid were isolated next morning and checked on the 1% Agarose gel using agarose gel electrophoresis. Restriction analysis was also done after plasmid isolation to check whether we got the bands of the expected desired length.

Plasmid Name	Description
pRS414	A Yeast centromere vector with a TRP1 marker, Ampicillin resistance, and an MCS derived from pBLUESCRIPT II.

Table 4.1 Plasmid used for constructing recombinant plasmid.

4.2.3 Plamsid isolation

The plasmid was borrowed from Dr. Ronald Hector's USDA lab and was first transformed into competent *E. coli* cells of the DH5α strain. The transformation mixture was plated on LB agar plates with the appropriate antibiotic. The positive colonies were grown in LB broth and incubated at 37 °C and 200 rpm overnight. The plasmid isolation was done using the Favorgen plasmid extraction mini-prep kit. The plasmid was isolated from 4.5 mL of well-grown bacterial culture distributed in three MCTs and centrifuged at 11000 x g for 10 minutes to pellet down the cells. The pellet was resuspended with 200μL of FAPD1 buffer containing RNase A by pipetting up and down. 200μL FAPD2 buffer was added to the resuspended cells, inverted very gently, and incubated at room temperature for 2 to 3 minutes to lyse the cells. To neutralize cell lysate, 300μL of FAPD3 buffer was added and inverted 10 to 15 times immediately. These tubes were centrifuged at 18500 x g for 5 minutes to clear the cell lysate. Using 200μL tips, the supernatant was carefully transferred into the FAPD column, kept in a collection tube, and centrifuged again at 11000 x g for 30 seconds. The flowthrough was discarded, 300μL of WP buffer was added to the FAPD column carefully without touching the column, and centrifuged at 11000 xg for 30 seconds. The flowthrough was discarded, and 700μL of wash

buffer(Ethanol added) was added, followed by centrifugation for 30 seconds at 11000 x g. The flowthrough was discarded. It was dried by centrifuging at 18500 x g for 4 minutes to ensure no remanent ethanol in the column. The FAPD column was then placed into a fresh microcentrifuge tube, properly labeled, and the 50 µl of elution buffer(pre-heated at 60°C for maximum yield) was added drop-by-drop to the center of the silica membrane in a column without touching it with the tip, centrifuged at 18000 x g for 2 minutes, the column was discarded, and MCT tube containing desired plasmid was stored at 4°C.

4.2.4 Agarose gel electrophoresis

Nucleic acids like DNA and RNA can be separated via agarose gel electrophoresis, in which agarose gel of appropriate concentration is prepared. The agarose matrix will separate the DNA sample on applying voltage. The shorter molecule travels the larger distance in the agarose gel, and the larger molecule travels the shortest distance. For preparing wells into the gel, molten agarose with a fluorescent dye Ethidium bromide (EtBr) for staining the sample was poured into the casting tray of the electrophoresis unit and allowed to solidify. EtBr intercalates into the rings of DNA, which can be seen under UV The electrophoresis unit was filled with the 1X TAE buffer, and the samples were mixed into the loading dye and then loaded into the wells. The loading dye contains glycerol to give density to the sample and bromophenol blue to track the movement of the sample into the gel. Electric fields were then applied to separate the sample of different sizes from the mixture. The run gel was then analyzed under a transilluminator.

4.2.5. Sequential restriction digestion

7 to 8 µg of freshly isolated pRS414 plasmid was sequentially digested with one µl each of *Xho*I and *Nae*I restriction enzymes by incubating the reaction mixture at 37 °C for 2 hours or more till complete digestion was achieved. The reaction mixture composition is given in the table. The progress of digestion was checked by agarose gel electrophoresis.

After complete digestion, the enzymes in the reaction mixture were heat inactivated at 65 °C and proceeded for gel elution

4.2.6. Gel elution

After restriction digestion and heat inactivation of the reaction mixture, it was loaded into 0.8% agarose gel and ran at 60V for about 2 hours till a clear and distinct band was resolved. Around 300 grams of the gel containing the desired resolved band is cut into tiny pieces and weighed in an MCT. Further steps in gel elution were carried out using FavorPrep™ GEL/PCR Purification Kit. 0.5 mL of FADF buffer was added to 300 mg gel slices and incubated at 55 °C in a water bath for 15 minutes by mixing the mixture by tapping the MCT every 3-5 minutes to ensure the complete dissolution of gel in FADF buffer. This mixture cooled to room temperature, transferred into the FADF column in a collection tube, and centrifuged at 11,000 x g for 30 seconds. The supernatant was discarded, and 0.75 mL of wash buffer(ethanol added previously) was added to the column by the walls and centrifuged at 11,000 x g for 30 seconds. The column was dried by centrifuging at 18,500 x g for 3 minutes to ensure no remnant ethanol was in the column. The flowthrough was discarded along with the collection tube, and the column was transferred 30 µl of elution buffer was added drop by drop to the center of the silica membrane in the column without touching it and centrifuged at 18,000 x g for 2 minutes. This was stored at 4 °C till further use.

4.2.7 Genomic DNA isolation.

This experiment is carried out in three steps:

1. Harvesting the cells from the liquid culture
2. Cell wall lysis and crude DNA extraction
3. Genomic DNA purification and quantification

4.2.7.1 Harvesting the cells from liquid culture:

Saccharomyces cerevisiae strain was grown on YPD agar and a single colony was inoculated from the plate into YPD media(yeast extract:1%

w/v, peptone:2% w/v, and Dextrose:2% w/v), incubated at 30 °C at 200rpm for 22 hours. The liquid culture was centrifuged at 3600rpm at 4 °C for 10 minutes, and the pellet was resuspended in distilled water and centrifuged again at 3600rpm for 10 minutes to get the cell pellet. This cell pellet was kept on ice.

4.2.7.2 Cell wall lysis and crude DNA extraction:

The cell pellet was resuspended in a lysis buffer. 450-600 μm glass beads were added to the resuspended cells and vortexed on a vortexer for 10 minutes to mechanically lyse the cells with the shear force of glass beads. After a white slurry-like solution was observed, Tris-EDTA buffer was added to solubilize the nucleic acid and protect it from DNases. This mixture is centrifuged at 18000 x g for 10 minutes at room temperature, and around 400μL of clear supernatant is transferred into a microcentrifuge tube. 1 mL of ice-cold ethanol is added to the mixture and inverted 5-10 times to precipitate the DNA. This mixture was centrifuged at 18,000 x g for 10 minutes at room temperature, and the pellet was washed with 70% ethanol and air-dried until the smell of ethanol was gone from the tube. Finally, the dried pellet is resuspended in 500 μL Tris-EDTA buffer.

4.2.7.3 Genomic DNA purification and quantification:

RNase A pretreatment is given to the resuspended pellet by adding 2 mg/mL RNase A and incubating at 37 °C for 30 minutes to degrade the RNA in the mixture. A 500 μL of phenol-chloroform-isoamyl alcohol(25:24:1) is added, vortexed for 5 minutes on a multi-tube vortexer, and centrifuged at 18000 x g at room temperature for 10 minutes. This step made DNA partitioned into the aqueous phase and lipids and other cell debris into the organic phase. The aqueous layer is transferred into another fresh microcentrifuge tube and treated with 4M ammonium acetate and ice-cold ethanol, and inverted to mix to precipitate the proteins and DNA. The whole mixture was incubated at

-20 °C for two hours to allow the maximum amount of DNA to be precipitated. After two hours of incubation, the mixture is centrifuged at maximum speed for 10 minutes, and the pellet is washed with 70% ethanol and air-dried until the smell of ethanol is entirely gone from the tube. The air-dried pellet was resuspended in 100 μL of Tris-EDTA buffer and stored at -20 °C for using it as a template for PCR amplification reactions.

4.2.8. Primer design for recombinant plasmid construction

All inserts are amplified with appropriate overlap regions/homology regions to assemble the multiple inserts into a vector. The primers were designed with nucleotide overhangs with 25 base pair overlaps with a melting temperature of over 48 °C to ensure correct insert annealing. Primers were designed to split the overlap region between the reverse primer of one insert and the forward primer of the next insert. After PCR, the resultant insert includes an overlap region of 25 base pairs. The pRS414 Vector, a shuttle vector, was digested with *Nae*I (at 1568th position; 5'GCC-GGC 3') and *Xho*I (at 1909th position; 5' C-TCGAG 3'). The forward primer of the first insert(ScTEF1 in case of first integration construct) and reverse primer of the last insert (DIT1 terminator in case of first integration construct) are designed to regenerate these restriction sites for cutting the whole insert from vector to use in the next cloning step. The list of long primers for the amplification of each fragment is given in the Appendix.

4.2.9 Polymerase chain reaction

Pfu DNA Polymerase amplified The template DNA using Forward and Reverse gene-specific primers. The master mix was prepared using primers, 1.5 mM MgCl2, 0.2 mM dNTPs, template DNA, and Pfu DNA polymerase. The master mix was aliquot into PCR tubes. The negative control was prepared similarly but without template DNA. The program

of thermocycler was set for a PCR reaction with an initial denaturation at 95C °C followed by 30 cycles of denaturation at 95 °C for 2 minutes and 30 seconds, respectively, annealing at the temperature according to primers for 30 seconds and extension at 72 °C for a time depending on the length of the gene of interest. Finally, the final extension was set at 72 °C for 10 minutes. The PCR products obtained after the reaction were estimated by agarose gel electrophoresis.

4.2.10 Insert preparation

All inserts(genes, promoters, and terminator sequences) were amplified using *Pfu* DNA polymerase with *Saccharomyces cerevisiae* genomic DNA isolated from the YRH396 strain as the template in the PCR reaction. These inserts were PCR purified and stored at 4 °C till further use. The PCR programs for the amplification of each gene are given below:

	Temperature	**Time**
Initial Denaturation	95 °C	5 Minutes
Denaturation	92 °C	30 seconds
Annealing	54 °C	30 seconds
Extension	72 °C	4minutes 15 seconds
Final extension	72 °C	10 minutes

Table 4.1 PCR program for the amplification of the PYC2 gene.

The PYC2 gene is of size 3.543kb. s_PYC2_F, s_PYC2_R are the short primers, and L_PYC2_F, L_PYC2_R are the long primers used for the amplification

	Temperature	Time
Initial Denaturation	95 °C	5 Minutes
Denaturation	92 °C	30 seconds
Annealing	50.5 °C	30 seconds
Extension	72 °C	67 seconds
Final extension	72 °C	10 minutes

Table 4.2 PCR program for the amplification of the MDH3 gene.

The MDH3 gene is of size 1.302 kb. s_MDH3_F, s_MDH3_R are the short primers, and L_MDH3_F, L_MDH3_R are the long primers used for the amplification

	Temperature	Time
Initial Denaturation	95 °C	5 Minutes
Denaturation	92 °C	30 seconds
Annealing	47 °C	30 seconds
Extension	72 °C	34 seconds
Final extension	72 °C	10 minutes

Table 4.3 PCR program for the amplification of the ScTEF1 promoter.

The ScTEF1 Promoter is of size 511bp. s_ScTEF1_F, s_ScTEF1_R are the short primers, and L_ScTEF1_F, L_ScTEF1_R are the long primers used for the amplification.

	Temperature	Time
Initial Denaturation	95 °C	5 Minutes
Denaturation	92 °C	30 seconds
Annealing	46.3 °C	30 seconds
Extension	72 °C	45 seconds
Final extension	72 °C	10 minutes

Table 4.4 PCR program for the amplification of the TDH3 promoter.

The TDH3 Promoter is of size 681bp. s_TDH3_F, s_TDH3_R are the short primers, and L_TDH3_F, L_TDH3_R are the long primers used for the amplification.

	Temperature	Time
Initial Denaturation	95 °C	5 Minutes
Denaturation	92 °C	30 seconds
Annealing	55.7 °C	30 seconds
Extension	72 °C	30 seconds
Final extension	72 °C	10 minutes

Table 4.5 PCR program for the amplification of the RPL41B terminator.

The RPL41B Terminator is of size 454bp. s_RPL41B_F, s_RPL41B_R are the short primers, and L_RPL41B_F, L_RPL41B_R are the long primers used for the amplification.

	Temperature	Time
Initial Denaturation	95 °C	5 Minutes
Denaturation	92 °C	30 seconds
Annealing	49.8 °C	30 seconds
Extension	72 °C	24 seconds
Final extension	72 °C	10 minutes

Table 4.6 PCR program for the amplification of the DIT1 terminator.

The DIT1 terminator is of size 434bp. s_DIT1_F, s_DIT1_R are the short primers, and L_DIT1_F, L_DIT1_R are the long primers used for the amplification.

4.2.11. PCR purification

After PCR amplification, all inserts were PCR purified using FavorPrep GEL/PCR Purification Mini Kit. Five times the volume of the PCR reaction mix of FADF solution is added to the PCR reaction mixture. The mixture was transferred into the FADF column, placed in a collection tube, mixed by tapping the tube 15-20 times, and centrifuged for 30 seconds. The flowthrough was discarded from the collection tube, and the column was placed back into the collection tube. 0.75 mL of wash buffer(ethanol added) was added into the FADF column by the walls of the column and centrifuged at 11,000 x g for 30 seconds. Flowthrough was discarded, and the column was dried by centrifuging at 18,500 x g for 3 minutes. Flowthrough was discarded, the FADF column was transferred to a fresh MCT, and 30 µL of elution buffer was added drop-wise onto the center of the silica membrane without touching it, centrifuged for 2 minutes at 18,000 x g and stored at 4 °C until further use.

4.2.12 Recombinant plasmid construction.

NEBuilder Hifi DNA Assembly master mix was used to assemble vector and multiple fragments of inserts in a single run with appropriate positive control. This method allowed us to assemble different sizes of DNA fragments with overlaps (25 bp). The reaction includes different enzymes that work together in the same buffer:

The exonuclease creates single-stranded 3' overhangs that facilitate the annealing of fragments that share complementarity at one end (the overlap region). The polymerase fills in gaps within each annealed fragment, and the DNA ligase seals nicks in the assembled DNA. The result is a double-stranded, fully sealed recombinant DNA molecule.

pmol = (weight in ng) x 1,000 / (base pairs x 650 daltons)

Equation 4.1 Formula for calculating the amount of DNA in pico moles to design the vector and insert assembly

A 20 µl reaction mixture was prepared by adding pRS414(sequential double digested using *Xho*I and *Nae*I) along with PCR purified inserts in a ratio of 1:1 with an overall DNA concentration of 5 pico moles and 10 µL of the NEBuilder Hifi DNA master mix was added to it. The detailed reaction volume of each component is given in the table. The reaction mixture was incubated at 50 °C for one hour and transformed into chemically competent DH5α *E. coli* cells.

	Length(in bp)	**Concentration**	**Volume (in μL)**	**The total amount in ng**	**DNA in pmol**
ScTEF1p	511	90	0.3	27	0.08
PYC2	3570	90	1.9	171	0.07
RPL41Bt	483	90	0.25	22.5	0.07
TDH3p	684	90	0.36	32.4	0.07
MDH3	1032	90	0.55	49.5	0.07
DIT1t	476	35	0.65	22.75	0.07
pRS414	4447	100	2.1	210	0.07
		Total volume (in μL)	**6.11**	Total DNA(in pmols)	**0.51**

Table 4.7 Inserts and vector concentrations calculated according to the assembly guidelines.

4.2.13. Transformation of *E. coli* DH5α cells.

The vail containing 200 μL of chemically competent DH5α cells of *E. coli* was taken from the -80 °C fridge and kept on ice for 5 minutes. 2 μL of the Vector and insert assembly mixture was added to the vial and mixed gently by pipetting up and down 3-5 times on the ice and incubating on ice for 30 minutes. A heat-shock treatment was given to these cells at 42 °C for 90 seconds, transferred immediately onto the ice, and incubated for 2 minutes. 800 μL of LB broth was added to the vial and incubated at 37 °C and 220 rpm for 90 minutes. After incubation, 100 μL of the overgrown culture from the vial was plated on the LB/ampicillin plates and incubated at 37 °C overnight.

4.2.14. Strain revival

The strains were received on a sterile filter paper wrapped in aluminum foil along with a small amount of media on the filter paper. The filter paper was kept on the agar plate, and 20 μL of sterile water was added on top of it. Incubate it at 30 °C overnight till visible colonies were observed. One of the colonies was inoculated into a 5 mL YPD media and incubated at 30 °C, 200 rpm overnight, and 15 % Glycerol stocks were prepared using this overnight incubate culture when the O.D600 reached 0.8 and frozen at -80 °C

Chapter 5

In-silico model testing of *Saccharomyces cerevisiae* using DFBA.

5.1 Introduction to FBA and DFBA:

Flux balance analysis is a mathematical approach to analyzing the flow of metabolites through a metabolic network[22]. It is based on the steady-state and mass balance principle, where metabolites' production rate must equal their consumption rate. FBA assumes that the cell optimizes a particular objective function, such as maximizing biomass production while satisfying all the stoichiometric constraints of the metabolic network. FBA ensures that the metabolic fluxes (reactions) are balanced around each node (metabolite). When the metabolic network is operating in a steady state, the mass balances are described by a set of linear equations,

$$\mathbf{S} \cdot \boldsymbol{v} = \mathbf{0}$$

Equation 5.1 Mass balance constraint imposed in FBA, which ensures steady state

where S is the m x n stoichiometric matrix of the reactions,m is the number of metabolites, n is the number of fluxes, and v is the flux vector of the network. Since there are more unknown fluxes than metabolites, an objective function formulated as a linear programming problem will be used to obtain a solution using a linear programming solver. COBRA(Constraint-based reconstruction analysis) toolbox takes care of solving the objective function and gives an optimized solution for FBA and DFBA[23]

Dynamic Flux Balance Analysis (DFBA) extends FBA by considering the dynamic behavior of metabolic networks at different time points. It incorporates the dynamics of metabolic reactions and transport processes, allowing for a more accurate representation of cellular behavior. This approach enables the simulation of time-dependent

metabolic fluxes and the prediction of how the metabolic network responds to external environmental changes and hence optimizes the performance of metabolic networks under these dynamic conditions[18]

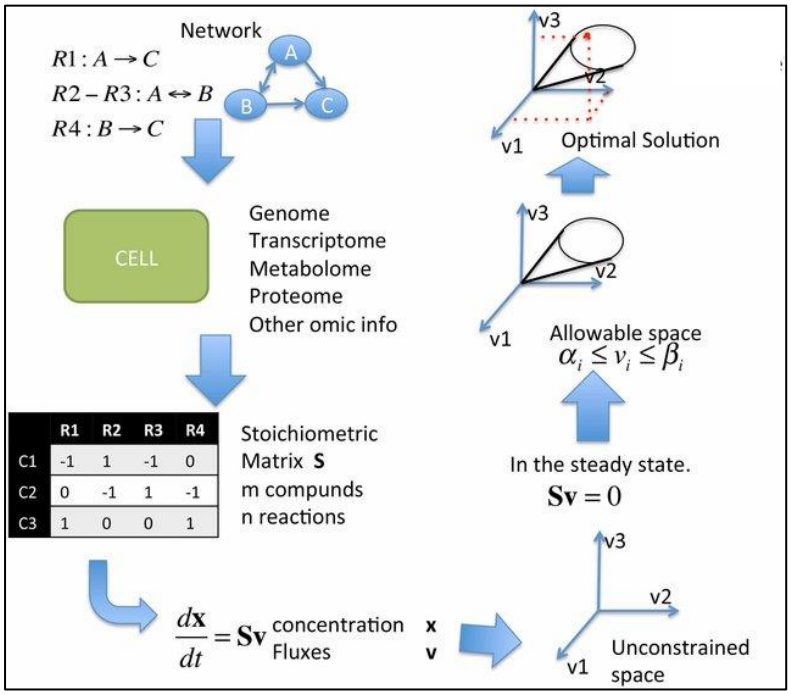


Figure 5.1 An illustration of Flux balance analysis[24]

5.2 Model testing

5.2.1 Basic model structure

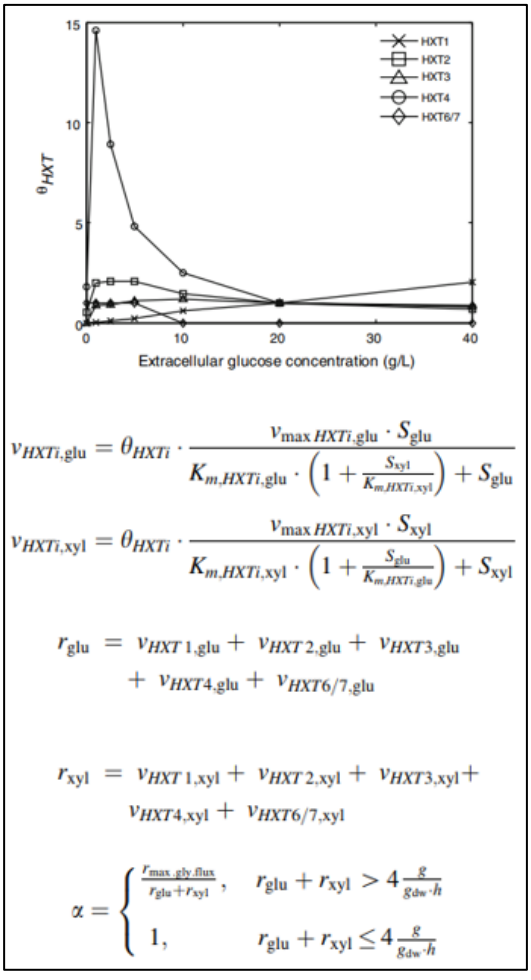
In this project, we used the iMM904 in-silico genome-scale metabolic model of S. *cerevisiae* that was developed by integrating data from various sources and high-throughput experimental data. The model iMM904 has 904 genes and 1,412 metabolic reactions and was used in this study to connect extracellular metabolomic measurements to intracellular flux states in yeast for predicting intracellular fluxes and identifying metabolic changes under different conditions[23].

Here we customized the model to run DFBA by including a loop that runs the FBA every 30 seconds. After each run, the flux bounds will be updated based on the results of the FBA solution in the preceding

iteration. This instantaneous objective function results in better predictions than a terminal-type objective function. In this study, we ran each DFBA for 48 hours.

5.2.2 Sugar Transport and media composition

The transport fluxes of Glucose and xylose were calculated based on the method developed by Bertilsson et al. [16]. According to this model, only Glucose and xylose are transported by HXT family transporters. Moreover, the transport rate by each transporter is decided by their relative expression level, which is given by θ_{HXTi} in the model equations. The total uptake of both xylose and Glucose is given in the Figure 5.2.



$$v_{HXTi,\mathrm{glu}} = \theta_{HXTi} \cdot \frac{v_{\max HXTi,\mathrm{glu}} \cdot S_{\mathrm{glu}}}{K_{m,HXTi,\mathrm{glu}} \cdot \left(1 + \frac{S_{\mathrm{xyl}}}{K_{m,HXTi,\mathrm{xyl}}}\right) + S_{\mathrm{glu}}}$$

$$v_{HXTi,\mathrm{xyl}} = \theta_{HXTi} \cdot \frac{v_{\max HXTi,\mathrm{xyl}} \cdot S_{\mathrm{xyl}}}{K_{m,HXTi,\mathrm{xyl}} \cdot \left(1 + \frac{S_{\mathrm{glu}}}{K_{m,HXTi,\mathrm{glu}}}\right) + S_{\mathrm{xyl}}}$$

$$r_{\mathrm{glu}} = v_{HXT1,\mathrm{glu}} + v_{HXT2,\mathrm{glu}} + v_{HXT3,\mathrm{glu}} + v_{HXT4,\mathrm{glu}} + v_{HXT6/7,\mathrm{glu}}$$

$$r_{\mathrm{xyl}} = v_{HXT1,\mathrm{xyl}} + v_{HXT2,\mathrm{xyl}} + v_{HXT3,\mathrm{xyl}} + v_{HXT4,\mathrm{xyl}} + v_{HXT6/7,\mathrm{xyl}}$$

$$\alpha = \begin{cases} \frac{r_{\max\ \mathrm{gly\ flux}}}{r_{\mathrm{glu}} + r_{\mathrm{xyl}}}, & r_{\mathrm{glu}} + r_{\mathrm{xyl}} > 4\frac{g}{g_{dw} \cdot h} \\ 1, & r_{\mathrm{glu}} + r_{\mathrm{xyl}} \leq 4\frac{g}{g_{dw} \cdot h} \end{cases}$$

Figure 5.2 HXT transporters kinetics given by Bertilson et al.[13]

As we run DFBA, the media composition may change at each iteration. Therefore, we should keep track of that dynamic media composition, including Glucose, xylose, xylitol, ethanol, acetic acid, succinic acid,

lactic acid, and glycerol. This was accomplished by the inclusion of rate-of-change equations in the form of Equation 5.2

$$\frac{dM}{dt} = r_M X dt$$

Equation 5.2 Equation for calculating the rate of change of metabolites during DFBA

Here, M is the tracked metabolite, and r_M is the metabolite's specific consumption or production rate. For this, we took the model developed by Hohenschuh et al.[22]. Further testing is also done on the same model.

5.2.3 Model testing with pure and mixed sugar concentrations

We simulated the model with pure and mixed sugars of different concentrations to analyze the metabolite fluxes of succinate, ethanol, xylitol, xylose, glucose, and others. With pure sugars, either glucose or xylose is given as a sole carbon source. With mixed sugars, glucose and xylose of different ratios are given as carbon sources for the model. This checks the dynamics of xylose uptake at different concentrations and whether the model would produce succinate and ethanol when provided with xylose alone.

5.2.4 Model testing for xylose uptake and succinate and ethanol production.

To know the reactions inside the model that limit the succinate production while utilizing xylose, we tested the model by turning on and off different genes involved in the succinate production.

A symport reaction and an extracellular export reaction were identified in the model, regulating the succinate export. The symport reaction transported succinate and a proton between the extracellular matrix and cytosol. The extracellular export reaction was exporting the succinate out from the extracellular matrix. The two reactions were turned off one at a time, and observed which reaction was regulating the succinate export in a significant way which is given in Table 5.1.

	Reaction I.D.	**Reaction formula**
Symort reaction	**SUCCt2r**	H^+[c] + succinate[c] <=> H^+[e] + succinate[e]
Extracellular exchange reaction	**EX_succ_e**	Succinate[e] --->

Table 5.1 Symport and extracellular reactions of succinate export and their reaction I.Ds and their reaction formulae as per the iMM904 model.

Twelve reactions were identified to produce succinate in both mitochondria and cytosol. All these genes were turned off except one at a time and observed for succinate production. By doing this, we can observe which reaction would limit the succinate production if the gene is turned off. When such a reaction is identified, we could say that the reaction is significantly responsible for succinate production. All these twelve genes are given in Table 5.2.

	Reaction I.D.	Reaction formula
Succinate production in the cytosol	**SHSL4r**	H2O[c] + succinate semialdehyde[c] <=> 2-oxobutanoate[c] + H[c] + NH4[c] + succinate[c]
	SHSL1	L-cysteine[c] + succinate semialdehyde[c] <=> L-cystine[c] + H[c] + succinate[c]
	TAUDO	2-Oxoglutarate[c] + O2[c] + taurine[c] <=> acetate[c] + CO2[c] + H[c] + sulfite[c] + succinate[c]
	FRDcm	FADH2[c] + Fumarate[c] <=> FAD[c] + succinate[c]
	ICL	Isocitrate[c] <=> Glyoxylate[c] + succinate[c]
Succinate production in mitochondria	**SUCOASm**	ATP[m] + CoA[m] + succinate[m] <=> ADP[m] + Phosphopantetheine[m] + succinyl-CoA[m]
	SUCFUMtm	Fumarate[m] + succinate[c] <=> fumarate[c] + succinate[m]
	SUCD2_ufm	succinate[m] + ubiquinone-6[m] <=> fumarate[m] + ubiquinol-6[m]
	SUCD1m	FAD[m] + succinate[m] <=> FADH2[m] + fumarate[m]
	SUCCtm	succinate[c] + Pi[m] <=> succinate[m] + Pi[c]
	MCITL2m	Malate/Isocitrate[c] <=> succinate[m] + Pyruvate[m]
	FRDm	FADH2[m] + Fumarate[m] <=> FAD[m] + succinate[m]

Table 5.2 Succinate production reactions along with their reaction I.Dsand their reaction formulae as per the iMM904 model.

The downstream reactions directing the flux towards succinate production were also identified, and the model was tested similarly for succinate export and succinate production. Among these reactions, the first one is involved in fumarate production, which is the substrate in the FRDcm reaction, which is shown to regulate succinate production. The second involves malate production, and the third is oxaloacetate production. These reactions are given in table 5.3.

	Reaction I.D.	**Reaction formula**
Fumarate production	**FUM**	Malate [c] <=> Fumarate[c] + H_2O[c]
Malate production	**MDH**	H+[c] + OAA[c] + NADH[c] <=> Malate[c] + NAD^+[c]
Oxaloacetate production	**PC**	ATP[c] + HCO_3^-[c] + Pyruvate[c] -> ADP[c] + H^+[c] + OAA[c] + Pi[c]

Table 5.3 The downstream reactions that direct the flux to the succinate production identified after performing DFBA, along with their reaction I.Ds and their reaction formulae as per the iMM904 model.

5.3 Results and Discussion.

5.3.1 Model testing with pure and mixed sugar concentrations

The results showed that when xylose is only sugar provided, there was no significant xylose utilization, and also, there was no significant amount of ethanol or succinate production. This is shown in Figures 5.3, 5.4 and 5.5

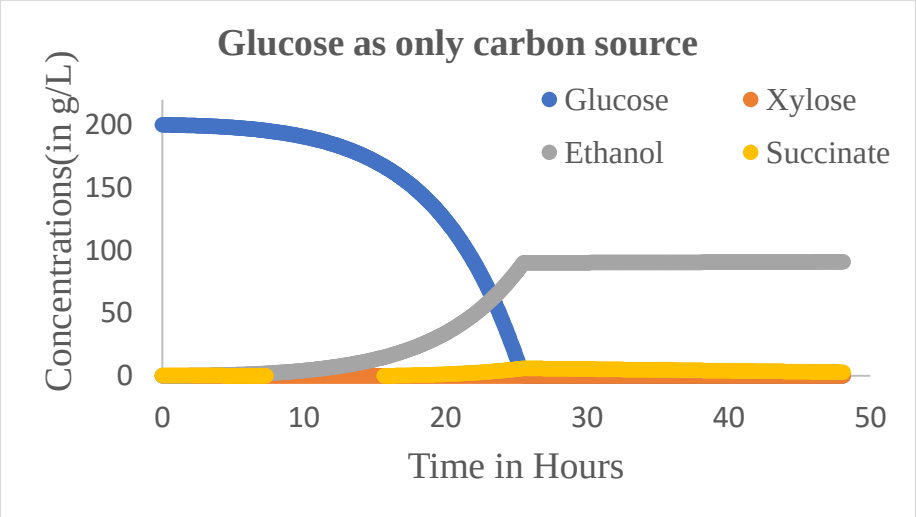


Figure 5.3 DFBA simulation of fermentation with Glucose as the sole carbon source

This data supports the study made by Bertilsson et al.[13]that in the absence of glucose, xylose uptake is significantly slower than glucose uptake due to the lower affinity of the sugar transporters for xylose compared to glucose

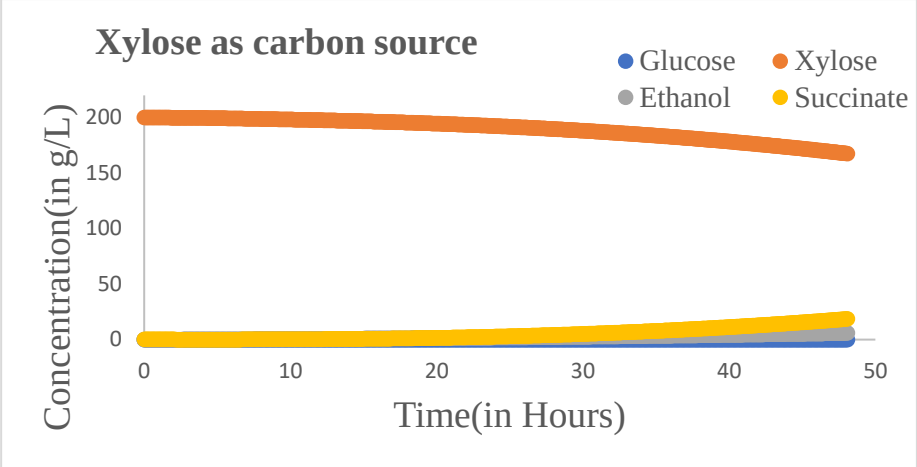


Figure 5.4 DFBA simulation of fermentation with xylose as the sole carbon source

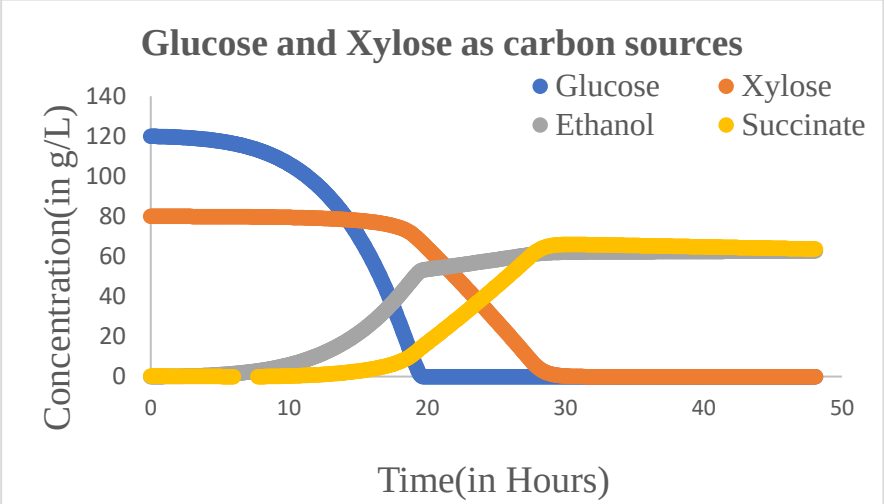


Figure 5.5 DFBA simulation of fermentation with glucose and xylose as carbon sources.

Production rates of glucose and xylose and utilization rates of ethanol and succinate at different pure and mixed sugar concentrations are given in Figure 5.6.

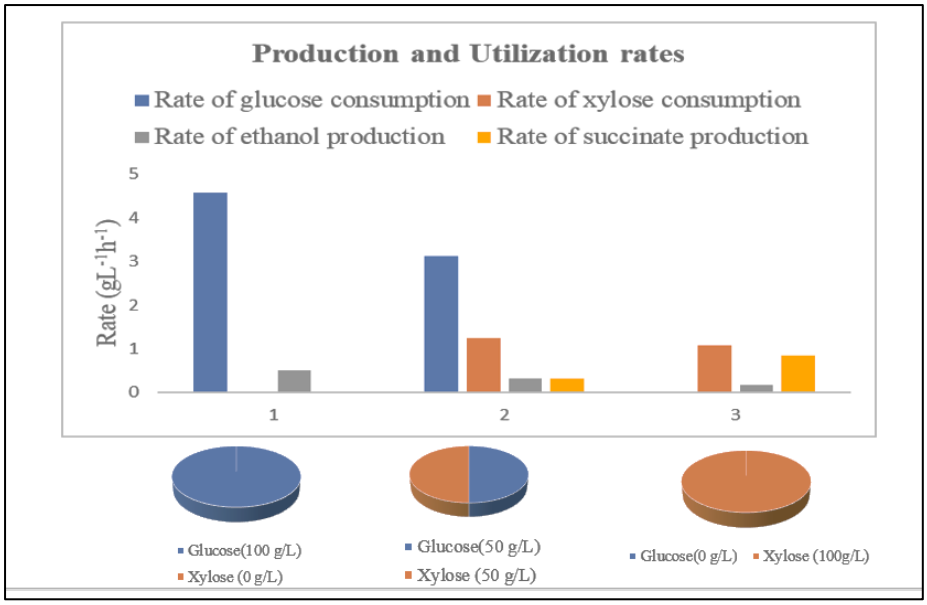


Figure 5.6 Production rates of glucose and xylose and utilization rates of ethanol and succinate.

5.3.2 Model testing for xylose uptake and succinate and ethanol production.

Model testing for succinate export showed that the symport reaction in section 5.2.5 regulates the succinate export. A DFBA was performed when this specific gene was turned off and turned on; the concentration of glucose, xylose, ethanol, and succinate was plotted against time. These graphs are shown in Figures 5.7 and 5.8.

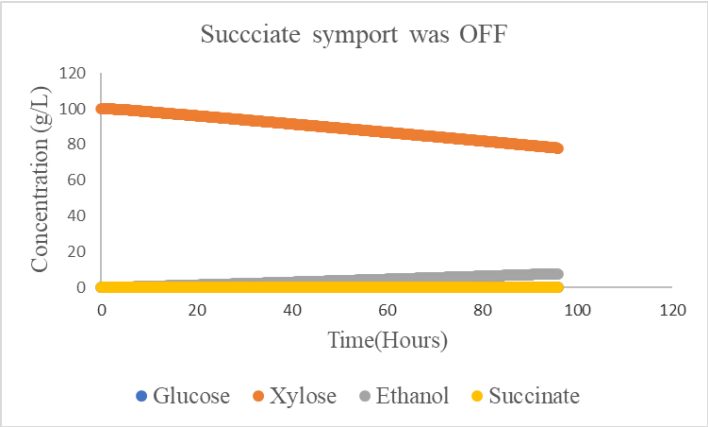


Figure 5.7 Graph plotted for metabolic concentrations observed in DFBA when succinate symport reaction was turned off.

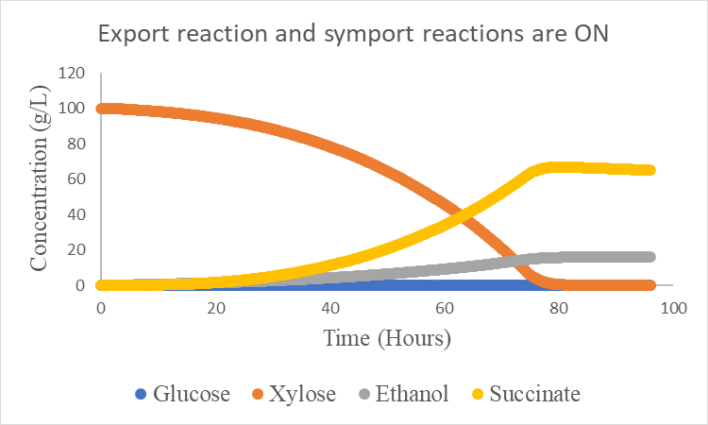


Figure 5.8 Graph plotted for metabolic concentrations observed in DFBA when succinate symport and succinate export reactions were turned on.

Model testing for succinate production showed that, among the twelve genes involved, the reaction FRDcm, given in the table, is shown to be regulating the succinate production. This inference was drawn after a DFBA was performed when this specific gene was turned off and turned on; glucose, xylose, ethanol, and succinate concentration were plotted against time. This graph is shown in Figure 5.9.

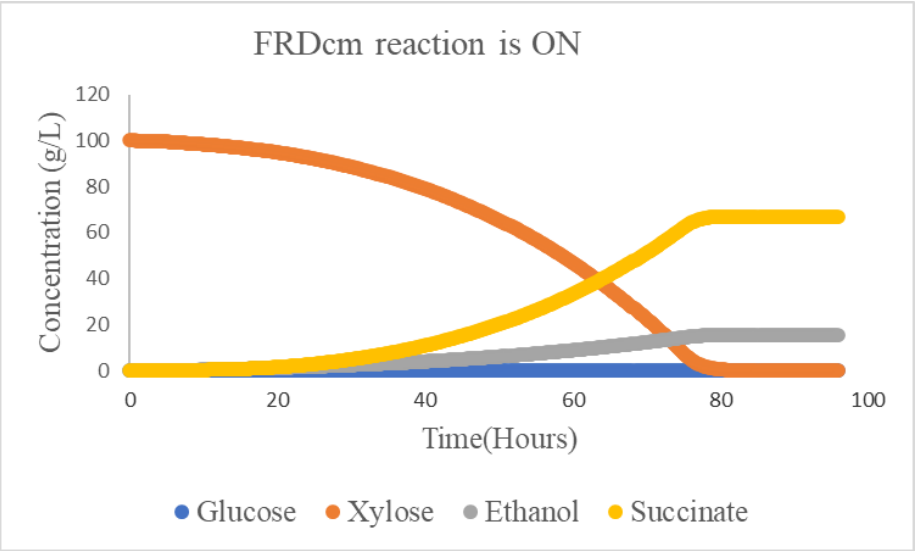


Figure 5.9 Graph plotted for metabolic concentrations observed in DFBA when the FRDcm reaction was turned on while the other eleven reactions were turned off.

In the FRDcm reaction, Fumarate is the substrate. So the reactions producing Fumarate were identified, and the FUM reaction was identified as the regulating reaction after performing DFBA when this reaction is turned off and turned on. Graphs 5.10 and 5.11 showed the concentrations of metabolites when these reactions were turned off and on.

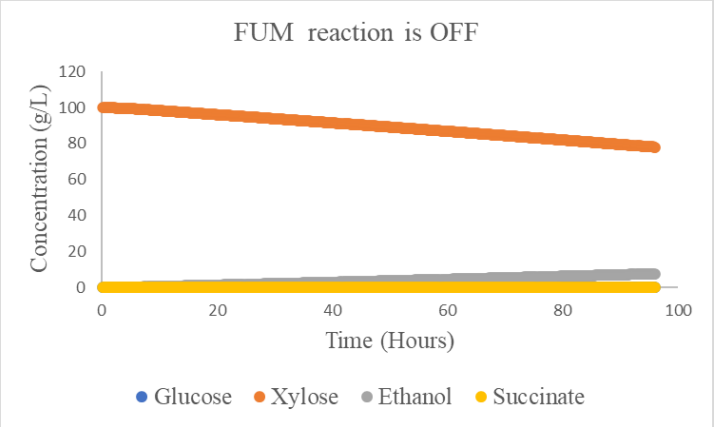


Figure 5.10 Graph plotted for metabolic concentrations observed in DFBA when the FUM reaction was turned off.

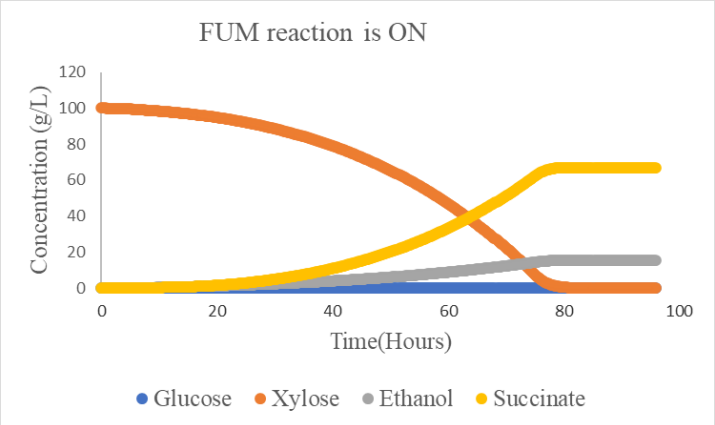


Figure 5.11 Graph plotted for metabolic concentrations observed in DFBA when the FUM reaction was turned on.

Malate is the substrate in the **FUM** reaction. So we investigated the reactions involved in the malate production. Among those reactions, after performing DFBA, the **MDH** reaction was observed to regulate malate production. The graphs for the concentrations plotted against time are shown in Figures 5.12 and 5.13.

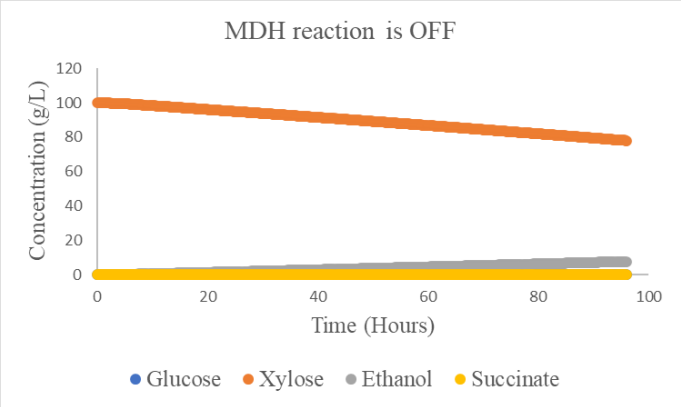


Figure 5.12 Graph plotted for metabolic concentrations observed in DFBA when the MDH reaction was turned off.

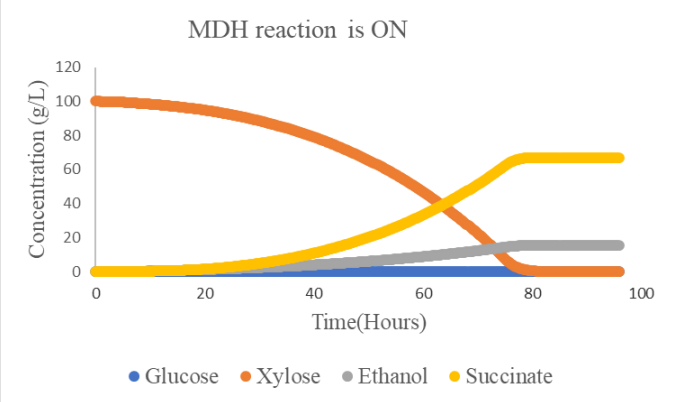


Figure 5.13 Graph plotted for metabolic concentrations observed in DFBA when the FUM reaction was turned on.

Oxaloacetate is the substrate in the **MDH** reaction. So we investigated the reactions involved in the malate production. Among those reactions, after performing DFBA, the **PC** reaction was observed to regulate oxaloacetate production. The graphs for the concentrations plotted against time are shown in Figures 5.14 and 5.15.

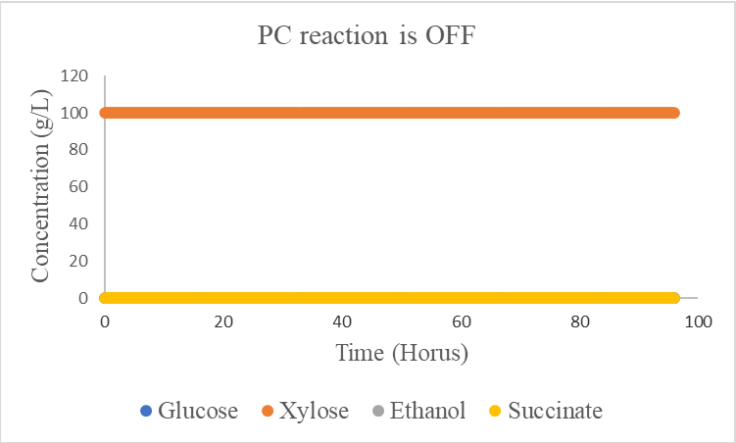


Figure 5.14 Graph plotted for metabolic concentrations observed in DFBA when the PC reaction was turned off.

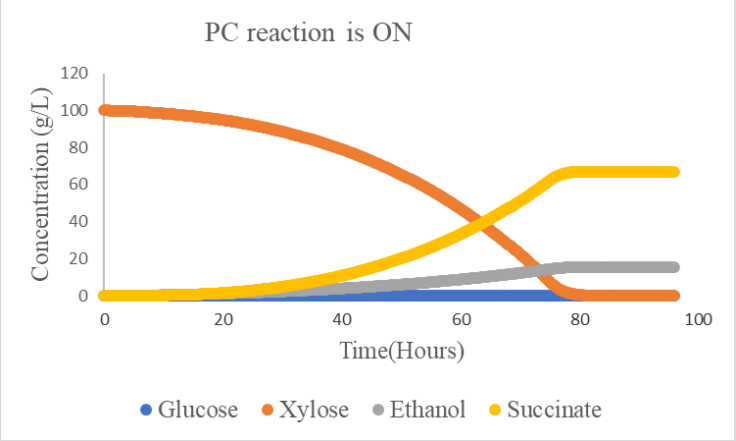


Figure 5.15 Graph plotted for metabolic concentrations observed in DFBA when the PC reaction was turned on.

Chapter 6

Recombinant plasmid construction for chromosomal integration

6.1 Introduction

In hosts with recombinant plasmids, there is a possibility for copy number variation, leading to different metabolic outputs at different times. These strains must constantly be maintained under selection pressure, which is not economically feasible at a biorefinery level. Therefore, genetic changes at a chromosomal level would increase the stability and longevity of the target gene's expression inside the host and remove the cost of antibiotics in maintaining the recombinant strains.

As we hypothesized, we had to integrate a set of five genes that encode a metabolic pathway that would efficiently produce, which would decrease co-factor imbalances and co-produce ethanol and succinate. Each of these five genes was assembled with strong promoter and terminator sequences. We used an appropriate promoter while expressing a gene with the antibiotic marker(MX cassette), as its over-expression may cause toxic effects on cells[25, 26]. We have selected the terminator sequences to terminate the transcription efficiently[27].

6.2 Cloning strategy

We have distributed the integration of all five genes into forming two integration constructs. After building each integration construct, right and left flanking homology sequences will be added, which decide the target site of chromosomal integration. A reusable antibiotic resistance marker cassette with Lox-m3 sequences[28] was also added along with the flanking homologies.

6.3 Results

6.3.1 Genomic DNA isolation.

Genomic DNA was isolated from YRH396 strains of *Saccharomyces cerevisiae*. Desired DNA band was observed when run on 0.8% Agarose gel, shown in Figure 6.1.

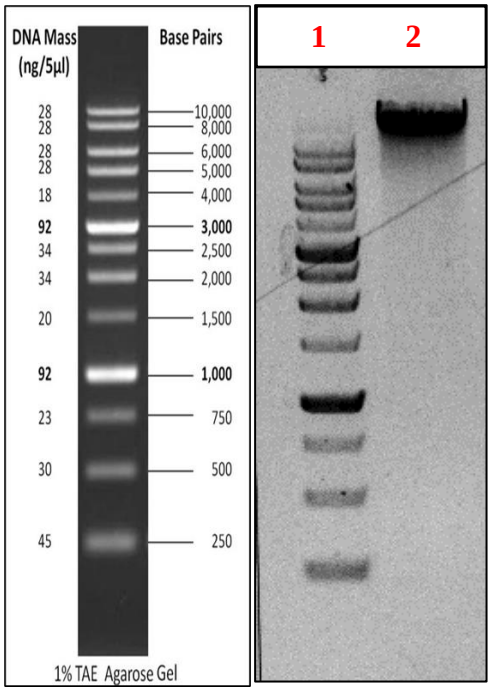


Figure. 6.1 Gel image of genomic DNA isolation

Lane 1: 1kb DNA ladder

Lane2: *Saccharomyces cerevisiae* genomic DNA

6.3.2 Vector and insert assembly

pRS414 vector was isolated from DH5α and ran on 1% agarose gel; desired bands were observed when compared with a 1kb DNA ladder. This isolated plasmid was digested sequentially with *Nae*I and *Xho*I enzymes and then run on 1% agarose gel. The gel image showed the desired band sizes compared with the 1kb DNA ladder. The enzymes were heat-inactivated and gel eluted. These are shown in Figures 6.2 (a) and 6.2 (b).

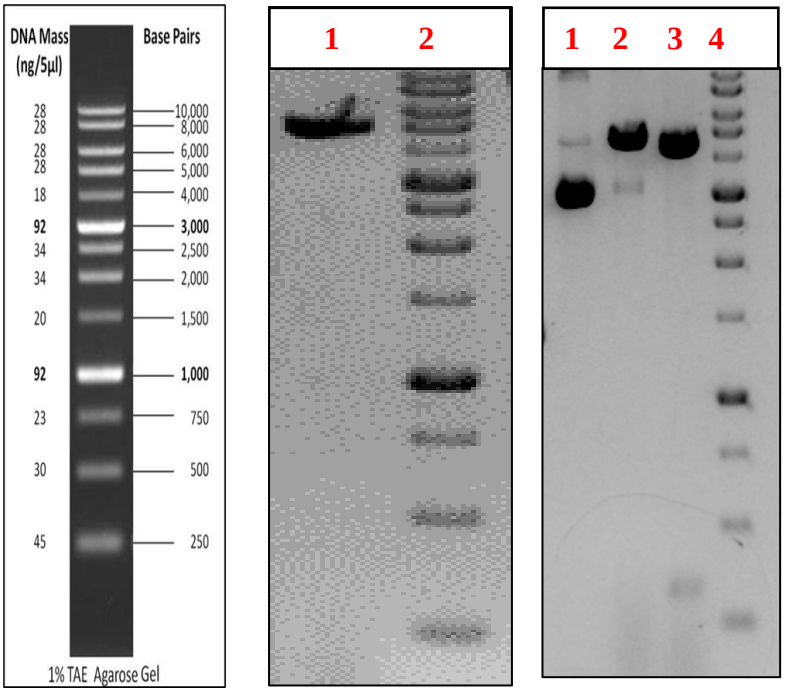
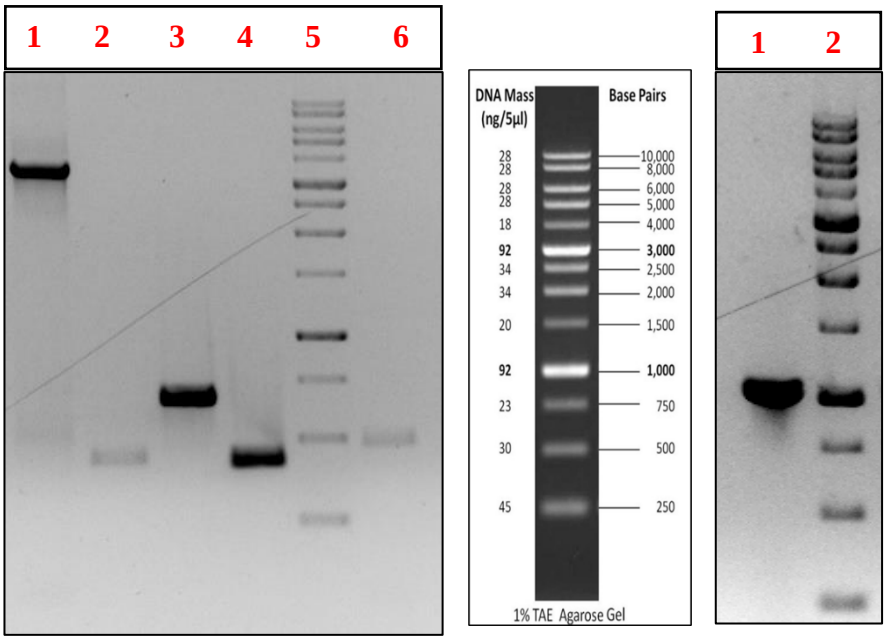


Figure. 6.2

(a) Gel image of pRS414 isolation Lane 1: pRS414 supercoiled DNA and **Lane2**: 1kb DNA ladder

(b) Gel image of pRS414 restriction enzyme digesiton. Lane 1: pRS414 supercoiled plasmid, **Lane2:** NaeI digested **pRS414** , **Lane 3**: NaeI and XhoI digested pRS414 and **Lane 4**: 1kb DNA ladder

All six inserts were amplified using PCR and ran on 1% Agarose gel. Desired bands with appropriate sizes were obtained, as shown in Figures 6.3 (a) and (b). Restriction analysis was performed with different enzymes to check the inserts. The table shows the enzymes used to perform restriction analysis on all the inserts. All the gel images in Figures 6.4 (a) and (b) showed desired bands, suggesting that inserts are correctly amplified.



(a) (b)

Figure. 6.3

(a)Gel image of PCR amplification of PYC2, TDH3, DIT1, ScTEF1 fragments

Lane1: PYC2 gene with expected size of 3543bp**; Lane2**: RPL41B terminator with the expected size of 454bp

Lane3: TDH3 promoter with the expected size of 681bp

Lane4: DIT1 terminator with the expected size of 433bp

Lane5: 1Kb DNA ladder

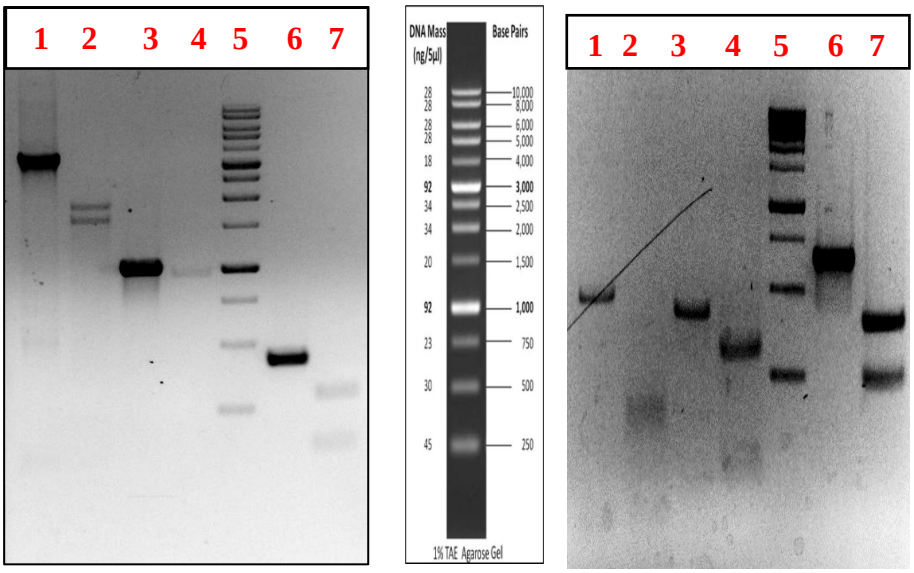
Lane6: ScTEF1 promoter with the expected size of 511bp

(b) Gel image of PCR amplification of MDH3 gene

Lane 1: MDH3 gene with expected size 1032bp

An analytical gel was run to determine the concentrations of the amplified inserts and restriction digested-gel eluted plasmid, as shown in Figure 6.5. The concentrations were determined, and accordingly, the NEBuilder Hifi DNA assembly reaction was set up for constructing a recombinant plasmid with all six inserts cloned directionally.

After 60 minutes of incubation at 50°C, a 2µL reaction mixture was used to transform chemically competent DH5α cells.



(a) (b)

Figure. 6.4

(a) Gel image of restriction analyis of PYC2, MDH3 and DIT1 fragments
Lane1: PYC2 gene Uncut (Size: 3543bp)
Lane2: PYC2 gene cut with SspI-HF with the expected band sizes: 1893 and 1650
Lane 3: MDH3 gene (Size: 1032bp)
Lane 4: MDH3 gene cut with: SspI-HF with the expected band sizes: 987 and 45
Lane 5: 1kb DNA ladder
Lane 6: DIT1 Uncut terminator (Size:433bp)
Lane 7: DIT1 terminator cut with SspI-HF with the expected band sizes of 378bp and 115 bp

(b) Gel image of restriction analysis of ScTEF1, RPL41B, and TDH3 fragments
Lane1: ScTEF1 promoter uncut (Size:511bp)
Lane2: ScTEF1 promoter cut with ApoI with the expected band sizes of 198bp and 110bp and 28bp
Lane3: RPL41B terminator uncut (Size:455bp)
Lane4:RPL41B terminator cut with SspI-HF with expected band sizes of 333bp and 121bp
Lane 5: 1kb DNA ladder
Lane 6: TDH3 promoter *(*Size:681bp)
Lane 7: TDH3 promoter cut with SspI-HF with the expected band sizes of 268bp and 413bp

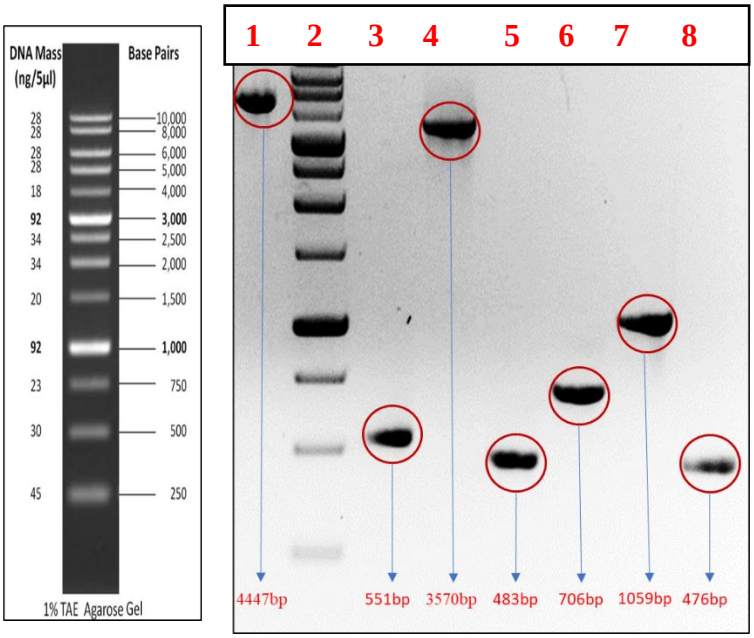


Figure. 6.5 Gel image of an Analytical gel for determination of concentratoins

Lane 1: Gel eluted pRS414 vector digested with *Nae*I and *Xho*I.

Lane 2: 1Kb DNA ladder

Lane 3: ScTEF1 promoter PCR product.

Lane 4: PYC2 gene PCR product.

Lane 5: RPL41B terminator PCR product.

Lane 6: TDH3 promoter PCR product.

Lane 7: MDH3 gene PCR product

Lane 8: DIT1 terminator PCR product

6.3.3. Recombinant plasmid isolation and analysis.

After transformation and plating on an LB agar plate with Ampicillin, four colonies were grown among these four colonies. Plasmids were isolated from all four colonies, as shown in Figure 6.6, and a single colony was shown to have the desired plasmid.

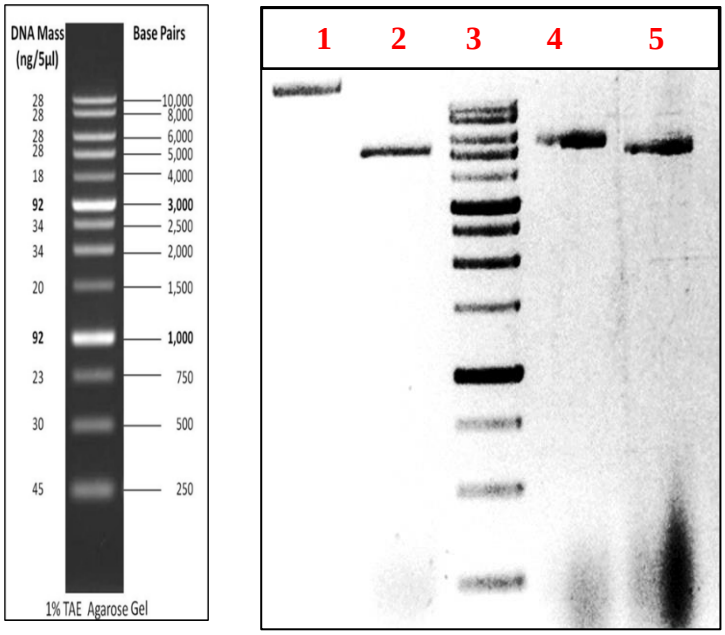


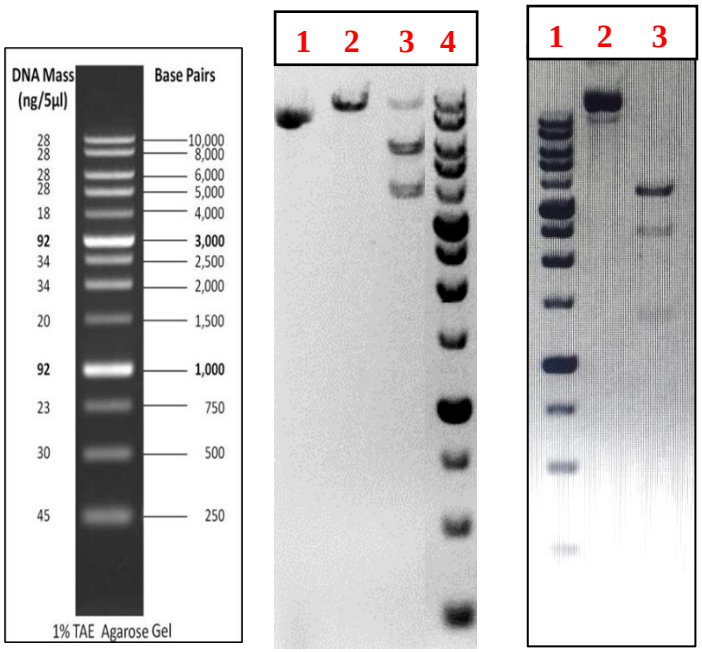
Figure. 6.6 Gel image of recombinant pRS414 plasmid isolation.

Lane 3: 1Kb DNA ladder

Lanes 1,2,4, and 5: Recombinant supercoiled plasmids isolated from four colonies obtained after transformation.

A set of restriction analyses was performed on the isolated recombinant plasmid. Desired bands were observed after the gel image was analyzed, as shown in Figure 6.7 (a) and (b), which suggested that this is our desired recombinant plasmid.

For further confirmation, the plasmid was sent for Sanger sequencing. The sequencing results showed multiple base substitutions but did not change the coding amino acid.



(a) (b)

Figure 6.7

(a) Gel image of restriction anlaysis of r-pRS414 vector with *Xho*I and *Nae*I

Lane1: uncut supercoiled r-pRS414 vector

Lane2: *Xho*I cut r-pRS414 vector

Lane3: *Xho*I and *Nae*I cut r-pRS414 vector

Lane4:1kb DNA ladder

(b) **Gel image of restriction anlaysis of r-pRS414 vector with *Eco*RV**

Lane 1: 1kb DNA ladder

Lane2: uncut supercoiled r-pRS414 vector

Lane3: *Eco*RV cut r-pRS414 vector

Chapter 7

Conclusion and Future Perspective

7.1 Conclusion

In conclusion, our project aims to modify *Saccharomyces cerevisiae* to improve the utilization of xylose and increase the production of ethanol and succinate. By implementing two objectives, we aimed to understand the metabolic fluxes involved in utilizing glucose and xylose and to create a recombinant vector for chromosomal integration in *Saccharomyces cerevisiae*.

The DFBA studies showed that the hypothesized pathway shown in Figure 7.1 gives a significant rate of succinate production and xylose utilization when xylose is provided as the sole carbon source of the model. This pathway also balanced the NAD^+ imbalance observed while xylose utilization by the organism. Integrating the genes regulating these reactions into the chromosome of *S. cerevisiae* would produce high amounts of succinate along with ethanol and utilize the xylose in high amounts.

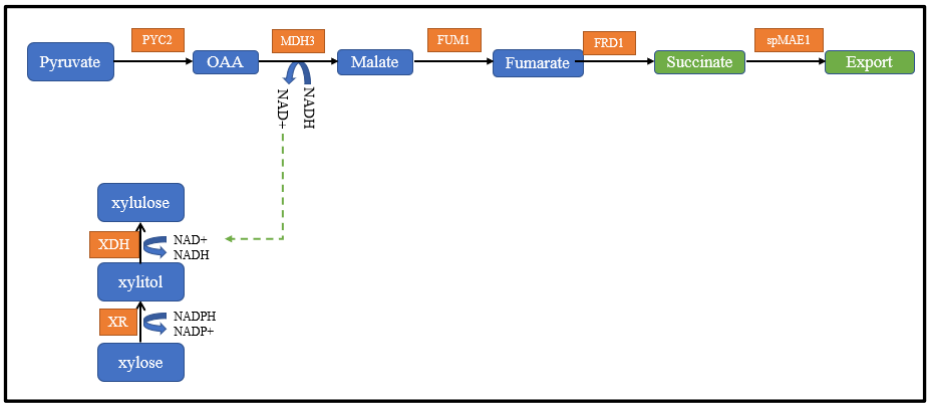


Figure 7.1 Overall hypothesized pathway regulating the succinate production utilizing xylose.

Based on our DFBA studies, we set our second objective to create a recombinant vector that can be used to integrate the genes along with strong promoters and terminators. We have created a recombinant vector with the *PYC2* and *MDH3* genes and the strong constitutive promoters. This can be used for chromosomal integration, enhancing the xylose uptake capability and co-production of ethanol and succinate in significant amounts. By performing the DFBA with various perturbations like the addition of transport reactions and modifying fluxes of different reactions, and changing type and concentration of the sugars, we were able to analyze the dynamics of metabolic fluxes, and we have gained a better understanding of the limitations in utilizing xylose and producing ethanol and succinate.

Moving forward, further experiments are necessary to achieve our objectives and optimize the production of ethanol and succinate. However, our work provides a foundation for future research in this area.

7.2 Future perspectives

Further research could optimize the recombinant vector and chromosomal integration process to improve the efficiency of xylose uptake and co-production of ethanol and succinate. Additionally, investigating the fermentation experiments on *Saccharomyces cerevisiae* can generate real-time data. This data can be used to perform model fitting of the current model that we are working on. This could provide valuable insights into the different bottlenecks and alternative pathways for utilizing xylose and other sugars.

Overall, our project represents a significant step forward in developing a systematic bioprocessing approach that is technically feasible, economically viable, and environmentally low-impact at a biorefinery level.

APPENDIX

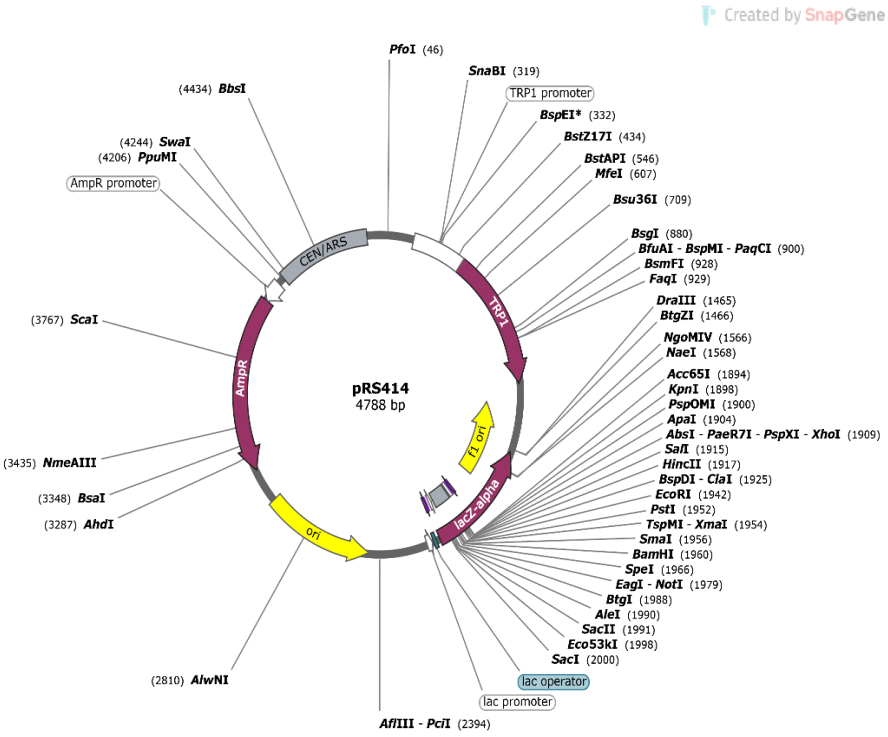


Figure 8. pRS414 Vector map (Procured form snapgene software)

pRS414 vector is a shuttle vector. We used it in our study to performing cloning for our desired genes.

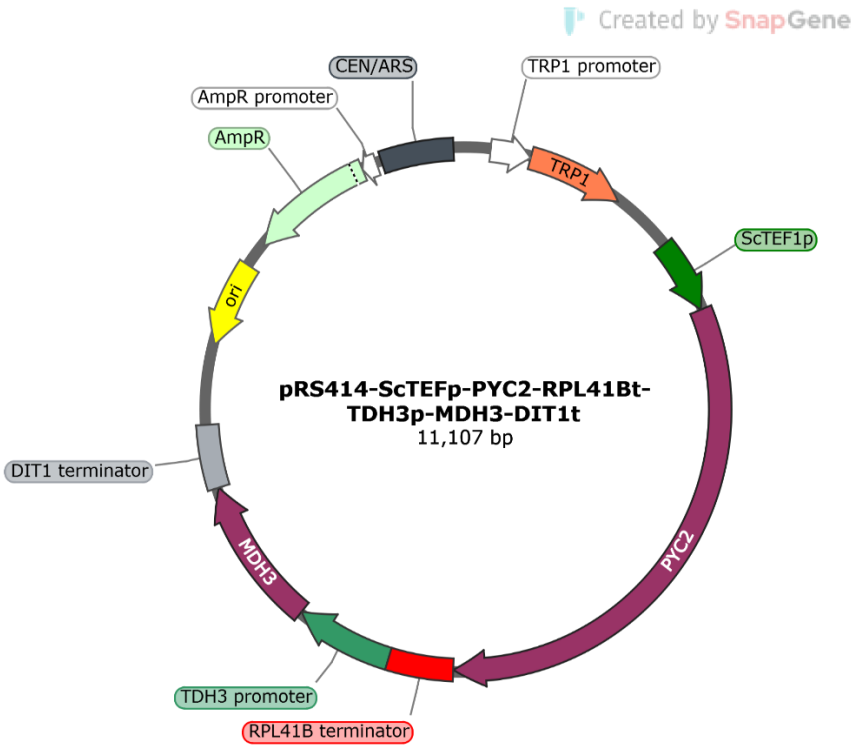


Figure 9 Recombinant pRS414 vector map (Procured form snapgene software)

This recombinant vector was constructed in our lab as a part of this study. This contains all six fragments that have been discussed in Chapter 6.

	Fragment size in (bp)	Restriction enzyme used to cut the fragment	Cut site of Restriction enzyme	Sizes of bands after restriction digestion(in bp)
PYC2 gene	3543	SspI	1893	1893, 1650
MDH3 gene	1032	SspI	987	987,45
ScTEF1 promoter	511	ApoI	198, 308, 336	198, 110, 28
TDH3 promoter	681	SspI	268	268, 413
RPL41B terminator	454	SspI	121	121, 333
DIT1 terminator	433	SspI	115	115,338

Table 6 of restriction enzymes used to perform restriction analysis of the PCR amplified fragments, their restriction sites, and expected band size

Primer Name	Primer sequences(5'→3')	Primer length(in bp)
L_PYC2_F	GTTTTAATTACAAAATGAGCAGTAGCAAGAAATTGG	36
L_PYC2_R	GCTCTCAATCCGCTTACTTTTTTTGGGATGGGG	33
L_RPL41B_F	CCAAAAAAAGTAAGCGGATTGAGAGCAAATCGTTAAG	37
L_RPL41B_R	GATAAACTCGAACTGAAGCCGAAAATCTTTCAAGCAC	37
L_ScTEF1_F	GATTTAGAGCTTGACGGGGAAAGCCGGCTATCACATAGGAAGCAACAGG	49
L_ScTEF1_R	GCTACTGCTCATTTTGTAATTAAAACTTAGATTAGATTGCTATGCTTTC	49
L_MDH3_F	CATAAACAAACAAAATGGTCAAAGTCGCAATTCTTGG	37
L_MDH3_R	CGCTCTTACTTTATCATAGCTTGGAAGAGTCTAGG	35
L_DIT1_F	CTTCCAAGCTATGATAAAGTAAGAGCGCTACATTGG	36
L_DIT1_R	TCAAGCTTATCGATACCGTCGACCTCGAGTTACTCCGCAACGCTTTTCTG	50
L_TDH3_F	GATTTTCGGCTTCAGTTCGAGTTTATCATTATC	33
L_TDH3_R	GCGACTTTGACCATTTTGTTTGTTTATGTGTGTTTATTCG	40

Table 7 List of long primers used for the PCR amplification of inserts

These long primers were used to amplify fragment-specific sequences along with overlapping homologies

Strain	Genotype/description/(reference)
YRH388	CEN.PK2-1C *ho*Δ::(*KanMX4*; P_{PGK1}-*XYL1*-T_{PGK1}; P_{ADH1}-*XYL2*-T_{ADH1}; P_{HXT7}-*XKS1*-T_{HXT7}) Haploid lab strain CEN.PK2-1C with *Scheffersomyces stipitis XYl1*, *XYL2*, and *S. cerevisiae XKS1* genes for xylose utilization integrated at the *HO* gene locus. (J Ind Microbiol Biotechnol. 2011 Sep;38(9):1193-202. doi: 10.1007/s10295-010-0896-1.)
YRH396	YB-2625 *ho*Δ::(*KanMX4*; P_{PGK1}-*XYL1*-T_{PGK1}; P_{ADH1}-*XYL2*-T_{ADH1}; P_{HXT7}-*XKS1*-T_{HXT7}) Diploid strain YB-2625 isolated from bagasse with *Scheffersomyces stipitis XYl1*, *XYL2*, and *S. cerevisiae XKS1* genes for xylose utilization integrated at the *HO* gene locus. Gene integration at *HO* locus on a single chromosome. (J Ind Microbiol Biotechnol. 2011 Sep;38(9):1193-202. doi: 10.1007/s10295-010-0896-1.)
YRH667	YB-2625 *ho*Δ::(*KanMX4*; P_{PGK1}-*XYL1*-T_{PGK1}; P_{ADH1}-*XYL2*-T_{ADH1}; P_{HXT7}-*XKS1*-T_{HXT7}) Diploid strain YB-2625 isolated from bagasse with *Scheffersomyces stipitis XYl1*, *XYL2*, and *S. cerevisiae XKS1* genes for xylose utilization integrated at the *HO* gene locus. Gene integration at *HO* locus on both chromosomes.
YRH1946	Haploid isolate from sporulation of diploid strain YB-2625 isolated from bagasse. Grows well on furfural. Does not have the genes for xylose integrated.

Table 8 List of strains used in genome isolation.

These strains were received from Dr. Ronald E. Hectors' lab. We used these to isolate the genomic DNA required for our PCR amplification. These shall also be used for fermentation experiments in the future.

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