Heterologous Expression of Xylose Isomerase from *Clostridium phytofermentans* in *Saccharomyces cerevisiae* for Single Step Conversion of Xylose into Xylulose and enhanced ethanol production.

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ACRONYMS

µg	Microgram
µg/ml	Microgram per milli-litre
µl	Microlitre
µM	Micromolar
ATP	Adenosine triphosphate
BLAST	Basic local alignment search tool
bp	Base pair
cDNA	Complementary deoxyribonucleic acid
CYC1	Cytochrome C1
DNA	Deoxyribonuleic acid
dNTP(s)	Deoxyribonucleotide triphosphate(s)
EDTA	Ethylene Di-amine Tetra-Acetic acid
EtBr	Ethidium Bromide
gm	gram
gm/ltr	Gram per liter
GAL	Galactose
GHG	Green House Gases
GPD	glycerol-3-phosphate dehydrogenase 2
GXS1	Glucose/Xylose Symporter
GXF1	Glucose/Xylose Facilitator
hr	Hour
hr^{-1}	Per hour
IDT	Integrated DNA Technology
kb	Kilo base pairs
L /ltr.	Liter
LB	Lauria Burtani Broth
LBA	Lauria Burtani Agar

M	Molar
MCS	Multiple Cloning Site
mg	Miligram
mg/ml	milligram per millilitre
Min.	Minute(s)
ml	milliliter
mM	Milimolar
NCBI	National center for Biotechnology Information
NFW	Nuclease Free Water
ng	nanogram
ng/ml	nanogram per millilitre
OD600	Optical Density at 600nm
ORF	Open reading frame
PCR	Polymerase Chain Reaction
PPP	Pentose Phosphate Pathway
Rcf/g	Relative centrifugal force/ g-force
RNA	Ribonucleic Acid
RNase	Ribonuclease
rpm	Revolutions per minute
RT	Room Temperature
RKI	Ribose 5-phosphate isomerase
RPE	Ribulose 5-phosphate epimerase
RT-PCR	Reverse transcriptase Polymerase chain reaction
Sec.	Second(s)
TAE	Tris- Acetate EDTA
TAE	Tris- Glacial Acetic acid – EDTA
TAL	Transaldolase
TE	Tris EDTA
TKL	Transketolase

Tm	Melting temperature
Tris	Tris-(Hydroxymethyl)-aminmethane
UV	Ultraviolet
V	Volts
XKS	Xylulokinase
XI	Xylose Isomerase
xylA	Xylose isomerase gene
YEp	Yeast expression plasmid
YEPD	Yeast-extract Peptone Dextrose
YIp	Yeast integrative plasmid
YNB	Yeast Nitrogen Base
YNBA	Yeast Nitrogen Base Agar
YNBX	Yeast Nitrogen Base with Xylose

ABSTRACT

Second generation bioethanol produced from lignocellulosic and hemicellulosic biomass presents more advantage as alternative to fossil fuels due to abundance of feed-stocks, its' renewable nature, less GHG emission and no concern of food vs. fuel unlike first generation bioethanol. *Saccharomyces cerevisiae,* an organism of choice for production of both first and second generation bioethanol is unable to use the pentose fraction (xylose) of lignocellulosic hydrolysates, the second most abundant sugar following glucose. The inability of *S. cerevisiae* to use pentose sugar on lignocellulosic hydrolysates may be due to lack of xylose specific transporter and enzyme system in yeast to drive xylose in central metabolic pathway. In this study, recombinant strain of *S. cerevisiae* MKY09 (MKY09B2) is generated by heterologous expression of codon optimized xylose isomerase (XI) gene from *Clostridium phytofermentans* in episomal plasmid construct pGPD2+XI, where XI gene is flanked by constitutive promoter GPD and CYC1 terminator (i.e. GPD-XI-CYC1). Transformation of yeast was confirmed by PCR, southern blotting and was also visualized by fluorescence microscopy using DAPI staining. Both control strain (MKY09D2) and XI-recombinant strain (MKY09B2) exhibited similar trend in glucose use and slightly different growth kinetics. The XI-recombinant strain used more amount of xylose accompanied by higher biomass and extended lag phase (and delayed stationary phase) in YNBX media. Strain MKY09B2 was found to yield slightly higher amount of ethanol than MKY09D2 irrespective of sugar supplemented in media. Strain MKY09B2 was also found to utilize higher amount of xylose may be through co-consumption with glucose when both sugars were provided in media followed by subsequent increase in ethanol yield.

Keywords: Bioethanol, *Saccharomyces cerevisiae*, Xylose, Xylose Isomerase.

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CHAPTER I

INTRODUCTION

1.1 Background

"Biofuel" is a representative term used for group of energy rich liquid, gas, and solid fuels which are predominantly produced from biomass. Biofuels include bio-methanol, bio-ethanol, bio-hydrogen, biodiesel, vegetable oils, bio-oil, bio-char, biogas, bio-synthetic gas named also as bio-syngas and Fisher–Tropsch liquids (Demirbas, A., 2008). Since the last decade, production of biofuels has increased in a remarkable way going from 6.4 in 2003 to 23.4 billion gallons in 2013 (Su, Zhang, & Su, 2015). It is estimated that, biofuels account for 8% of global oil volumes used for transportation (Kagan, 2015). The world leaders in biofuel development, production and use are Brazil, United States, Germany, France, Sweden and China (https://en.wikipedia.org/wiki/Biofuel). These countries differ in use of substrate for biofuel production that ranges from energy rich dedicated crops, sugarcane, agricultural solid waste, algal biomass and so on (Woiciechowski et al., 2016). Also, some forms of biofuel are being produced in industrial scale while some are still under experimentation (*REN21, 2011,* Renewables 2011 GLOBAL STATUS REPORT.

Focus on biofuels began with increasing cost of crude oil, concerns about energy security, greenhouse gas (GHG) emissions (Jambo et al., 2016a), and the realization for significance of renewable energy for economic growth (Soccol et al., 2010; Soccol & Editors, 2016). Especially after the oil crisis in the 1970s which put an end to abundance and low-cost fossil fuel, biofuels are considered as a promising sustainable renewable alternative to fossil fuel from economic, social, and environmental points of view (Cremonez et al., 2015). The primary interest for development of biofuels are to ensure the energy security, to reduce the greenhouse gas (GHG) emissions by generating alternatives to fossil fuels, and to promote rural agricultural development (Su et al., 2015).

1.2 Bioethanol

Bioethanol is simply an ethanol produced from natural sources mostly plant biomass or feedstock, e.g., wheat, wood, corn, straw, and sugar beet, agricultural, forestry or industrial waste, or from parts of dedicated energy crops. As the production depends on most abundant biomass on earth, it presents a way to reduce GHG emission unlike in case of crude oil thereby addressing environmental pollution and as alternative to crude oil consumption (Demirbas, 2008). Since the energy crisis in 1970s, it has been regarded as the liquid transport fuel and has been produced mostly in Brazil and USA. Bioethanol in Brazil has been produced almost exclusively from sugarcane while in USA the feedstock is wheat and

corn. Beside these two countries, Germany, Sweden, China, India are also contributing for the global bioethanol production to some extent (Balat et al., 2008; Balat & Balat, 2009; Balat, M., 2011).

Ethanol has been produced and used as an intoxicating agent in the preparation of alcoholic beverages since time immemorial which dates back to 6000 BC. Dried traces of ethanol was also found in China on 9000 years old pottery (Soccol & Editors, 2016). On the basis of nature of substrates used for the production, biofuels and/or bioethanol are categorized into three main groups: first, second and third generation (http://biofuel.org.uk/types-o f-biofuels.html, 2017). Recently, there is another class of biofuel named fourth generation that not only aims for biofuel production but also accounts for capturing and storing CO_2 (http://energyfromwasteandwood.weebly.com/generations-of-biofuels.html). Characterization is based not only on their sources of biomass but also their limitations as a renewable source of energy, their technological progress as well as transition of ethanol production period and expansion of the technology (http://biofuel.org.uk/types-of-biofuels.html, 2017).

Biofuels are energy sources made from recently grown biomass (plant or animal matter) that have been around for a long time. Petroleum and coal have been used primarily as energy sources due to their high abundance, high energy value, and cheap prices. These fossil fuels such as coal and petroleum are also the derivatives of biomass over millions of years for their formation (Dias et al., 2011). Biofuels are making a resurgence due to increasing oil prices, dwindling fossil fuel reserves, the desire to have a sustainable, renewable, reliable source of energy and as a way to mitigate the effects of global warming and climate change due to burning of petroleum fuels (Tye et al., 2016). Biofuels are a renewable resource because they are continually replenished in terms of biomass substrates unlike the fossil fuels that are not renewable since they require millions of years to form (Dias et al., 2011).

The bioethanol produced through carbon neutral approach has reduced greenhouse gas emission (GHG) which can simultaneously substitute crude oil for energy source and lead to economic and environmental benefits (Kuhad et al., 2011). Utilization of lignocellulosic biomass for ethanol production is ideal over the traditional procedure that uses glucose rich hydrolysate from sustenance crops like wheat and corn (Leandro, Gonçalves, & Spencer-Martins, 2006; Leandro, Spencer-Martins, & Gonçalves, 2008). The major focus had been drawn on bioethanol as alternative fuel source after the major energy crisis in 20th century. Possibility of bioethanol as transport fuel has been introduced since 1970s when energy crisis was an eminent problem. Ever since, much research have been focused on the bioethanol production to cope energy crisis concerning with declining fossil fuels (Favela-Torres, Allais, & Baratti, 1986). Since then, the field of bioethanol is broadly explored and used as ethanol blends to fossil fuel as E10, E15 and E85 that comprises 10%, 15% and 85% of ethanol mix to

fossil fuel respectively (http://www.eere.energy.gov/afdc/e85toolkit/specs.html). Scientists are also attempting to build an engine that works on 100% ethanol (Kelly et al., 1996; Lajoie et al., 2016). The bio-ethanol from sugarcane in Brazil, from corn in U.S. and China and biodiesels from oilseed in Germany are well known example of commercial bio fuels (Ben-Iwo, Manovic, & Longhurst, 2016).

1.2.1 Bioethanol usage as transport fuel

Brazil has been leading alongside USA for bioethanol production and sales in the world. More than 80 % of vehicles have been reported to be using bioethanol blended fuels (Soccol et al., 2016) in transport sector in Brazil. Brazilian government launched National Ethanol Fuel Program in 1970s at the time of oil crisis which was followed by series of measures to improve bioethanol production with an aim to reduce the oil import and to address the decrease of the national sugar price (Su et al., 2015). From 2000 till today, about 98 % of ethanol is used in transport sector, therefore, ethanol-gasoline cars are emerging (Su et al., 2015). In Colombia, biofuel policies include mandatory blending of 10 % bioethanol in cities whose population is above 500,000 inhabitants (Offermann et al., 2011; Sorda, Banse, & Kemfert, 2010). The law was applied in 2005 complete execution came true 4 years after when 75 % of total consumed gasoline had 10 % ethanol content (Rutz et al. 2009; Offermann et al., 2011). Colombian government offers many facilities to support and to encourage bioethanol production: such as, fixing of prices for sugarcane on the basis of international sugar prices, VAT exemption on bioethanol in order to fulfill the aim of increasing ethanol content up-to 25% (Sorda et al., 2010).

The bioethanol produced through carbon neutral approach has reduced greenhouse gas emission (GHG) which can simultaneously substitute crude oil for energy source and lead to economic and environmental benefits (Kuhad et al., 2011). Ethanol is being used as an additive to fossil fuel designating the name such as E10 that contains 10% ethanol and 90% gasoline and E15 (15%ethanol and 85% gasoline). Production of flexible – fuel vehicles (FFV) or dual-fuel vehicle that can use high ethanol blend such as E85 (85 % ethanol and 15 % gasoline) has already begun. Use of such technology subsequently would reduce petroleum use and reduce greenhouse gas emission (Gárdonyi & Hahn-Hägerdal, 2003; Gu et al., 2010; Saini, Saini, Tewari, et al., 2015). The US renewable fuel standard (RFS2) under energy independency and security act (EISA) 2007, aims to replace 36 billion gallon of fossil fuels per year (BGY) with biofuel by the year 2022. The EISA policies mandate to produce 21 BGY of second generation bio-ethanol from renewable source other than corn starch to mitigate 50% of GHG emission (EISA, Energy independence and security act, 2007).

1.3 Classes of Biofuels

Since the introduction of bioethanol as a blend to petrochemicals, it has been taken as the candidate for alternative fuel. Production of biofuel was initially based on the energy rich substrates such as corn, wheat and sugarcane etc. These conventional technique is basically dependent on the products that are also the food source for human and other animals. As alternative to this, other possibilities are under extensive study that focuses on using non-food materials as substrate for ethanol production. This in turn is expected to address the food vs feed controversy along with increased and efficient ethanol yield. The technique for ethanol production basically follows the fermentation of sugar content in substrate by yeast, *Saccharomyces cerevisiae*. Based on the substrate used for the fermentation, bioethanol has been classified as first, second and third generation bioethanol/biofuels. (http://energyfromwasteandwood.weebly.com/generations-of-biofuels.html) (http://biofuel.org.uk/types-of-biofuels.html)

1.3.1 First Generation Biofuels

Plants contain high-energy organic matter that is the basis of energy for all types of renewable biofuel. The biofuels sector began to exploit this knowledge by producing renewable fuel as an alternative to the dominant fossil fuel industry (Nova Institute, 2017). This kind of biofuel production based on crops with high energy content also referred as dedicated crops is termed "first-generation" (Burton & Forer, 2015; Marcelo et al., 2005).

In simple terms, first-generation biofuels are made from feedstock that can also be consumed as human food. Whether it is sugar, starch, or vegetable oil, all of them are also human food products which makes them a first-generation fuel (Naik et al., 2010). The feedstock for first-generation fuels includes food crops like corn, sugarcane, sugar beet, wheat and sorghum. Since they are easily extracted using conventional technology, they are also known as "conventional biofuels"(Millinger, Ponitka, Arendt, & Thrän, 2017).

Most common first-generation biofuels include:

- Biodiesel - extraction of vegetable oils, with or without esterification, from the seeds of plants like soybean, rapeseed (canola) and sunflower.
- Ethanol - fermentation of simple sugars from sugar crops (sugarcane) or starch crops (corn, wheat).
- Biogas - anaerobic fermentation of organic waste and crop residues as energy crops. (Dias et al., 2012; 2013).

First generation biofuels, also known as conventional biofuels, are made from sugar, starch or vegetable oil and are produced through well-understood technologies and processes, like

fermentation, distillation and trans-esterification. These processes have been in practice for hundreds of years for various purposes, most common use being alcohol production. Sugars and starches are fermented to primarily produce ethanol, and in smaller quantities, butanol and propanol. Ethanol has one-third of the energy density of gasoline, but is currently used in many countries, including the United States, Brazil, China and European countries as an additive to gasoline. One benefit of ethanol is that it burns cleaner than gasoline and therefore produces less greenhouse gases (Demirbas, 2008; Demirbaş, 2005; Naik et al., 2010). Biodiesel is another first generation biofuel which is produced by trans-esterification of plant oil or animal fat. Trans-esterification involves exposing oils with an alcohol such as methanol in the presence of a catalyst (Naik et al., 2010). Biodiesel can be used in place of petroleum diesel in many diesel engines alone or as mixture of two.

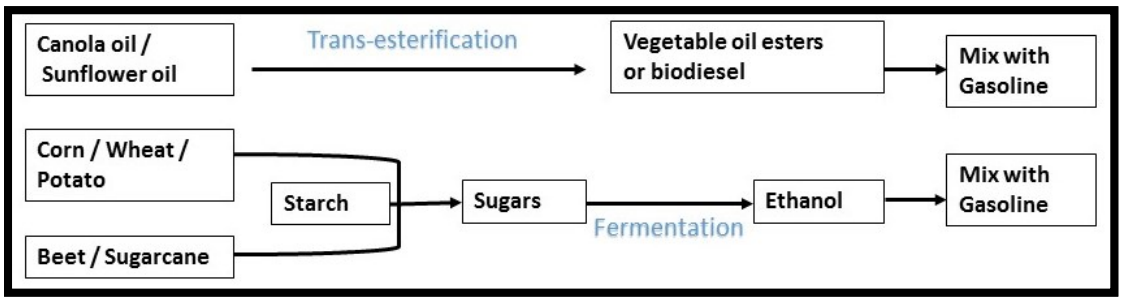

Figure 1.1 Process depicting the steps for 1st generation biofuel production.

First generation biofuels symbolizes a step towards energy independence and waning of fossil fuels for energy demands. These biofuels also support agricultural industries and rural communities through increased demand for crops. Beside these usefulness, 1st generation biofuels also have several disadvantages (Mohr & Raman, 2013). They pose a threat to food prices since substrate or the biomass used are energy rich food and feed crops (Goh & Lee, 2009). First generation biofuel production contributed to increase in world prices for food and animal feeds (Joshua Kagan, n.d.; Martin, 2010). They also have the potential to have a negative impact on biodiversity and competition for water in some regions. Additionally, biomass for first generation biofuels requires lots of arable land to grow, and this provides only limited greenhouse gases reduction. They also only provide a small benefit over fossil fuels in regards to greenhouse gases since they still require high amounts of energy to grow, collect, and process (Muktham, et al., 2016). And by default, current production practices use fossil fuels for power. Hence, first generation biofuels are a comparatively expensive option than gasoline, making it economically unfavorable. Finally, biodiesel almost always comes from recycled oils from restaurants, as opposed to virgin oils, so the supply is limited by restaurants' oil use (Sims et al., 2008).

1.3.2 Second Generation Biofuels

As with any industry, certain concerns related to cost and inefficiency arose for first-generation biofuel production. This evolution naturally led to second-generation biofuels. Like first-generation fuels, second-generation fuels are also produced from sustainable feedstock but, in this case, these feedstocks are not normally used for human and animal consumption (Sims et al., 2008; Soccol et al., 2010a). That is, second-generation feedstock does not focus on crop as substrate biomass, though certain food crops may become substrate for second-generation fuels if and when they're no longer useful for consumption (Nigam, 2002; Nigam & Singh, 2011). Second-generation non-food feedstocks include woody crops and agricultural residues or waste, forest litters, baggage rich in lignocellulose which are a little more difficult to extract. For this reason, advanced conversion technologies are needed in the process to extract fermentable sugars (Tye et al., 2016; Woiciechowski et al., 2016). Hence, second-generation biofuels are also known as "advanced biofuels" (NNFCC, 2017).

Second-generation technologies cover a wider range of biomass resources, from agriculture to forestry and waste materials. One well-known second-generation technology is called lignocellulosic processing, which uses plant and plant derived matters mostly agricultural and forest materials (Bezerra & Ragauskas, 2016). Second-generation biofuels have increased efficiency that is attributed to the feature enabling it to use vast majority of biomass feedstock which avoids waste seen in first-generation biofuel production. Advantage of second generation biofuel also relies on its renewable nature and vast abundance of such biomass (Dias et al., 2013). The biomass sources for 2^{nd} generation biofuels include wood, organic waste, food waste and specific biomass crops, fast growing trees such as poplar trees that need to undergo a series of physical and/or chemical pretreatment steps to break down lignin, that subsequently frees up the cellulose and hemicellulose which in turn is followed by hydrolysis of these sugar polymers to fermentable sugar residues. This pretreatment is achieved by thermochemical or biochemical reactions that unlock the sugars embedded in fibers of the plant (Stephen et al., 2012). After hydrolysis, ethanol production from the hydrolysates resemble that of 1^{st} generation ethanol production. In addition to this, straw and other forest residues can go through a thermochemical step that produces syngas (a mixture of carbon monoxide, hydrogen and other hydrocarbons). Hydrogen can be used as a fuel and the other hydrocarbons can be used as additives to gas oil.

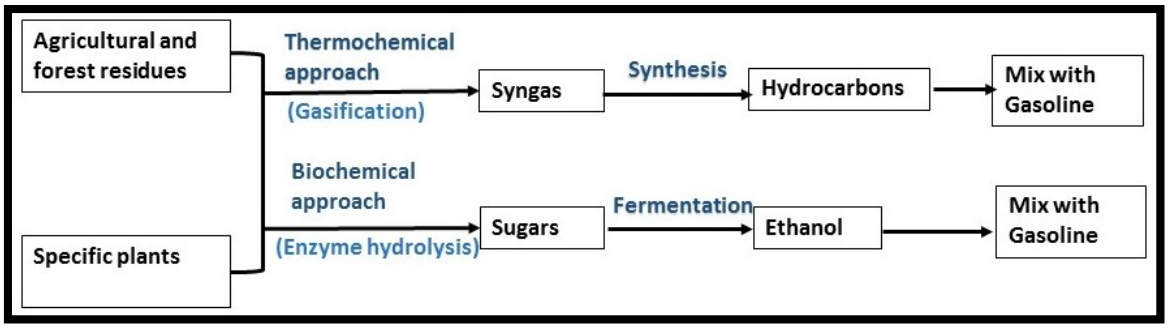

Figure 1.2 Diagrammatic representation of the workflow for production of 2nd generation biofuels (Naik et al., 2010)

Second generation biofuels addresses many issues associated with limitation of 1st generation biofuels. They don't compete between fuels and food crops since they come from distinct biomass. Second generation biofuels also generate higher energy yields per acre than 1st generation fuels (Soccol et al., 2010a, 2016). They allow for use of poorer quality land where food crops may not be able to grow. The technology is fairly immature, so it still has potential of cost reductions and increased production efficiency as scientific advances occur. However, some biomasses for second-generation biofuels still compete with land use since some of the biomass grows in same climate as food crops (Woiciechowski et al., 2016). This leaves farmers and policy makers with hard decision of which crop to grow. Cellulosic sources that grow alongside food crops could be used for biomass, such as corn stover (leaves, stalk, and stem of corn). However, this would take away too many nutrients from the soil and would need to be replenished through fertilizer. In addition, the process to produce 2nd generation fuels is more elaborate than 1st generation biofuels because it requires pretreatment of biomass to release sugars residues. This requires more energy and materials that in turn adds economic burden to final product (Sims et al., 2008; The Royal Society, 2008).

1.3.3 Third Generation Biofuels

Third-generation biofuel is based on the use of algae as biofuel producer or using algal biomass as substrate for biofuel production (Goh & Lee, 2009). Whichever be the case, key word in third-generation fuels is algae. Algae's use in biofuel production was formerly relegated to second generation but some key differences such as diversity: Algae can produce such fuels as biodiesel, butanol, gasoline (petrol), ethanol, and even jet fuel; higher yields: Algae is capable of producing much more biomass than its other feedstock counterparts with lower resource inputs forced to place it into separate category (Dragone et al., 2010).

The capital and operating costs of third-generation biofuel production are the highest and requires additional research and development to reach the point of being a sustainable method of consistent biofuel production on a commercial scale (Jambo et al., 2016b).

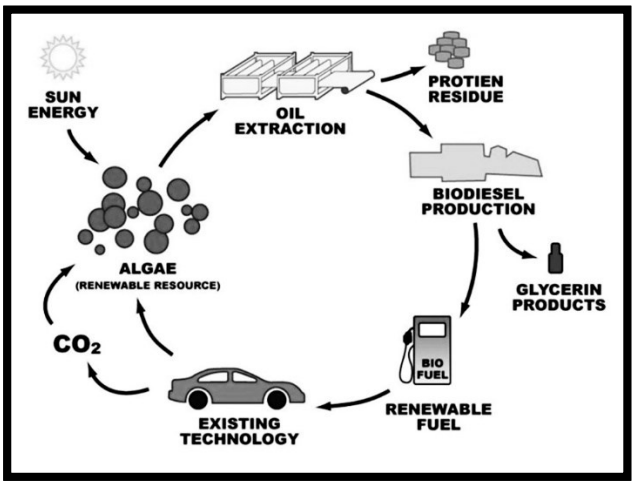

Figure 1.3 General Steps for production of third generation biofuels (adapted from Jambo et al., 2016).

Third generation biofuels are based on specially engineered algae as the energy source. These algae are grown and harvested to extract oil within them. The oil can then be converted into biodiesel through a similar process as 1st generation biofuels, or it can be refined into other fuels as replacements to petroleum-based fuels. Third generation biofuels are more energy dense than 1st and 2nd generation biofuels per area of harvest. They are cultured as low-cost, high-energy, and completely renewable sources of energy (Carere et al., 2008; Jambo et al., 2016a; Saïdane-Bchir, et al., 2016).

Algae are advantageous in terms of their growth rate and biomass generation. It can be grown using sewage, wastewater, and saltwater, such as oceans or salt lakes and unlike biomass for first and second generation biofuel it does not depend on land (Jambo et al., 2016a). Hence, resources, which could be utilized for human consumption, need not be allocated for the sole purpose of production of environment friendly algae derived biofuel. However, further research still needs to be done to enhance extraction process in order to make it financially competitive to petro diesel and other petroleum-based fuels (Saïdane-Bchir et al., 2016).

1.4 Lignocellulosic Biomass (Final Billion ton Vision report 2, 2005; USDA &DOA)

Biomass — including all plant and plant-derived materials along with animal manure, not just starch and sugar, but also oil crops already used for food and energy has great potential to provide renewable energy (Sun & Cheng, 2002). In addition to many benefits attributed to renewable energy, biomass is particularly attractive because it is the only current alternative renewable source of liquid transportation fuel (Tye et al., 2016). This, of course, makes it invaluable in reducing oil imports. Biomass also has great potential to provide heat and power to industry and to provide feedstock to make a wide range of chemicals and materials or bio-products (Woiciechowski et al., 2016).

Biomass mostly referring to organic material derived from plant matter derived from agricultural, forestry or industrial waste, or from parts of dedicated energy crops, has been identified as a possible alternative sustainable energy source for the production of biofuels due to its vast abundance and renewable nature. The global production of plant biomass amounts to approximately $2x10^{11}$ Mt per annum, of which between 8 and $20x10^{9}$ Mt is potentially accessible for processing (Lin & Tanaka, 2006). It is estimated that replacing fossils fuels with biofuels could decrease CO_2 emissions by 60-90% (Wang, Wu, & Huo, 2007). By far, the most successfully utilized commercial biofuel is bioethanol (Jeffries & Jin, 2004; Soccol et al., 2016; AFDC, (https://www.afdc.energy.gov/fuels/ethanol_fuel_basics.html) 2017) .

Ethanol production from lignocellulosic biomass is an alternative renewable energy source for liquid fuel, termed as bioethanol and/or biofuel. This approach is considered as carbon neutral as it has reduced greenhouse gas (GHG) emission which leads to significant socio-economic and environmental benefit (Kuhad *et al*., 2011) over fossil fuels. Use of second generation biomass for ethanol production is preferable over the traditional process that uses glucose rich hydrolysates derived from food crops like wheat and corn (Leandro *et al.,* 2006). This advantage comes into play because hemicellulose and lignocelluloses are major component of low value agriculture products and waste, other plant biomass, paper industries and wood pulping waste. Ethanol thus produced has various other uses besides liquid fuel for vehicles, such as organic solvent, germicide, antifreeze, a depressant and chemical intermediate for organic chemicals. Focus on ethanol as fuel source began after major energy crisis in 1970s. Since then it has been striking as the low-cost, sustainable and renewable energy source that has motivated researchers towards development of ethanol production (Favela *et al*., 1986). Production and use of bioethanol from biomass potentially reduces consumption of crude oil and environmental pollution.

In the context of Nepal, technology for modern bioenergy or biofuel production does provide promise of alternative energy. The agricultural wastes, plant biomass available, other organic wastes, all rich in lignocellulosic biomass can be used as raw materials or substrates for ethanol production. It also contributes to rural development along with addressing current energy crisis (Baral *et al.* 2013).

1.5 Components of lignocellulose

Lignocellulosic biomass comprising forestry, agricultural and agro-industrial wastes are abundant, renewable and inexpensive energy sources. Such wastes include a variety of materials such as sawdust, poplar trees, sugarcane, bagasse, waste paper, brewer's spent grains, switchgrass, straws, stems, stalks, leaves, husks, shells and peels from cereals like rice, wheat, corn, sorghum and barley, among others. Lignocellulose wastes are accumulated every year in large quantities, causing environmental problems, although being temporary in nature due to their eventual decomposition. (Shahzadi et al., 2014). However, due to their chemical composition based on sugars and other compounds of interest, they are frequently being used for production of various value added products, such as ethanol, food additives, organic acids, enzymes, and others. Therefore, accumulation of such biomass in nature not only creates problem but also loss of potentially valuable sources. The major constituents of lignocellulose are cellulose, hemicellulose, and lignin, polymers that are closely associated with each other constituting the cellular complex of vegetal biomass (McMillan, 1994; Sun & Cheng, 2002). Basically, cellulose forms a skeleton which is surrounded by hemicellulose and lignin where amount of each component varies on the basis of source and type of biomass (Weber et al., 2010; Farwick et al., 2014).

Cellulose is linear polysaccharide composed of up to 15,000 repeating units of the disaccharide cellobiose (two glucose molecules) linked by α β 1-4 glycosidic linkage and makes up most portion of dry weight, which is available for microbial fermentation (Mussatto & Teixeira, 2010).

Hemicellulose is a heteropolymer of pentose (xylose and arabinose) and hexose (glucose, mannose, and galactose) sugars. It is the second most abundant component in biomass that is classified into four groups (xylan, xyloglucan, mannan, and glucomannan) based on the sugar moieties making up the polysaccharide. Xylan is a homopolymer of xylose linked by β 1-4 glycosidic bonds, and is a major component of hardwood trees. Xyloglucan contains α,β 1-4 linked glucose backbone with mainly β 1-6 linked xylose and sometimes sugar moieties as side chains. Xyloglucans play role in linking adjacent cellulose microfibrils, via hydrogen bonding between xyloglucans and cellulose. The final types of hemicellulose mannans and glucomannans are composed of mannose homopolymers and heterpolymers of mannose

and glucose respectively that makes up the principal components of softwoods (Mussatto & Teixeira, 2010).

Lignin, the third major component of lignocellulosic biomass is responsible for the structural rigidity of plants. It is a heteropolymer comprised of p-hydroxyphenyl, guaiacyl, and syringyl monolignol units that form a complex branched network around cellulose microfibrils (Kricka, Fitzpatrick, & Bond, 2015; Mussatto & Teixeira, 2010).

1.6 *Saccharomyces cerevisiae* and novel xylose fermenting yeasts

Saccharomyces cerevisiae has larger size, thicker cell walls, better growth at low pH, less stringent nutritional requirements and greater resistance to contamination which provides it with the advantage as a commercial fermenter to produce ethanol from glucose. *Saccharomyces cerevisiae* is also preferred microorganism used to produce ethanol due to its excellent ability to ferment glucose in addition to its high tolerance to ethanol and inhibitors present in lignocellulosic hydrolysates after pretreatments (Limayem & Ricke, 2012; Weber et al., 2010). However, *S. cerevisiae* cannot naturally ferment pentose sugars like xylose and arabinose (Fonseca et al., 2011) for ethanol production. Therefore, various approaches including but not limited to genetic/metabolic engineering, cross breeding, and evolutionary engineering etc. are under investigation to integrate xylose and arabinose utilization pathways into *S. cerevisiae* (Kuyper et al., 2003, 2005; Jeffries, 2006).

There are some yeasts such as *Pichia stipites, P. segobiensis, Candida shehatae, Pachysolen tannophilus* that has the ability to ferment xylose to ethanol (Jeffries, 2006). Some microflora isolated from hindgut of Beetles also have shown xylose fermenting capabilities (Suh et al., 2005). Suh and Blackwell also isolated another novel xylose fermenting yeast *Enteroramus dimorbhus* from *Odondotaenius disjunctus* (a beetle species) which feeds on white rotten hardwood.

1.7 Xylose Isomerase for xylose to xylulose conversion

Xylose isomerase (EC 5.3.1.5) is an enzyme of family 'isomerase' that catalyzes inter conversion of D-xylose into D-xylulose (Mitsuhashi & Lampen, 1953), specifically those intra-molecular oxidoreductases interconverting aldoses and ketoses. Xylose-isomerases are also commonly called fructose-isomerases due to their ability to isomerize glucose and fructose (Marshall, Kooi, & Moffett, 1957). The systematic name of this enzyme class is D-xylose aldose-ketose-isomerase. Other names in common use include D-xylose isomerase, D-xylose ketoisomerase, and D-xylose ketol-isomerase. There are different species of bacteria that have been reported to produce xylose isomerase among them current study focuses on use

of codon optimized xylose isomerase from *Lachnoclostridium phytofermentans (strain ATCC 700394 / DSM 18823 / ISDg) (Clostridium phytofermentans).*

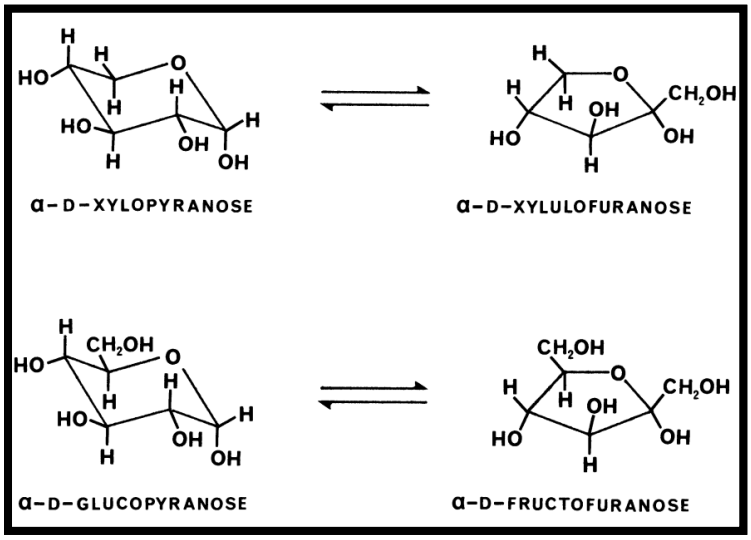

Figure 1.4 Reversible isomerization of D-xylose and D-glucose to their respective ketoses.

Xylose isomerase is a homomeric tetramer with varying molecular weight depending on the microbial source: 180 kD for *Streptomyces griseofuscus* (Kasumi et al., 1981), 165 kD for *Streptomyces albus* (Hogue-Angeletti, 1975), 120 kD for *Streptomyces olivochromogenes,* 130 kD for *Bacillus stearothermophilus* (Suekane et al., 1978), 183 kD for *Lactobacillus xylosus* (Yamanaka and Takahara, 1977), 185 kD for *Arthrobacter* strain N.R.R.L. B3728 (Smith et al., 1991), 196 kD for *Thermus aquaticus* HB8 (Dekker, Yamagata, Sakaguchi, & Udaka, 1991); Lehmacher and Bisswanger, 1990A), and 200 kD for *Clostridium thermohydrosulfuricum* (Dekker, 1991A) and for *Thermus thermophilus* (Dekker et al., 1991B). The E. *coli* isomerase is a dimer of subunits with a MW of 44 kD (Tucker et al., 1988). It is interesting that irrespective of variation in size of enzyme, their function remains same, this may be attributed to the conserved active site of enzyme.

1.8 Xylose Utilization by Microbes

In bacterial systems the xylose from degradation of hemicellulose is converted to xylulose by xylose isomerase and phosphorylated to xylulose-5-phosphate by xylulokinase, which is then channeled to the pentose phosphate pathway (Li et al., 2016). Most fungi and yeasts, however, require two enzymes in a two-step process to convert xylose to xylulose, Xylose reductase using NADPH for reducing xylose to xylitol, and NAD^+ linked D-xylitol dehydrogenase for the oxidation of xylitol to xylulose (Jeffries, 2006). There is considerable interest in introducing a bacterial xylose isomerase gene into yeast cells to achieve direct conversion of biomass (Chan, Ueng, & Chen, 1989). An alternative approach involves cloning

the genes for the ethanol pathway into *E. coli* to achieve a complete conversion of xylose to ethanol in a single stage process (Beall, Ohta, & Ingram, 1991). The most important utilization of xylose isomerase is recognized in the corn sweetener industry. For the industrial production of high-fructose corn syrup, the enzyme is immobilized in various ways, including whole cell entrapment, covalent crosslinking, and adsorption on cellular materials. The enzymes used in these processes are usually products of microorganisms obtained from sophisticated screening procedures to optimize thermal stability and operation conditions (Antri & Auterinen, 1986). Recent approaches include not only the cloning of native XI genes (*xyl*A) but also the cloning of codon optimized genes with or without adaptive mutagenesis and evolutionary adaptation for optimization of better growth and fermentation.

1.9 Problem Statement

The use of food and feed products as a substrate for biofuel has brought out concerns regarding increasing population and demand of food while cultivable land remains constant. Lignocellulosic biomass, especially when considered as a waste material from agriculture, paper industries, forest litters, animal wastes etc. offer an attractive alternative. However, the recalcitrant nature of these materials and inability of microorganisms to efficiently ferment sugars in lignocellulosic hydrolysates still prevent the efficient and economic production of bio-alcohols from these plentiful sources. There is no known organism which combines all the properties necessary to be a sustainable bio-alcohol producer. Therefore, breeding technologies, genetic engineering, evolutionary engineering and the search for undiscovered species are promising means to provide a microorganism that exhibits high alcohol productivities and yields, converting all lignocellulosic sugars or are even able to use carbon dioxide or monoxide, and being highly resistant to inhibitors and fermentation products which will ease up cultivation in huge bioreactors (Weber et al., 2010). The inability of *S. cerevisiae* to ferment other components of the lignocellulosic biomass with exception of hexose sugars like glucose into ethanol by endogenous metabolic system acts as the roadblocks in the way of making economically viable second generation bioethanol as substitute for fossil fuels. When it comes to the pentoses like xylose and arabinose, their use in metabolism by yeast is absent and /or insignificant (Kuyper et al., 2005). This reluctance in xylose metabolism by yeast is attributed to poor import of xylose from surrounding to cell due to lack of efficient transporters as well as effective enzyme system to drive the process towards xylose fermentation (Jeffries, 2006; Kuyper et al., 2003).

1.10 Objectives / research questions

General objective

The aim of this research is to genetically improve xylose metabolism in *Saccharomyces cerevisiae* for enhanced ethanol production.

Specific objectives

i. Construction of xylose isomerase yeast expression system by sub-cloning codon optimized synthetic XI gene from *Clostridium phytofermentans* into *Saccharomyces cerevisiae.*

ii. Transformation of *Saccharomyces cerevisiae* with Xylose isomerase yeast expression construct.

iii. Confirmation of Xylose isomerase expression/transformation by PCR, Restriction Digestion/fluorescence microscopy and Southern Blotting.

iv. Comparative analysis of the transformed yeast and control yeast, with regard to their growth kinetics, xylose metabolism and ethanol production.

1.11 Research Hypothesis

"Genetically modified *Saccharomyces cerevisiae* with the expression of xylose isomerase can metabolize xylose and the yeast can ferment xylose of lignocellulosic biomass with increased efficiency in bio-ethanol production."

1.12 Significance / Rationale of the study

The major limitation or shortcomings of *S. cerevisiae* in ethanol production is its inability to metabolize hemicelluloses such as xylose. This is attributed to lack of efficient transport system and intrinsic metabolic pathway for yeast to utilize xylose. Hence, efficient xylose conversion to ethanol can be attained by introducing xylose transporters for example GXS1 and GXF1of *Candida intermedia* and optimization of successive downstream metabolic pathway for xylose metabolism. GXS1 and GXF1 are the glucose/xylose symporter and glucose/xylose facilitator genes from *Candida intermedia* which results in the uptake of xylose while Xylose isomerase converts the xylose to xylulose, which is further utilized in pentose phosphate pathway. Previous studies with individual transformation of *S. cerevisiae* with GXS1 and GXF1 showed significant uptake of xylose from the medium with only slight increase in ethanol production, which could be attributed to the inability of the yeast to metabolize xylose. For this purpose, a synthetic gene, Xylose isomerase (XI) from *Clostridium phytofermentans* has been synthesized and currently being cloned into yeast expression plasmid. The proposed study will help to understand the ability of a recombinant strain of *S. cerevisiae* expressing xylose isomerase to metabolize xylose even in the absence of specific transporters. In absence of xylose transporters, xylose can still enter the yeast cell through hexose transporters. Thus transported xylose can only be utilized after it is converted to xylulose which is then phosphorylated by xylulose kinase into xylulose-5-phosphate.

CHAPTER II

LITERATURE REVIEW

2.1 Lignocellulose structure and composition

Lignocellulose, as the name suggests comprise of three different homo and heteropolymers namely Cellulose, Hemicellulose and Lignin (Maurya, Singla, & Negi, 2015; Saini, Saini, & Tewari, 2015). Cellulose and hemicellulose is bound together by lignin to allow structural integrity and protection against degradation, thereby acting as recalcitrant (Saha, 2003).

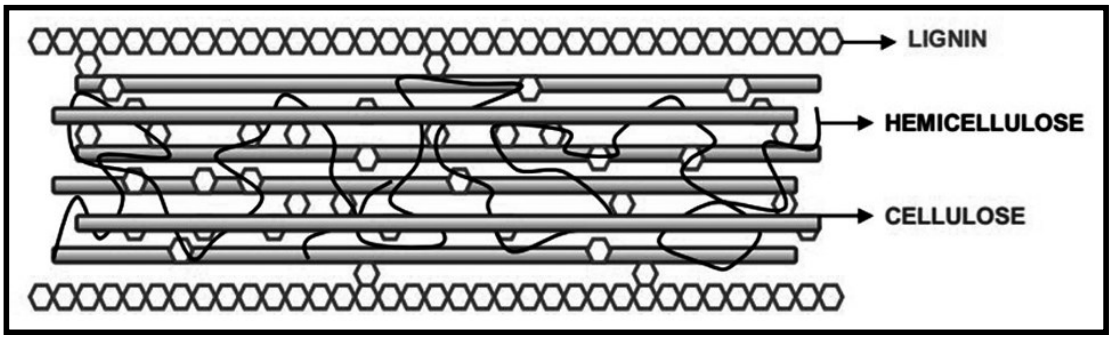

Figure 2.1: Pictorial representation of Lignocellulose structure (Adapted from Muktham et al., 2016)

Lignin is a very complex molecule made up of phenylpropane units linked in a large three-dimensional structure. Three phenyl propionic alcohols exist as monomers of lignin: p-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol (Boerjan, Ralph, & Baucher, 2003; Vanholme et al., 2010).

In vascular plants, Lignin follows cellulose in abundance. Lignin has major importance for plant physiology and development: mechanical support to plant organs, upright growth and large sizes, provides strength and rigidity, transport of water and solutes in the vascular system due to its hydrophobicity and mechanical resistance and protection against pathogens (Boerjan et al., 2003; Boudet, 2000; Lourenço et al., 2016; Rencoret et al., 2011). Lignin is closely bound to cellulose and hemicellulose that provides rigidity and cohesion to cell wall, which confer water impermeability to xylem vessels, and to form a physico-chemical barrier against microbial attack (Dietrich, Fengel & Wegener, 1989). Molecular configuration of lignin confers it with extreme resistance to chemical and enzymatic degradation (Palmqvist & Hahn-Hägerdal, 2000b). The amounts of carbohydrate polymers and lignin vary from one plant species to another and within same plant depending on condition and growth stage of plant. On hydrolysis, lignin is reported to yield inhibitors like phenolics which present challenges for cellulase activity and in turn reduces ethanol yield.

In all plant derived biomass, cellulose is usually the dominant structural polysaccharide of plant cell walls (35–50%), followed by hemicellulose (20–35%) and lignin (10–25%) (Mussatto & Teixeira, 2010; Saha, 2003). The relative proportion of lignin monomers varies among plants and variation depends on the tissue, cell location or environmental conditions. The lignin molecule has a high chemical flexibility, i.e., the plant produces a lignin with a specific composition depending on the precursors that are been deposited in the lignifying zone (Boudet, 2000). Average Values Of The Main Components In Some Lignocellulose Wastes Are Shown In Following Table (Jørgensen et al., 2007) .

Table 2.1: Percent composition of lignocellulose components in various lignocellulosic materials (Adopted from Iqbal et al., 2013).

Lignocellulosic material	Lignin (%)	Hemicellulose (%)	Cellulose (%)
Sugar cane Bagasses	20	25	42
Sweet sorghum	21	27	45
Hardwoo	18-25	24-40	40-55
Softwood	25-35	25-35	45-50
Corn cobs	15	35	45
Corn stover	19	26	38
Rice straw	18	24	32.1
Nut shells	30-40	25-30	25-30
Newspaper	18-30	25-40	40-55
Grasses	10-30	25-50	25-40
Wheat straw	16-21	26-32	29-35
Banana waste	14	14.8	13.2
Bagasse	23.33	16.52	54.87
Sponge gourd fibers	15.46	17.44	66.59

Cellulose is a high molecular weight linear homopolymer of repeated units of cellobiose (two anhydrous glucose rings joined via a β-1,4 glycosidic linkage). Degree of polymerization can range approximately from 4000-8000 glucose residue giving the estimated size of 200-2000 kDa (Aristidou & Penttila, 2000). The long-chain cellulose polymers are linked together by hydrogen and Van der Walls bonds, which cause the cellulose to be packed into microfibrils (M.-A. Ha et al., 1998; Ho, Chen, & Brainard, 1998). By forming these hydrogen bonds, the chains tend to arrange in parallel and form a crystalline structure. Therefore, cellulose microfibrils consists both highly crystalline regions around 2/3 of the total cellulose and 1/3 of less-ordered amorphous regions. More ordered or crystalline cellulose is less soluble and less degradable (Taherzadeh & Karimi, 2007; Zhang & Lynd, 2006; Zhang & Lynd, 2004).

Hemicellulose is a linear and branched heterogeneous polymer typically made up of five different sugars L-arabinose, D-galactose, D-glucose, D-mannose, and D-xylose as well as other components such as acetic, glucuronic, and ferulic acids. The backbone of the chains of hemicelluloses can be a homopolymer (generally consisting of single sugar repeat unit) or a heteropolymer (mixture of different sugars) (D Fengel & Wegener, 1984). Based on main sugar residue in the backbone, hemicellulose are classified as xylans, mannans, glucans, glucuronoxylans, arabinoxylans, glucomannans, galactomannans, galactoglucomannans, β-glucans, and xyloglucans. Hemicelluloses differ from cellulose by composition of sugar units, presence of shorter chains, branching of main chain molecules, and nature being amorphous (Fengel & Wegener, 1989), that makes it more hydrolysable than cellulose.

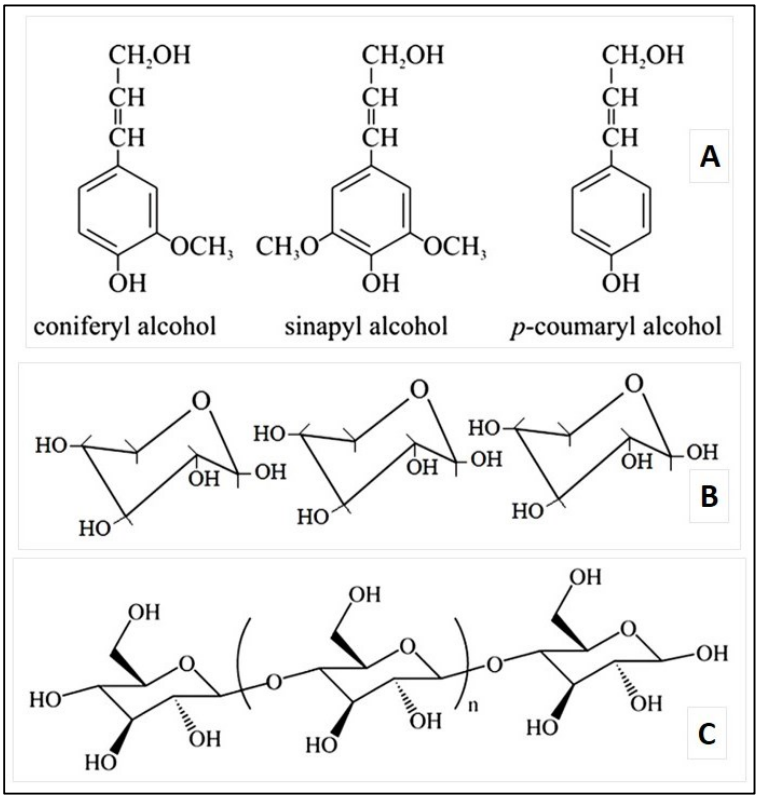

Figure 2.2: Chemical structure of lignocellulosic material- (A) Building blocks/units of Lignin; (B) Xylose unit of hemicellulose; and (C) Cellulose. (Adopted from Iqbal et al., 2013).

2.2 Lignocellulose and Second Generation (advanced) bioethanol

Second generation biofuels, referred as advanced biofuels, are those that can be manufactured from various types of biomass. Mostly, the second generation biofuel refers to biofuel produced from lignocellulosic and hemicellulosic biomass including but not limited to agricultural waste, forest litters, black liquor from paper manufacturers, animal wastes, remains of crop and/or any organic wastes other than dedicated energy crops like corn, wheat, sugarcane etc. used in traditional technology of fermentation. The term 'Biomass'

here signifies any source of organic carbon that is renewed rapidly as part of the carbon cycle derived from plant materials but can also include animal materials (Azadi et al., 2013; Ramirez et al., 2015). Aristidou and Pentilla in 2000 listed out possible substrates for second generation bio-ethanols which include agricultural residues (corn stalks, corn stover, wheat straws, potato or beet wastes etc.), wood residue (leftovers from harvested wood, forest litters etc.), specifically grown plants (hybrid poplar, black locust, willow, silver maple, sugarcane, sugar beet, extensively growing grasses like switch-grass etc.), waste streams (municipal solid waste, paper baggages) etc.

First generation biofuels are made from sugars and vegetable oils found in arable crops, which can be easily extracted using conventional technology. In comparison, second generation biofuels that are made from lignocellulosic biomass or woody crops, agricultural residues or waste, are difficult to extract. A series of physical and chemical treatments are required to convert lignocellulosic biomass to liquid fuels suitable for transportation (Ramirez et al., 2015). Even though the starting material in this approach is much more complex than that used in first-generation biofuel production, and requires thermochemical and/or biological pretreatments, the approach is of greater interest due to renewable nature and possible replacement of fossil fuel. The pretreatment step aims to increase cellulose and hemicellulose accessibility, generally through alteration of physical structure of cellulose and solubilization of hemicellulose and lignin fractions of biomass. These polymers are further hydrolysed to release fermentable sugars hexoses and pentoses (Chandel et al., 2015; Chen et al., 2013; Harmsen et al., 2010).

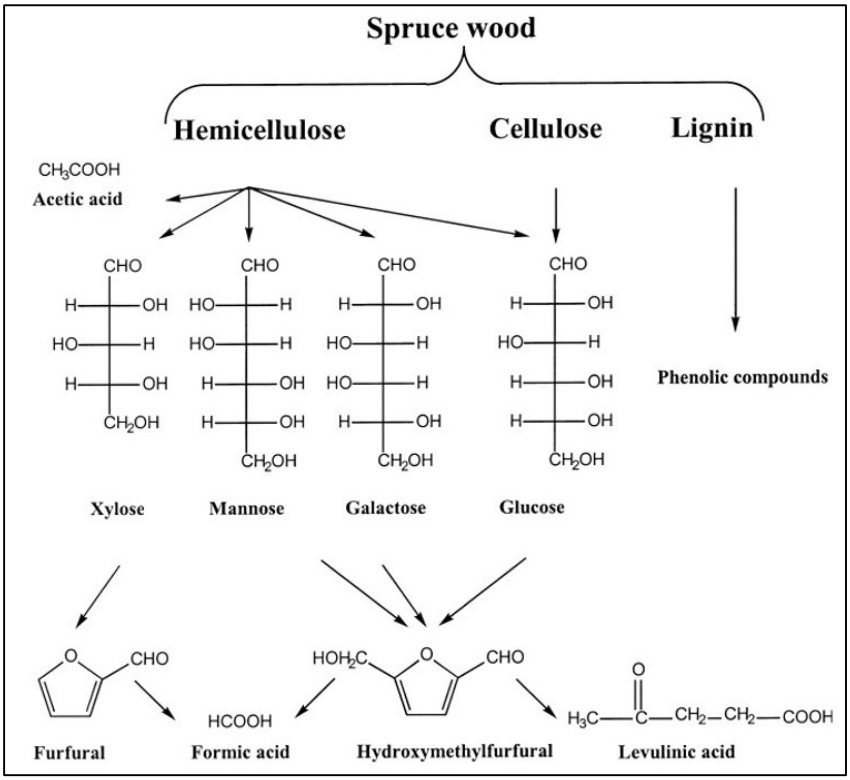

Figure 2.3: Components of lignocellulosic hydrolysates and their derivatives (Palmqvist & Hahn-Hägerdal, 2000a).

Bio-alcohols produced by microbial metabolism from renewable materials are promising substitutes for traditional source of fossil fuels. Ethanol has been produced in large amounts from feed-stocks such as cereals or sugarcane and used as a blend for gasoline or even as a pure biofuel. Use of dedicated energy crops brings out the social and ethical issue regarding food available and demand of present and future (Goh & Lee, 2010; Kagan, 2015).

There are several strategies under investigation for the production of second generation/advanced bioethanol. These strategies differ slightly from one another in terms of approach and steps used for fermentation of feedstock (Chung et al., 2014). Conventional model of second generation bioethanol production begins with pretreatment of lignocellulosic/hemicellulosic biomass followed by chemical or enzymatic hydrolysis to derive fermentable hexose and pentose sugars. These sugars are then fermented to yield ethanol. There are number of suitable organisms that have been identified or genetically engineered for the process with number of optimized conditions, *Saccharomyces cerevisiae* being the most studied organism (Brat et al., 2009; Kuyper et al., 2003). Similarly, Consolidated Bio-Processing models for multistep and single step conversion of biomass into bioethanol are also under investigation. These all concept are of significance to reduce the economic price in final product (Chung et al., 2014).

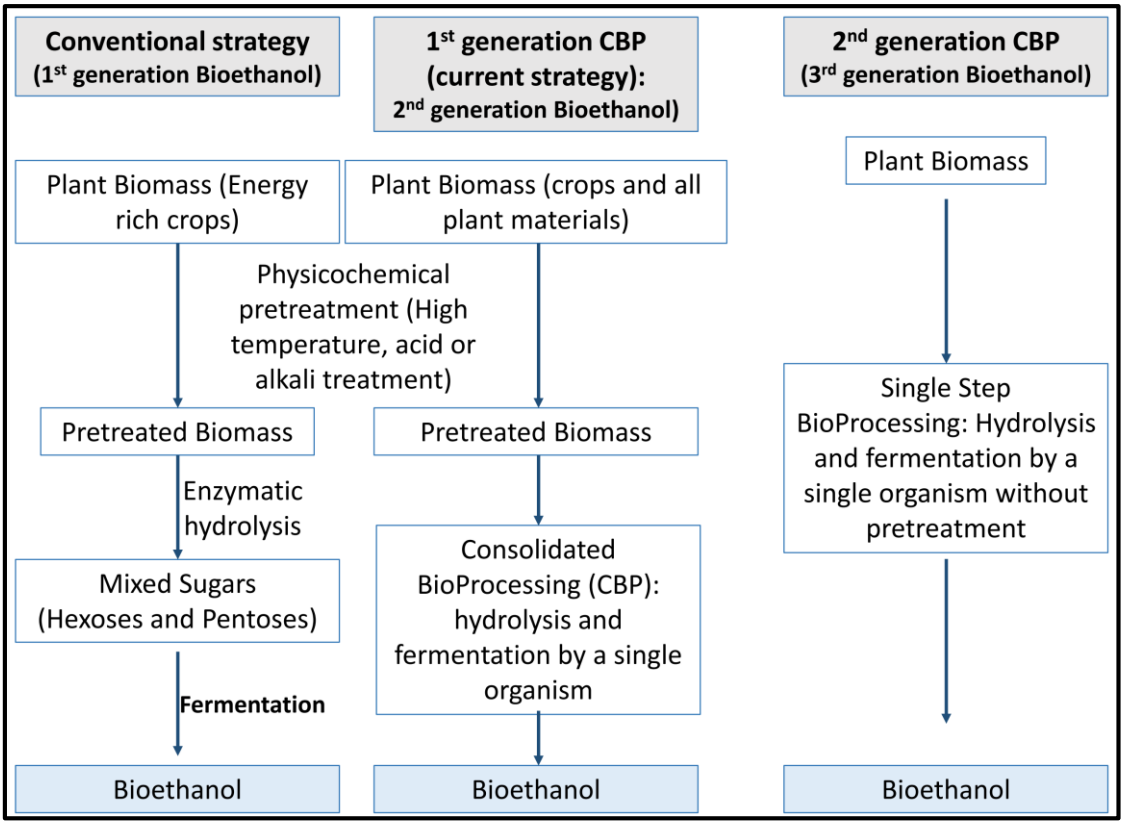

Figure 2.4*:* Different strategies of second generation bioethanol production from lignocellulose (Adapted from Chung et al., 2014).

2.4 Ethanol production from Lignocellulose

Bacteria such as *Zymomonas mobilis* and engineered *Escherichia coli* strains are capable of homoethanolic fermentation of sugars (Bi et al., 2009; Förster et al., 2014) however *Saccharomyces cerevisiae* remains the organism of choice for large-scale industrial production of ethanol (Soccol et al., 2010b, 2016). Factors contributing to the popularity of *S. cerevisiae* as an industrial ethanol producer include its high ethanol tolerance, its ability to grow under strictly anaerobic conditions and an important characteristic distinguishing it from prokaryotic organisms i.e. its insensitivity to bacteriophage contaminations. Moreover, *S. cerevisiae* grows well at low pH, reducing problems with contamination of industrial processes such as lactic acid bacteria (Maris et al., 2007). *S. cerevisiae* is naturally able to convert glucose into ethanol but it is unable to convert xylose, which is one of the predominant pentose in lignocellulose/hemicellulose (Leandro et al., 2006) and the study focuses on enabling the yeast with ability to use xylose for ethanol production.

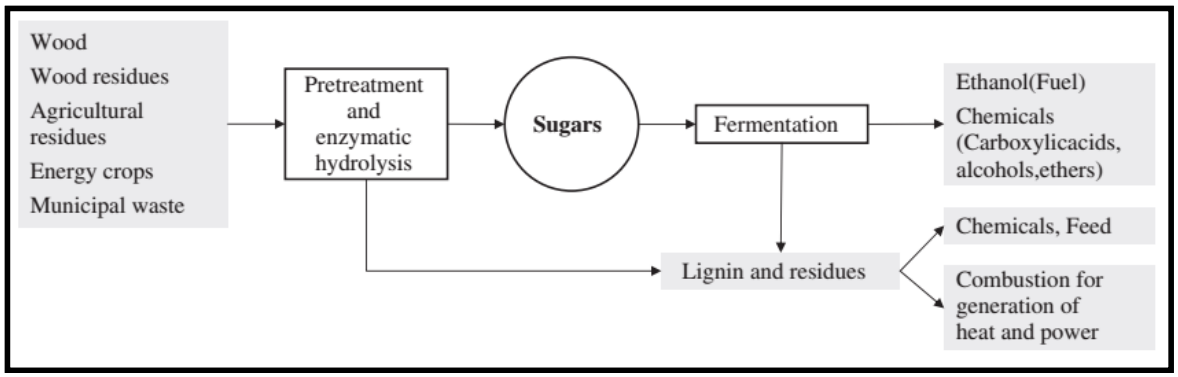

Figure 2.5: Overview of an integrated bio-refinery producing fuel, chemicals and energy from lignocellulosic materials.

Efficient conversion of xylose to ethanol in *S. cerevisiae* is limited by multiple issues including cellular redox imbalance, low flux of xylose into the pentose phosphate pathway, and lack of efficient xylose transport into the cell (Hector et al., 2008).

Since the completion of genome sequencing of *Pichia stipites* and development of efficient transformation system, metabolic engineering of xylose fermentation is proceeding rapidly (Jin et al., 2003).

Technologies are being developed for converting non-food biomass to ethanol. It is estimated that current biomass waste could be converted to 50 billion gallons of ethanol with the potential for significant increases (up to 350 billion gallons) using dedicated energy crops (Mosier et al., 2005; Wyman, 1994).

2.5 Ethanol production pathway

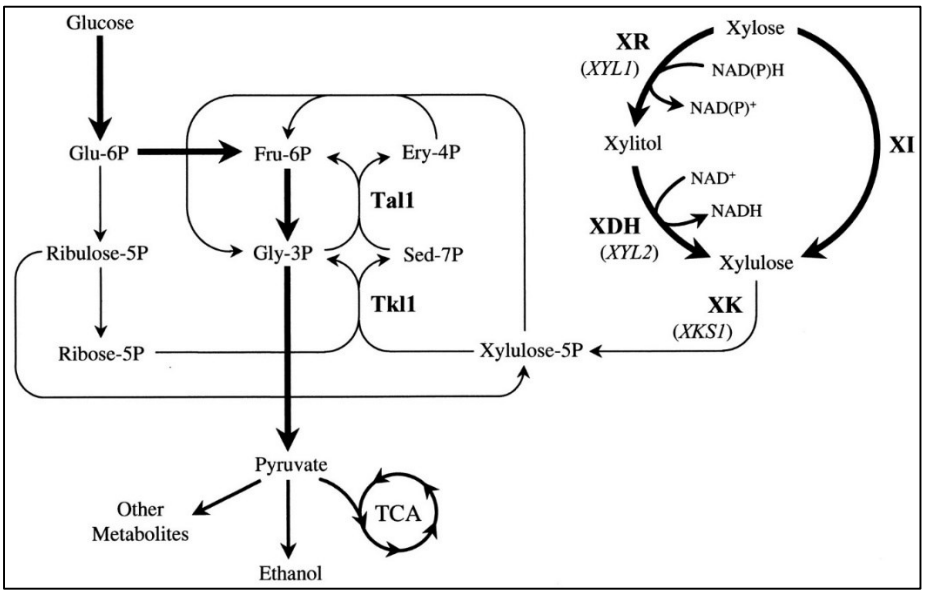

Figure 2.6: Overview of ethanol production by microbes (Adapted from McMillan, 1993).

2.6 Xylose as substrate for Ethanol production

Catabolism of both xylose in all microorganisms requires conversion of sugar to D-xylulose-5-phosphate. However, the pathways to convert D-xylose to D-xylulose-5-phosphate are distinctly different in bacteria and fungi. In bacteria, D-xylose is converted to D-xylulose by an isomerase (EC 5.3.1.5) and then phosphorylated by xylulokinase (EC 2.1.7.53). In fungi, pentose sugar goes through oxidation and reduction reactions before they are phosphorylated by xylulokinase. D-xylose is reduced to xylitol by a reduced nicotinamide adenine dinucleotide phosphate (NADPH)-consuming reaction and xylitol is then oxidised by an NAD+-consuming reaction to form D-xylulose. D-xylulose then enters the Pentose Phosphate Pathway (PPP) after phosphorylation to D-xylulose-5-phosphate by Xylulokinase (Fernandes & Murray, 2010; Dragone et al., 2010).

Xylose can be used by bacteria *E. coli* and some fungi like *Orpinomyses* sp. and yeast *Pichia stipitis*. But the use of xylose for ethanol production is limited in terms of the feedback effect of the ethanol produced as it tends to exhibit the antimicrobial effect (Agbogbo & Coward-Kelly, 2008; Govindaswamy & Vane, 2007). However most dominant and widely used ethanol producer *S. cerevisiae* is able to withstand the effect of ethanol but is unable to use it unless is converted to Xylulose. Xylulose is then phosphorylated into Xylulose 5 phosphate by xylulokinase (XKS) in expense of an ATP and enters into pentose phosphate pathway. Finally, it moves towards ethanol formation as Glycealdehyde 3 phosphate via EMP pathway or Glycolysis (Jeffries, 2006; McMillan, 1993b). Possibility for fermentation of D-xylose to equimolar amounts of ethanol and CO_2 under anaerobic conditions in engineered *S. cerevisiae* is reported by Fernandes & Murray, 2010 with optimal aeration.

2.7 *Saccharomyces cerevisiae* as bioethanol producer

Saccharomyces cerevisiae, also known as baker's yeast and brewer's yeast, is commonly used organism for the production of ethanol. It is named so because of its usual involvement i.e. use in baking and brewing industries since time immemorial (Kanan *et al.* 1998). *S. cerevisiae* is an eukaryotic microorganism investigated thoroughly to aid our understanding of the biology of the eukaryotic cells and organisms and hence, ultimately, human biology. For several centuries, *S. cerevisiae* has been used in the production of food and alcoholic beverages, and today this organism is also used in a number of different processes within the pharmaceutical industry. *S. cerevisiae* is a very attractive organism to work with since it is nonpathogenic, and due to its long history of application in the production of consumable products such as ethanol and bakery items, it has been classified as a GRAS (Generally Regarded As Safe) organism (Ostergaard, 2000). Also, the well-established fermentation and process technology for large-scale production with *S. cerevisiae* makes this organism ideal

for several biotechnological purposes. Applicability of *S. cerevisiae* within the field of biotechnology is also due to its high susceptibility to genetic modifications by recombinant DNA technology, rising tool for genetic engineering, further facilitated by the completion of whole genome sequence of *S. cerevisiae*, published in 1996 (Goffeau et al., 1996). Although *S. cerevisiae* can grow well even at relatively low pH preventing contamination by other bacteria, native strains are unable to utilize xylose for growth or fermentation which comprises of about 30-40% in hemicellulosic hydrolysates (Chandrakant & Bisaria, 1998; Katahira et al., 2004). Instead, it metabolizes D-xylulose, an isomerization product /a keto-isomer of D-xylose (Matsushika et al., 2009; Richard et al., 2000).

Conventional strain improvement method for baker's and brewer's yeasts is based on random mutagenesis or classical breeding and genetic crossing of two strains followed by screening for mutants exhibiting enhanced properties of interest. With the developments of sophisticated methods in the field of recombinant DNA technology, genetic manipulation in pathway of interest to improve the cell efficiency by a more directed approach has been made feasible. It is now possible to introduce specific genetic modifications in terms of promoter strength of a given gene, gene deletions, and insertion of new genes or metabolic pathways into the cell. Directed improvements of the cellular properties from interdisciplinary theoretical analysis and modelling relying on biochemical information, and application of genetic engineering is referred as metabolic engineering (Bailey, 1991; Stephanopoulos, Aristidou, & Nielsen, 1998). Metabolic engineering approach consists of two important parts: (i) the analytical side of metabolic engineering dealing with analysis of the cells to identify most promising target(s) for genetic manipulation, and (ii) genetic engineering of cells, where cell with genetic modifications is constructed (Ostergaard, 2000).

Saccharomyces cerevisiae is currently used to produce ethanol from glucose, but it cannot utilize five-carbon sugars contained in the hemicellulose component of biomass feed-stocks. Hemicellulose can make up to 20–30% of biomass and is primarily composed of xylose (Saha, 2003). Enzymes from native xylose-assimilating organisms have been transferred to *S. cerevisiae* allowing fermentation of xylose (Jeffries, 2006; Karhumaa et al., 2007). *S. cerevisiae* lacks efficient transporter for xylose, instead sugar enters into cell by non-specific hexose transporter (HXT) (Leandro et al., 2006, 2008) with low affinity (Km approx. 100mM). Attempts have been made to develop recombinant S. cerevisiae with high affinity xylose-H^+ symporter in order to increase fermentation of xylose (Sedlak & Ho, 2004a, 2004b).

2.8 Approaches for Ethanol production from Xylose

There are two pathways known for metabolism of xylose in nature (Katahira et al., 2006). Prokaryotes mainly bacteria uses an enzyme system of xylose isomerase encoded by *xyl*A

gene for isomerization of xylose to xylulose. Beside bacteria, fungi like *Piromyces* and *Orpinomyces* species are also reported to use this pathway. On the other hand, second pathway common in yeast (eg. *Pichia stipitis*, *Candida Shehatae*) and some fungi and few plants include two enzymes Xylose reductase (XR) and Xylitol dehydrogenase (XDH) encoded by *XYL*1 and *XYL*2. Enzymes XR and XDH are differentially dependent on cofactors NADPH and on NAD+ respectively for their activity (Chiang & Knight, 1960a; Katahira et al., 2006; Madhavan et al., 2009).

Based on these facts, two heterologous xylose-assimilating pathways are currently under construction in order to engineer xylose-fermenting *S. cerevisiae*. First one involves an enzyme xylose isomerase (XI) (Brat et al., 2009; S. J. Ha, Kim, Choi, Park, & Jin, 2011; Karhumaa et al., 2007), another uses xylose reductase (XR) and xylitol dehydrogenase (XDH) (Ho et al., 1998; Y.-S. Jin et al., 2003; Meaden et al., 1994). Both pathways intend on conversion/isomerization of xylose into xylulose which then require overexpression of xylulokinase (XKS) that mediates xylulose to the endogenous pentose phosphate pathway of *S. cerevisiae* by phosphorylation (Jin & Alper, 2005; Richard et al., 2000; Wahlbom et al., 2003).

In any hydrolysis methods for lignocellulose biomass, glucose and xylose are the most abundant sugars in cellulosic hydrolysates i.e. 60–70% glucose and 30–40% xylose (Mosier et al., 2005). Therefore, efficient and rapid utilization of xylose is a pre-requisite for producing biofuels and chemicals from renewable biomass sustainably and economically (Hahn-Hägerdal et al., 2006). Naturally, xylose-fermenting microorganism are unable to ferment xylose under industrially relevant fermentation conditions of high osmotic levels, strict anaerobic environment and/or with fermentation inhibitors that are inevitably present in cellulosic hydrolysates from pretreatments (Jeffries & Jin, 2004; Jeffries et al., 2007; Rudolf et al., 2008). Therefore, numerous approaches including but not limited to metabolic engineering, evolutionary and strain engineering are in the pipeline to introduce xylose metabolic pathways into *Saccharomyces cerevisiae*, which is the most widely used microorganism for industrial production of ethanol from corn or sugarcane.

A number of attempts to express bacterial *XYL*A coding for XIs were have been heterologously expressed in *S. cerevisiae*, including *XYL*A from *Escherichia coli* (Sarthy et al., 1987), *Clostridium thermosulfurogenes* (Moes, Pretorius, & Zyl, 1996), *Bacillus subtilis* or *Actinoplanes missouriensis* (Amore & Hollenberg, 1989), *Thermus thermophilus* (Walfridsson et al., 1996) and *Streptomyces rubiginosus* (Gárdonyi & Hahn-Hägerdal, 2003).

2.9 Genetic manipulation in *Saccharomyces cerevisiae* to enable xylose metabolism

The conversion of xylose to xylulose can be mediated by two different pathways. Most xylose-assimilating eukaryotes (e.g., *Pichia stipitis*, *Candida shehatae*, and *Pachysolen tannophilus*) convert xylose to xylulose by two step redox reactions, catalyzed by the predominantly NADPH dependent xylose reductase (XR) followed by NAD+ dependent xylitol dehydrogenase (XDH), with xylitol as the pathway intermediate (Chiang & Knight, 1960b; Katahira et al., 2006). Such yeasts and recombinant *S. cerevisiae* strains producing these enzymes can ferment xylose to ethanol. However, under anaerobic conditions, the different coenzyme specificities of XR and XDH generate a cofactor imbalance which results in considerable accumulation of xylitol as a by-product and reduces the yield of ethanol (Jeffries & Jin, 2004; Pitkänen et al., 2003). In the second pathway, the metal ion-dependent isomerization of xylose to xylulose, catalyzed by the enzyme xylose isomerase (XylA), alleviates the excessive production of xylitol (Chandrakant & Bisaria, 1998).

The XylA pathway is functional in a majority of prokaryotes (e.g., *Escherichia coli, Streptomyces* sp.), a few fungi (e.g., *Piromyces, Orpinomyces*), and plants (*Hordeum vulgare*, *Oryza sativa*, and *Arabidopsis thaliana*) (Gárdonyi & Hahn-Hägerdal, 2003; Hamacher et al., 2002; Harhangi et al., 2003; Sarthy et al., 1987). Most of the attempts to functionally express bacterial xylose isomerases in yeast have failed. In case of heterologous expression of *Clostridium thermosulfurogenes* and *Actinoplanes missouriensis xyl*A genes, no protein product or enzyme activity could be detected, although xylose isomerase-specific mRNA was present (Amore & Hollenberg, 1989; Moes et al., 1996). The expression of *Bacillus subtilis, E. coli*, and *Streptomyces rubiginosus xyl*A in yeast resulted in predominantly insoluble, catalytically inactive proteins, possibly due to improper folding of prokaryotic enzymes by eukaryotic host (Amore & Hollenberg, 1989; Gárdonyi & Hahn-Hägerdal, 2003; Sarthy et al., 1987). The enzyme from *Thermus thermophilus* is the only bacterial XylA to be heterologously expressed in active form in yeast (Walfridsson et al., 1996). However, the optimum temperature of the *T. thermophilus Xyl*A was 85°C and it retained only 4% of its activity at 30°C, resulting in the poor fermentation of xylose by the recombinant yeast (Walfridsson et al., 1996). Recently, the xylA gene from *Piromyces* was functionally expressed at high level in *S. cerevisiae* and the recombinant strain exhibited slow growth on xylose medium (Kuyper et al., 2003a).

Recombinant strains of *S. Cerevisiae* with the ability to co-ferment glucose and xylose have been constructed by heterologous expression of the genes XYL1 and XYL2 encoding for an NADPH-dependent Xylose Reductase and a NAD^+ dependent Xylitol Dehydrogenase, and by enhancing expression of the endogenous Xylulokinase converting xylose into xylitol and xylulose, respectively. Xylulose is then phosphorylated by yeast xylulokinase and guided into

the pentose phosphate pathway (Ho et al., 1998). However, a large amount of xylitol was produced in these recombinant strains reducing ethanol yields. This has been primarily attributed to the difference in coenzyme specificities between the strictly NAD^+ dependent xylitol dehydrogenase and the mainly NADPH dependent xylose reductase, resulting in an intracellular cofactor imbalance (Kötter & Ciriacy, 1993).

Metabolic flux analysis suggests that the redox cofactor imbalance limits ATP production and thus growth. The redox imbalance has recently been addressed by either adding an $NADP^+$ dependent D-glyceraldehyde-3-phosphate dehydrogenase to help regenerate NADPH or by replacing xylose reductase with a mutant enzyme that has a higher affinity for NADH and thus uses less NADPH. Alternative approach in order to bypass the redox imbalance issue can be met by replacing the reductase and dehydrogenase with one enzyme Xylose isomerase (XI) that can be expressed in yeast. The isomerase enzyme inhibited by xylitol can be prevented by deletion of aldose reductase (gre3) coding gene to minimize xylitol production from xylulose and achieve higher yields and rates (Gray *et al*., 2006).

In currently available recombinant yeasts, xylose uptake occurs solely via the hexose facilitators (Hxt proteins and Gal2) that also accept xylose as a substrate but with very low affinity Km 100 mM (Hamacher et al., 2002). Hence, xylose uptake is slow and dependent on the concentration of glucose present in the environment or media. On the contrary, an engineered *S. cerevisiae* strain selected for efficient xylose fermentation exhibits significantly improved xylose transport kinetics (Kuyper et al., 2005). Heterologous expression of a high-affinity xylose transporter and xylose isomerase is therefore expected to represent an asset for the xylose-fermenting *S. cerevisiae* strains in terms of xylose import followed by elevated ethanol production as limiting factors have already been optimized (Karhumaa et al., 2007; Karhumaa et al., 2005) in it.

The kinetics of an anaerobic fermentation of glucose-xylose-arabinose mixtures were greatly improved by using a novel evolutionary engineering strategy. This strategy included a routine of repeated batch cultivation with repeated cycles of consecutive growth in three media with different compositions (glucose, xylose, and arabinose; xylose and arabinose; and only arabinose) followed by rapid selection of an evolved strain (IMS0010) exhibiting improved specific rates of consumption of xylose and arabinose. Engineered *S. cerevisiae* strain (strain IMS0003) was capable of fermenting mixtures of glucose, xylose, and arabinose with a high ethanol yield (0.43 g g-1 of total sugar) without formation of the side products xylitol and arabinitol (Wisselink et al., 2009).

Park et al. in 2013 prepared recombinant *Saccharomyces cerevisiae* which expressed xylose isomerase (XI) gene isolated from *Clostridium phytofermentans* that render it with ability to metabolize xylose and use it as a carbon and energy source. Engineered strain was able to

use up 23.48 gm/lit. of xylose from media containing 40gm/lit. xylose and 20 gm/lit. galactose.

Madhavan et al. on 2008 reported for the heterologous expression of xylose isomerase from rumen fungus *Orpinomyces* species. In the study, gene of 1314 bp was cloned and expressed in *Saccharomyces cerevisiae* resulting enzyme of 437 amino acid chain with character similar to family II xylose isomerase. Enzyme was found to be homodimer with a subunit of 49 kDa mass. In the study, expression of xylose isomerase was accompanied by overexpression of endogenous xylulokinase gene and *Pichia stipitis SUT*1 gene for sugar transport in order to attain production of ethanol from xylose efficiently.

2.10 Sources of gene(s) for genetic manipulation of *S. cerevisiae* for xylose metabolism

2.10.1 *Candida intermedia*

C. intermedia has shown to express two genes GXS1 (glucose/xylose symporter1) and GXF1 (glucose/xylose facilitator 1), responsible for transport of xylose into the cell (Leandro et al., 2006, 2008). Hence, the transporter genes are retrieved from it and introduced in *S. cerevisiae* to enhance the xylose import into cell. Runquist et al., at 2008 also reported to transform glucose/xylose facilitator Gxf1 gene from *C. intermedia* to *S. cerevisiae.*

2.10.2 *Clostridium phytofermentans*

Clostridium phytofermentans is an obligate anaerobic, rod-shaped, gram-positive bacterium forming spherical spores. Strains of this species are able to produce ethanol, acetate, carbon dioxide, and hydrogen gas by fermentation of cellulose, polygalacturonic acid, and other compounds common in plant biomass. Ethanol producing ability of this strain is attributed to endogenous metabolism with production of Xylose isomerase (XI) enzyme (Kuyper et al., 2005; Lee et al., 1990). This enzyme is studied in detail and gene responsible *xyl*A has been successfully synthesized in-vitro. The gene is also codon-optimized so that it is functionally expressed in eukaryotes like *S. cerevisiae* (Brat et al., 2009; Jin et al., 2012).

2.10.3 *Orpinomyces* and *Piromyces* species

Heterologous Introduction of xylose isomerase into *S. cerevisiae* is not limited to the bacterial origin. Some fungi are found to express bacterial like xylose isomerase for xylose metabolism instead of XR/XDH enzyme system. Among them most commonly used are Xylose isomerase from polycentric fungus *Orpinomyces* species (Madhavan et al., 2009) and anaerobic

cellulolytic fungus *Pyromyces* species (Kuyper et al., 2003b, 2005) are mostly studied ones for their expression in *S. cerevisiae*.

2.11 Xylose isomerase

Xylose isomerase (EC 5.3.1.5) is an enzyme of family 'isomerase' that catalyzes inter conversion of D-xylose into D-xylulose (Mitsuhashi & Lampen, 1953), specifically those intra-molecular oxidoreductases interconverting aldoses and ketoses. Xylose-isomerases are also commonly called fructose-isomerases due to their ability to isomerize glucose and fructose (Marshall, Kooi, & Moffett, 1957). The systematic name of this enzyme class is D-xylose aldose-ketose-isomerase. Other names in common use include D-xylose isomerase, D-xylose ketoisomerase, and D-xylose ketol-isomerase. *Lachnoclostridium phytofermentans (strain ATCC 700394 / DSM 18823 / ISDg) (Clostridium phytofermentans)* xylose isomerase is an enzyme of 438 aminoacid residue with mass of 48,713 Da (UniProt).

A thermostable xylose (glucose) isomerase cloned from *Streptomyces chibaensis* J-59 with open reading frame of xylA (1167 bp) encoding protein of 388 amino acids and calculated molecular mass of about 43 kDa showed high sequence homology of 92% identity with that of *S. olivochromogenes.* The gene was expressed in *Escherichia coli* and purified whose apparent molecular mass was determined to be 45 kDa, which corresponds to the molecular mass calculated from the deduced amino acid and that of the purified wild-type enzyme. The enzyme was highly stable at relatively high temperature with optimal activity at 85^{o}C (Joo et al., 2005). The xylA gene coding for xylose isomerase from the hyperthermophile *Thermotoga neapolitana* 5068 was cloned, sequenced, and expressed in Escherichia coli. The gene encoded a highly thermostable polypeptide of 444 residues with a calculated molecular weight of 50,892 (Vieille et al., 1995).

The recombinant *Orpinomyces xyl*A was made up of a single type of subunit with a molecular mass of approximately 49 kDa, which was significantly close to calculated molecular mass of 49.4 kDa based on amino acid sequence. The native molecular mass of the recombinant *Orpinomyces Xyl*A was 108 kDa, which indicated that the active enzyme was possibly homodimeric (Madhavan et al., 2009). The sequence was found to exhibit 94% identity to the *Piromyces* sp. strain E2 xylose isomerase. Zhau et al. at 2012 approached the rational metabolic engineering of a *S. cerevisiae* strain, which involved overexpression of the *Piromyces* xylose isomerase gene (XYLA) along with *Pichia stipitis* xylulose kinase (XYL3) and genes of the non-oxidative pentose phosphate pathway (PPP) to enable it to grow on xylose.

2.12 Xylose metabolism pathway

The metabolic pathway involves xylose reductase (XR) and xylitol dehydrogenase (XDH) in fungi and Xylose Isomerase (XI) in bacteria to convert xylose to xylulose which then enters into pentose phosphate pathway, which is the chief biochemical route for xylose metabolism found in all cellular organisms (McMillan, 1993a, 1994). D-xylulose is first phosphorylated by Xylulokinase enzyme before it enters into metabolic pathway. This pathway provides D-ribose for nucleic acid biosynthesis, D-erythrose-4-phosphate for the synthesis of aromatic amino acid and NADPH for anabolic reactions (Thomas W. Jeffries, 2006). This pathway operates in two phases including oxidative and non-oxidative.

Oxidative phase converts hexose (D-glucose 6P) into pentose (D-ribulose 5P) with release of CO_2 and NADPH while non-oxidative phase converts D-ribulose 5P into D-ribose-5P, D-xylulose 5P, D-sedoheptulose 7P, D-erythrose 5P, D-fructose 6P and D-glyceraldehyde 3P. D-xylulose involved in the pathway is also provided by D-xylose and L-arabinose (McMillan, 1993a).

Various strains of Bacteria, fungi and yeast are available in nature that can ferment xylose to ethanol. Production of ethanol from xylose involves mostly two pathways in microbes. Yeast and most fungi uses pathway involving enzymes xylose reductase (XR) and xylitol dehydrogenase (XDH) for converting xylose to xylitol and then xylitol to xylulose respectively. On the other hand, Bacteria use Xylose isomerase (XI) to isomerize xylose into xylulose. Both of these pathway requires Xylulokinase for phosphorylation of Xylulose before it enters into the pentose phosphate pathway (PPP) followed by EMP pathway and glycolysis to yield ethanol (Karhumaa et al., 2007; Kuyper et al., 2005).

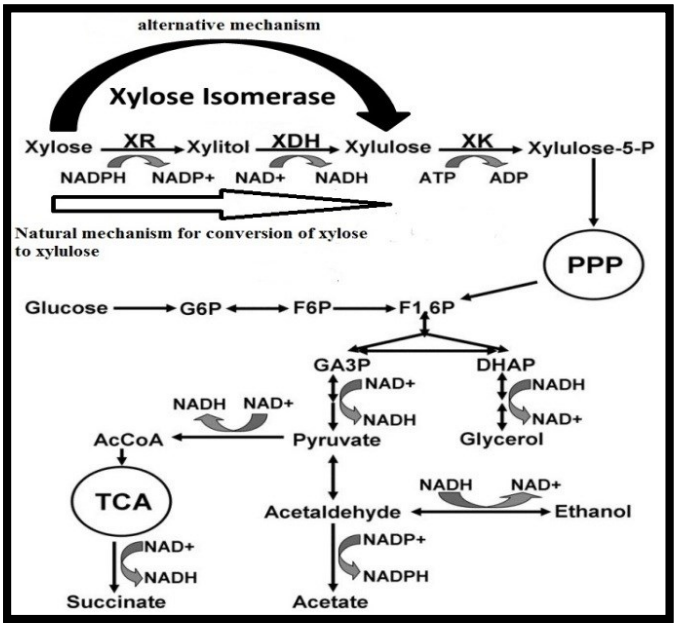

Figure 2.7: Bioconversion of xylose and entry into the pentose phosphate pathway (PPP) (adapted from McMillan, 1994).

Different studies have suggested that, xylose transport into the cell may be one of the rate limiting steps in xylose metabolism (Kuyper et al., 2005) and ethanol production. Hence, several works have been performed to introduce the xylose transport system into the cell. Xylose transport was shown to be increased via increased number of hexose transporters. Hector *et al.* engineered strains of *S. cerevisiae* able to utilize xylose by introducing transporter genes from *Arabidopsis thaliana* and XYL1, XYL2 and XKS1 encoding for XR, XDH and Xylulokinase from *P. stipites* that showed increased xylose consumption.

Genome-scale modeling of engineered *S. cerevisiae* metabolic pathways predicted that a balanced cofactor system of XR/XDH would increase ethanol yield by 25% and xylose consumption rate by 70%. On the other hand, site-directed mutagenesis of cofactor-binding sites and computational redesign of proteins successfully altered the cofactor preference of XR or XDH to balance their requirements (Matsushika et al., , 2008; Watanabe et al., 2007).

Overexpression of endogenous *S. cerevisiae* XyluloKinase gene (*XKS*1) or the introduction of heterologous XK gene (*XYL*3 from *Scheffersomyces stipitis*) is required to facilitate xylose metabolism through the pentose phosphate pathway (Toivari, 2007; Toivari et al., 2004; Wahlbom et al., 2003). However, over expression of the XK genes could lead to growth inhibition on xylose (Jin et al., 2003) or reduction in a xylose consumption rate. In a strain overexpressing XYL1 and XYL2, moderate expression of XKS1 led to basal xylitol accumulation and more ethanol production (Matsushika & Sawayama, 2008).

Another approach involved XI from bacteria with deletion of a non-specific aldose reductase gene GRE3 in *S. cerevisiae* to block xylitol fermentation. Brat *et al*. in 2009 reported that xylose isomerase from *Clostridium phytofermentans* enables yeast to metabolize xylose as carbon source with superior activity than previously used XI enzyme gene (xylA) from *Thermus thermophilus* and *Piromyces sp*. (Brat et al., 2009) and thermostable xylose (glucose) isomerase cloned from *Streptomyces chibaensis* J-59 (Joo et al., 2005).

Conversion of D-xylose into D-xylulose is crucial in metabolic engineering of xylose fermentation by *S. cerevisiae*. Heterologous expression of xylose reductase and xylitol dehydrogenase does enable xylose utilization, but intrinsic redox constraints of this pathway resulted in undesirable byproduct formation in the absence of oxygen (Xi, 2007). On the contrary, expression of xylose isomerase (XI), which directly isomerizes D-xylose and D-xylulose, does not present with such constraints (Kuyper et al., 2003a; McMillan, 1993b).

Glucose/Xylose transporters

In *S. cerevisiae* there are no specific transporters for import of pentose into the cell, instead they are transported inside by native hexose transporters/facilitators (hxt proteins and Gal2) but with very low affinity (Km.100 mM; (Hamacher et al., 2002)). Xylose uptake is thus slow and dependent on the concentration of glucose present in the environment. *S. cerevisiae*

strain with heterologous expression of a high-affinity xylose transporter including GXS1 and GXF1 from *C. intermedia* has been engineered with significant rate of xylose transport into the cell (Jeffries, 2006; Kuyper et al., 2005; Leandro et al., 2008), which can be first step to metabolize xylose for ethanol production. Heterologous expression of other xylose transporters such as At5g59250 and At5g17010 from *Arabidopsis thaliana*, XylE from *E. coli* and XUT gene from *S. stipitis* have been practiced (Young et al., 2011).

Expression of the transporters from *Arabidopsis thaliana* increased xylose uptake and xylose consumption up to 46% and 40%, respectively (Hector et al., 2008). In the experiment, xylose co-consumption rates (prior to glucose depletion) were also increased by up to 2.5-fold compared to the control strain. Increased xylose consumption correlated with increased ethanol concentration and productivity and engineered strains expressing the transporters had up to a 70% increase in ethanol production rate (Hector et al., 2008).

Candida intermedia PYCC 4715 was shown to grow well on xylose and was able to transport this sugar by two different transport systems: high-capacity and low-affinity facilitated diffusion (gxf1p) and a high-affinity xylose–proton symporter (gxs1p), both of which accept glucose as a substrate. These genes encoding both transporters, GXF1 (glucose/xylose facilitator 1) and GXS1 (glucose/xylose symporter 1) respectively were isolated and expressed in *S. cerevisiae* and the kinetic parameters of glucose and xylose transport were determined. In the study Km values of GXF1 and GXS1 were found to be 50 and ~0.4 mM respectively (Leandro et al., 2006, 2008).

Characterization of Gxs1 and comparison of its amino acid sequence with those available in public databases led to the identification of closely related, uncharacterized putative sugar transporters from three other yeast species (*Debaryomyces hansenii, Candida albicans* and *Yarrowia lipolytica*). *D. hansenii* and *C. albicans* are known to produce xylose - H^+ symporters, whereas *Y. lipolytica* is able to grow on xylose but the biochemical characteristics of sugar transport had not previously been investigated (Leandro et al., 2008; Apel et al., 2016). In addition, Gxs1 also shares a high degree of identity with several putative sugar transporters from *Ascomycetous* filamentous fungi, such as MSTA, a recently characterized broad-specificity sugar - H^+ symporter from *Aspergilus niger* (Sedlak & Ho, 2004a, 2004b). Hence a novel subgroup within the fungal sugar transporter family, which includes monosaccharide - H^+ symporters with overlapping substrate specificity, seems to be emerging. This will give a new impetus to the identification of protein regions that determine the mechanism of transport (H^+ symport versus facilitated diffusion) as well as substrate specificity (Leandro et al., 2006, 2008). Further studies, involving additional members of this subfamily of high-affinity xylose transporters, are expected to yield an effective molecular tool to improve xylose transport in *S. Cerevisiae* (Farwick et al., 2014a; Reider Apel al., 2016; Weber et al., 2010).

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2.13 Transport of Pentose Sugars into *Saccharomyces* Species

Native xylose metabolizing yeasts possess both xylose-specific transport utilizing a proton symport system and nonspecific transport systems mediated by facilitated diffusion through other low-affinity sugar transporters. *Saccharomyces* species can uptake xylose via glucose transporters encoded by the HXT gene family, though it lacks xylose specific transporter. However, in mixed sugar medium, glucose is preferentially transported into cell due to a 100-fold lower affinity of xylose for the transporters. This problem has been addressed by the introduction of genes encoding xylose transporters into *S. cerevisiae* (Kricka et al., 2015).

In different studies involving overexpression of transporter genes such as GXF1/GXS1 from *Candida intermedia*, Trxlt1 from *Trichoderma reesei*, SUT1 from *P. stipitis*, and At5g17010 from *Arabidopsis thaliana* have met with mixed results with some improvements of xylose uptake reported in mixed sugar fermentations (Leandro et al., 2006; Runquist et al., 2009). The overexpression of various native hexose transporters (HXT1, HXT7, HXT13, and GAL1) can also increase xylose transport into the cell, however, ethanol production was not significantly increased (Tanino et al., 2010, 2012).

The Transporters GXF1 and GXS1 from *Candida intermedia* has been separately transformed into *Saccharomyces cerevisiae* by Regmi P., 2016 and Nepal N., 2016 respectively in their M. Sc. dissertation work. During the study, both genes were reported to be expressed successfully in yeast using episomal yeast expression system.

2.14 Expression of xylose isomerase in *S. cerevisiae*

Xylose isomerase (XI) genes were identified from bacteria such as *Thermus thermophiles, Clostridium phytofermentans* (Brat et al., 2009) and *Bacteroides* stercoris (Ha et al., 2011) or anaerobic fungi such as Piromyces sp. E2 (Karhumaa et al., 2007; Kuyper et al., 2005) and *Orpinomyces sp.* which are functionally expressed in *S. cerevisiae*. Many other XI genes failed to show their functional expression in *S. Cerevisiae*. Screening for a novel xylose isomerase was performed recently using *Escherichia coli* expressing a soil metagenomic library. *S. cerevisiae* strains expressing newly isolated xylose isomerase genes (xym1 and xym2), however, exhibited only 25% of the growth rate of a strain expressing *Piromyces* XylA (Kim, Park, Jin, & Seo, 2013; Parachin & Gorwa-Grauslund, 2011). For the successful expression of bacterial xylose isomerase genes in *S. cerevisiae*, the gene search has to be done directly in *S. cerevisiae* or the gene synthesis for optimizing their codon usage might be required. Codon optimization is performed by changing the original codons of the heterologous genes to those of highly expressed genes in *S. cerevisiae*, like glycolytic enzymes, through a

commercial service for gene synthesis. Expression of prokaryotic genes in *S. cerevisiae* often requires their codon-optimization to achieve desired phenotypes (Brat et al., 2009). Recently, the strain expressing the codon-optimized XI gene from *C. phytofermentans* showed a 46% improved specific growth rate on xylose in comparison to the strain expressing the original gene (Brat et al., 2009).

Kaisa Karhumaa et al. in 2005 developed the xylose fermenting recombinant *S. cerevisiae* strain TMB 3045 able to express xylose isomerase. In the study, XI gene form *T. thermophilus* was introduced in yeast strain lacking GRE3 gene that codes for unspecific aldose reductase responsible for xylitol accumulation by reducing xylose into xylitol (kuhn et al., 1995), which is a concentration dependent inhibitor of XI activity (Dekker et al., 1991). Deletion of GRE3 gene is based on low activity of *T. thermophilus* XI in yeast reported by Lonnet et al. in 2003, where activity of the XI gene was found to improve when endogenous GRE3 gene was deleted. Recombinant strain was also provided with overexpression of XK (xylulokinase) gene and enzymes of non-oxidative pentose phosphate pathway (PPP) ie. Transaldolase (TAL), Transketolase (TKL), ribose 5-phosphate ketol-isomerase (RKI) and ribose 5-phosphate epimerase (RPE) to enhance xylose utilization in yeast. Recombinant strain TMB 3045 only presented with very low amount of xylose consumption and hence was subjected to adaptation and evolution by adaptive mutagenesis to result strain TMB 3050. Thus adapted strain TMB 3050 showed the xylose consumption rate of 2.5mg/gm cells/hr with ethanol yield of 0.29 gm/gm of consumed xylose. Along with ethanol, xylitol was also produced with traces of acetic acid and glycerol as confirmed by HPLC (Karhumaa et al., 2005).

Expression of XI from *Piromyces* sp. in *S. cerevisiae* (Kuyper et al., 2003a) was also found to result slow xylose growth which was later improved after adaptation in continuous culture (Kuyper et al., 2004).

Kaisa Karhumaa et al., in 2005 engineered recombinant stain of yeast *Saccharomyces cerevisiae* TMB 3045 by expressing XI gene from *Thermus thermophilus* complemented by over-expression of gene encoding XK (Xylulokinase) and genes of non-oxidative pentose phosphate pathaway i.e. TAL (Transaldolase), TKL (Transketolase), RKI (Ribose 5-phosphate ketol-isomerase) and RPE (Ribulose 5-phosphate epimerase) alongwith deletion of GRE3 gene coding for non-specific aldose reductase. Recombinant strain TMB 3045 was further improved into TMB 3050 for xylose metabolism by evolutionary engineering through repeated culture. Study involved integration of heterologous gene into genome of the yeast and improvement of xylose metabolism of recombinant strain by combination of genetic modification beneficial for xylose fermentation which involved i) overexpression of XKS1 gene coding for XK that enables increased ethanol production from xylose/xylulose, ii) deletion of GRE3 gene coding for unspecific aldose reductase that reduces xylitol, a potential inhibitor for activity of XI and XR/XDH, iii) overexpression of genes for non-oxidative pentose

phosphate pathway enzymes (TAL, TKL, RKI and RPE) that improves xylose growth and fermentation. Recombinant TMB 3045 had low xylose growth and consumption which was improved by evolutionary engineering/repeated culture for adaptation that induced adaptive spontaneous mutation. The adapted recombinant strain had the xylose consumption rate of 2.4 mg/gm cells/hr in media with 50 gm/lit xylose and ethanol yield of 0.29 gm/gm consumed xylose. Hence, over-expression of non-oxidative PPP genes and adaptation is needed for strain engineered with heterologous XI gene to achieve significant xylose utilization (Karhumaa et al., 2005).

Tanino et al. (2010) reported multicopy integration of *Orpinomyces* sp. XI overexpression cassettes into genome of *S. cerevisiae* MT8-1 strain, with increased ethanol production from both xylose as sole carbon source and mixed sugar consisting of xylose and glucose without any adaptation. Expression of XI, complemented with GRE3 knockout and XKS1 overexpression enhanced xylose utilization during the study. Over-expression cassette of XI and XKS was integrated in genome of *S. cerevisiae* MT18-1 strain under GAPDH (Glyceraldehyde 3- phosphate dehydrogenase) promoter by delta integration method as described by Anjali Madhavan et al., 2009a and Yamada et al., 2010. The recombinant strain was able to use xylose and produce ethanol along glycerol and xylitol as byproducts with enzyme activity of 0.78 IU/mg protein, xylose consumption rate of 0.019gm/gm dry cell/hr. Ethanol concentration after 120 hr of fermentation by recombinant strain was determined to be 6.93 gm/lit. In the study, ethanol production from mixed sugar substrate (containing equal amount of glucose and xylose) was observed where ethanol production associated with glucose consumption was rapid while that of xylose consumption was slow. GRE3 knockout strain was found to produce reduced amount of xylitol as byproduct.

XI gene from bovine rumen metagenomic library designated as Ru-*xyl*A and from *Sorangium cellulosum* designated as Sc-*xyl*A were recently transformed and heterologously expressed into *S. cerevisiae* by Hou et al., (2015). Expression was functional with enzyme activities of 1.31 U/mg protein for Ru-*xyl*A and 0.35 U/mg protein for Sc-*xyl*A which were comparable to those of *Piromyces* sp. XI and efficiency was further improved by mutagenesis and growth based screening. The gene size of Ru-*xyl*A and Sc-*xyl*A were determined to be 1320 bp and 1332 bp coding for protein of molecular mass of 49.5 kDa and 49.6 kDa which corresponds to the calculated approximate mass of 49.4 kDa Xylose isomerase enzyme as reported by Madhavan et al. in 2008. XI from *Arabidopsis thaliana* was also expressed in *S. cerevisiae* by Hou et al., at 2015.

Xylose isomerase gene of 1314 bp length from *Orpinomyces* was cloned and expressed constitutively in *S. cerevisiae* coding for 437 aa similar to family II XI, closely related to *Piromyces* sp. E2 *Xyl*A and some prokaryotes. Expression of XI was accompanied by overexpression of *S. cerevisiae* endogenous xylulokinase gene and *Pichia stipitis* SUT1 gene

for sugar transport in recombinant yeast to facilitate with efficient production of ethanol from xylose. The genes XI from *Orpinomyces* sp., XKS from *S. cerevisiae* and SUT1 from *Pichia stipitis* were constructed to express constitutively under GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) promoter. Transformation of the construct was performed by Lithium aetate/single stranded DNA/PEG method as described by Gietz and Schiestl, 2007. Positive transformants were selected on Synthetic Complete glucose minimal medium plates based on aminoacid auxotrophy after 48 hrs culture at 38^{o}C.

Growth rate of recombinant yeast expressing *Orpinomyces Xyl*A was 0.01 hr^{-1} on synthetic media with 20 gm/lit xylose. On fermentation of 50 gm/lit xylose, recombinant yeast with only *Xyl*A used 5.91 gm/lit xylose to produce 2.33 gm/lit ethanol after 140 hrs fermentation. On the other hand recombinant yeast with *XKS* and *Xyl*A consumed 10.41 gm/lit of xylose and produced 4.06 gm/lit ethanol. Xylose consumption and ethanol production was further elevated to 15.55 gm/lit and 6.05 gm/lit respectively when *Xyl*A was accompanied by *XKS* and *SUT*1. It lead to the conclusion that co-expression of *XKS* and *SUT*1 along *Xyl*A significantly improves xylose consumption and ethanol production. This implies for involvement of xylose transport system in order to increased usage in metabolism. So, along the expression of *C. phytofermentans* XI gene in S. cerevisiae, co-expression of xylose specific transporters such as GXS1 and GXF1 from *C. intermedia* and overexpression of XKS and those of PPP could be promising alternative to significantly promote the xylose utilization followed by ethanol yield.

*Xyl*A gene from *Piromyces* sp. E2 was isolated by PCR from cDNA library and cloned into pYES2 and pPICZα under GAL1 promoter and trasformed into *S. cerevisiae* CEN.PK 113-5D MATα Ura3-52 by chemical method (LiAc/SS carrier DNA/PEG) as described by Gietz and Woods, 2002 by Marko Kuyper et al., at 2003. Recombinant stain displayed slow growth on xylose, but was able to consume xylose in both aerobic and anaerobic condition. Engineered strain in the study showed low XI activity of 0.025 U/mg protein in cell extracts which was improved to 1.1 U/mg protein when construct with original stop codon was transformed into the yeast. Low activity of protein may be attributed to absence of stop codon at original position as that of native gene resulting the misfolded or truncated protein. Growth rate of recombinant yeast was determined to be 0.005 hr^{-1} when engineered strain was grown on synthetic medium with % xylose (Marko Kuyper et al., 2003). Unlike this, our strain MKY09B2 expressing *C. phytofermentans* XI has significantly higher growth rate of 0.195 hr^{-1} and 0.155 hr^{-1} in YNB and YNBX media respectively. Growth rate of the recombinant strain expressing bacterial XI was also higher than that of strain expressing *Orpinomyces* XylA where growth rate was reported to be 0.01 hr^{-1} when grown on synthetic media with 20 gm/lit xylose (Madhavan et al., 2009).

XI gene from *C. phytofermentans* was also successfully transformed into *S. cerevisiae* MBL-JY by Yong et al. at 2013, where expression of XI was enhanced by supplementation of galactose under GAL promoter. In the study, amount of xylose consumption and ethanol production by recombinant strain over 159 hrs. were 23.48 gm/lit xylose and 15.89 gm/lit respectively when grown on media containing 40 gm/lit xylose supplemented with 20 gm/lit of galactose.

Zhau et al., 2012 overexpressed *Piromyces* xylose isomerase gene (*XYL*A), *Pichia stipitis* xylulokinase (*XYL*3) and genes of non-oxidative PPP to develop recombinant strain H131-A3 and subjected to evolutionary engineering to attain increased aerobic growth rate of 0.203 ± 0.006 hr^{-1} and xylose consumption rate of 1.866 gm/gm/hr with ethanol conversion yield 0.41gm/gm. Engineered strain had the specific growth rate of 0.031 ± 0.022 in SDX (synthetic defined xylose) media under aerobic condition. Xylose consumption rate was 1.464gm/gm/hr with ethanol yield of 0.438gm/gm of xylose. High xylose assimilation rate was observed with multicopy insertion of XYLA genes in presence XK and PPP genes complemented with adaptation or evolutionary engineering. Here, increased xylose transported is attributed to changes in expression of transporter during evolution.

CHAPTER III

MATERIALS AND METHODS

3.1 Materials, reagents and chemical used for this study

TECHNE ^{5}Prime-G/02 PCR machine was used for amplification of DNA. Electroporation was carried out using Bio-Rad MicropulserTM. Restriction enzymes used in this study were EcoR I HF (Thermo-scientific), *Hin*dIII (Promega), *Xho*I (Promega) and *Nde*I HF (Thermoscientific). Taq DNA polymerase HF (Thermoscientific) and Long-amp DNA polymerase (NEB) were used for amplification. The dNTPs and T4- DNA ligase were obtained from Thermo-scientific, USA. The DNA amplifying primers were purchased from Macrogen, Korea. The UV-transilluminator from major science was used to visualize Ethidium Bromide (EtBr) stained DNA. All the chemicals and reagents were obtained from Hi-media, Thermo-fischer and Merck.

3.2 Xylose isomerase gene

Codon optimized synthetic Xylose Isomerase (XI) gene originally from *Clostridium phytofermentans* was obtained in cloning vector pUC57 from Genescript. The vector with XI was termed as pUC57XI.

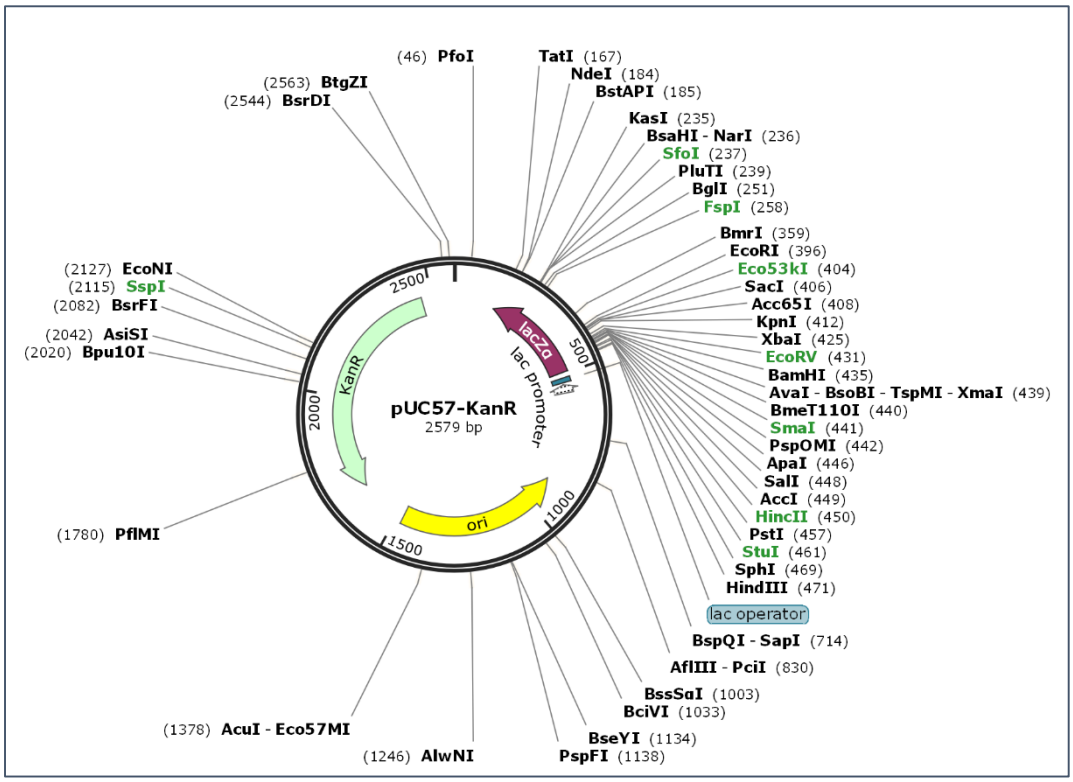


Figure 3.1: Detailed vector map of pUC57 cloning vector showing promoter and multiple cloning sites (MCS).

3.3 Primer designing and primer sequence

The Xylose isomerase gene specific set of ORF primer (TUXIF and TUXIR) was designed manually using different online tool namely OligoCalc (The oligonucleotide properties calculator), m-fold (the m-fold web server) and IDT (The integrated DNA technology). The forward and reverse primers were designed with restriction sites for *Hin*dIII and *Xho*I respectively.

A set of XI gene specific primer sequences for full length amplification of total length 1369bp.

Fw: 5'-GACAGTGAGCTCGGATCCTAC**AAGCTT*ATG***AAGAACTATTTTCCAAACG -3' 49 MER

Tm 79.8

Rv: 5'-CAGTGTCTGCAG**CTCGAG**GGATAA***TTA***TCTGAATAATATGTTGTTTACG-3' 49 MER

Tm 79.8

3.4 Bacteria and Yeast used in the study

3.4.1 *Escherichia coli*

E. coli DH5α was used as host for cloning of plasmid DNA and construct prepared. The genotype of the DH5α is F – Φ80 lacZ ΔM15 Δ(lacZYA-argF) U169 recA1 endA1 hsdR17 (rK–, mK+) phoA supE44 λ– thi-1 gyrA96 relA1.

3.4.2 *Saccharomyces cerevisiae* MKY09

The *S. cerevisiae* MKY09 was used as heterologous host for expression of Xylose Isomerase gene. It is a haploid strain kindly obtained from Boles lab stock, University of Frankfurt, Germany. The genotype of MKY09 is *MATa leu2-3,112 ura3-52 trp1-289 his3-Δ1MAL2-8^c SUC2* (Brat et. al., 2009).

3.5 Culture media

Bacterial host DH5α was cultured in Luria Bertani medium (Broth and Agar) with or without antibiotics as per requirements. Similarly, Recombinant yeasts were cultured in Yeast Nitrogen Base medium with necessary amino-acid supplements 20 gm/lit Glucose only (YNBG or YNB) and 20 gm/lit Glucose plus 20 gm/lit Xylose (YNBX) as carbon source. On the other hand, wild/native yeast was grown in YEPD media.

3.6 pGPD2 expression system and cloning

The pGPD2, a bacterial and yeast expression vector of size 5772 bp purchased from Addgene was obtained as bacteria in an agar stab. This vector possesses an ampicillin resistance gene (encoding β-lactamase) as a selection marker for transformed bacteria and URA3 gene (encoding Oritidine - 5 phosphate decarboxylase, an enzyme essential for biosynthesis of uracil) as a selection marker for transformed uracil auxotrophic yeast strain. The back bone of this vector is p416-GPD of size 5500bp and it is modified by inserting new multiple cloning site (MCS) between CYC terminator and GPD promoter. It has a T7 bacterial promoter for bacterial expression and GPD constitutive promoter for yeast expression. It possesses a pBR322-ori (origin of replication) for replication of plasmid in bacteria while CEN6- ARS4 to replicate plasmid in yeast.

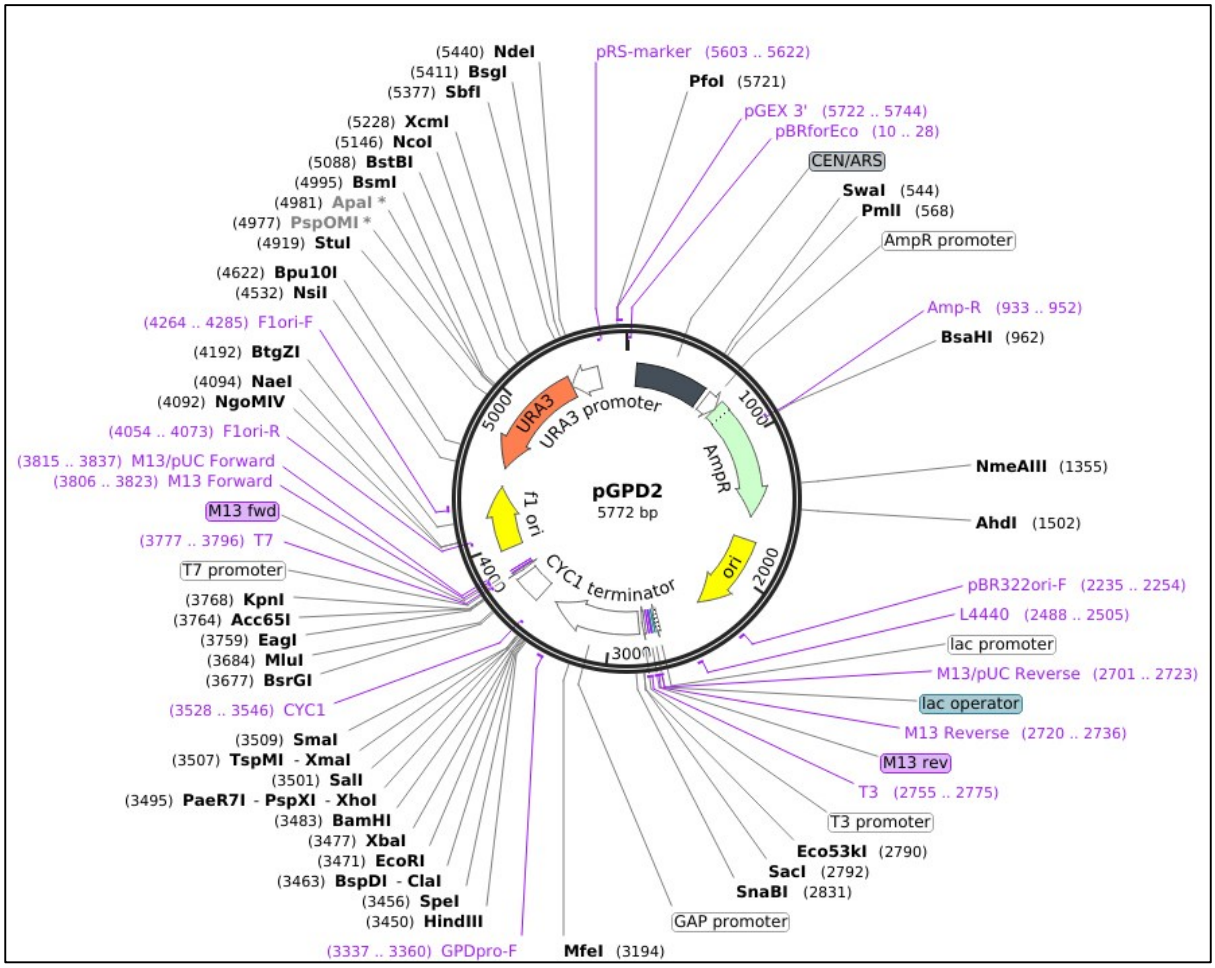


Figure 3.2: Detail vector Map for pGPD2 yeast expression system showing promoter and multiple cloning sites.

3.7 Plasmid Extraction from *E. coli* by Alkaline lysis method mini-preparation (Sambrook & Russell, 2000; modified)

3.7.1 Bacterial cell culture and cell harvesting

Isolated colony from was sub-cultured in 10 ml broth medium overnight at 37^{o}C and 200 rpm. The culture was aliquoted into 1.5 ml Eppendorf tubes and centrifuged at 13000 rpm for 1 minute. Supernatant was discarded completely followed by draining with micropipette.

3.7.2 Cell lysis and plasmid recovery

Cell pellet was suspended in 200 µl of Alkaline lysis solution-I with pipette and vortexing. Then 200 µl of freshly prepared Alkaline lysis solution-II was added and inverted gently for five times (at this stage denaturation of cell components occurs indicated by viscous nature developed in suspension). Immediately after this, 200 µl re-naturation solution i.e. Alkaline lysis solution-III was added and inverted few times and incubated in ice for 5 minutes (at this point re-naturation of nucleic acid occurs).

3.7.2.1 Plasmid precipitation

After 5 minutes incubation in ice, tubes were centrifuged at 13000 rpm for 8 minutes. The supernatant was then transferred in fresh tube with the help of micropipette. Double volume of supernatant i.e. 1 ml of ice cold isopropanol was added to the solution and left in ice for 30 minutes allowing precipitation of nucleic acids. Then mixture was centrifuged at 13000 rpm for 10 minutes and supernatant was discarded. Pellet was washed with 1ml of 70% ethanol and centrifuged again for 5 minutes at 13000 rpm. Supernatant was discarded and completely drained with help of micropipette and allowed to air dry. Then pellet was dissolved in 20 µl of nuclease free water (NFW) and confirmed by subjecting to agarose gel electrophoresis and visualization in UV-trans-illuminator.

3.7.2.2 Gel Electrophoresis and Visualization

Extracted plasmid was subjected to 0.8% agarose gel electrophoresis at 50 V for 60 minutes. DNA was stained with EtBr and visualized on UV-trans-illuminator.

3.8 Enrichment of Carrier plasmid with target Xylose isomerase gene

Codon optimized synthetic XI gene was obtained in cloning/transfer vector pUC57. The plasmid construct was transformed into *E. coli* DH5α for enrichment. Transformation was performed by Heat-shock method on competent cells prepared by chemical method (Sambrook & Russell, 2000). Transformed plasmid was then extracted and confirmed by restriction digestion and polymerase chain reaction for Xylose Isomerase.

3.8.1 Preparation of competent cell of *E. coli*

Pure colony of *E. coli* was inoculated into 5 ml LB broth and incubated overnight at 37 °C with constant shaking at 200 rpm, as a seed culture. One ml of this culture was then used for inoculation into 100 ml LB broth. The culture was left for incubation at 37 °C until the OD of the culture reached 0.3 – 0.4. Following this, the culture was kept on ice for 10 minutes to retard the growth and then transferred to two chilled 50 ml falcon tubes. Then cells were harvested at 4100 rpm for 10 minutes at 4 °C. The supernatant was discarded and the pellet was resuspended in 30 ml of ice cold 80mM $MgCl_2$ and 20mM $CaCl_2$ solution. The cells were recovered by centrifuging cell suspension at 4100 for additional 10 minutes at 4°C. The supernatant was drained out carefully and each of the cell pellets were resuspended in 2 ml of chilled 100mM CaCl2 solution from which 200 µl of competent cells was aliquoted in 1.5 ml centrifuge tube and used for transformation by heat shock.

3.8.2 Transformation by Heat Shock

200 µl of competent cells were taken in a fresh ice chilled Eppendorf tube/ micro-centrifuge tube and 1 µl (100ng) plasmid (pUC57+XI) was mixed with it. The mixture was chilled in ice for 30 minutes. The tube was then placed in pre-heated water bath for heat shock at 42°C for 90 seconds. Immediately after heat shock, 1 ml of LB media was added in tube and incubated at 37°C and 200 rpm agitation for 1 hour. After incubation, tubes were centrifuged at 8000 rpm for 1 minute and supernatant was discarded. Pellet was suspended in 100 µl of fresh LB medium and plated on LB Agar supplemented with 50µg/ml Kanamycin. The plates were then incubated at 37°C overnight.

3.8.3 Selection of Transformants

Plasmid containing XI had Kanamycin resistance gene as selection marker, indicating that only those colony harboring pUC57+XI plasmid will grow on LBA Kanamycin plate. The colony

growing on LB-Kanamycin agar media were sub-cultured in LB-Kanamycin agar plate and in LB Kanamycin (50µg/ml) broth.

3.8.4 Confirmation of Transformants

Transformants were selected on the basis of Kanamycin resistance on LBA plate. Isolated colonies were randomly selected among transformants and sub-cultured in LB broth supplemented with 50 µg/ml Kanamycin for plasmid extraction by alkaline lysis method. Plasmid was first subjected to preliminary screening by restriction digestion and confirmed by PCR using ORF primer set of TUXIF and TUXIR.

3.8.5 Preliminary screening of transformants by restriction digestion

Isolated plasmid was subjected to restriction digestion by single enzyme *Hin*dIII so as to linearize and calibrate the plasmid size more approximately for preliminary screening. Restriction digestion was performed in the reaction mixture as follows-

Table 3.1 Restriction digestion Reaction mixture

Components (stock concentration)	Volume (working concentration)
Buffer (10X)	2 µl (2X)
*Hin*dIII (10U/µl)	1µl (1U/µl)
Template (plasmid pUC57XI)	5 µl (625ng)
NFW	2 µl
Total	10 µl

Reaction mixture was incubated at 37^{o}C for 4 hours to allow complete digestion. After incubation, tube was kept at -20^{o}C for 30 mins. to stop the digestion reaction and directly used for 1 % agarose gel electrophoresis along 1 kb DNA ladder from FERMENTAS.

3.8.6 Confirmation of transformation of XI gene by PCR

For confirmation of transformation harboring plasmid with target gene, full length PCR (polymerase chain reaction) was performed using the primer set TUXIF and TUXIR. PCR reaction mixture was prepared as follows-

Table 3.2 Master-mix (for volume of 20 µl, 18 µl MM + 2µl template)

Components (stock concentration)	Volume (working concentration)
Tango Buffer (5X)	5 µl (1X)
dNTPs (2mM)	0.5 µl (200 µM)
Forward primer (10pM/µl or 10µM)	1 µl (0.4 µM)
Reverse primer (10pM/µl or 10µM)	1 µl (0.4 µM)
Taq-polymerase* (5U/µl)	1 µl (1U/25 µl)
NFW	9.5 µl
Total	18 µl#

* Added at the last during preparation of master-mix.

without volume of template

Master mix was aliquoted 9 µl each in two different PCR tubes and 1 µl of diluted plasmid template was added in each tube as mentioned in following table.

Table 3.3 PCR mixture

Components (stock concentration)	**Volume (working concentration)**
Master Mix	9 µl
Plasmid template (diluted)	1 µl (12.5ng)
Total	10 µl

PCR was done in TECHNE ^{5}PRIME G/02 thermocycler as the condition given below-

Table 3.4 PCR condition

Stage	Temperature (°C)	Time (minutes)	Cycle
I	95	5	1
II	95	0.5	5
	50	0.5	
	72	1.5	
III	95	0.5	25
	63	0.5	
	72	1.5	
IV	72	5	1
V	4		

3.8.7 Gel Electrophoresis of PCR product

Gel Electrophoresis of the PCR Product was performed in 1 % Agarose gel prepared in 1X TAE. Ethidium Bromide was used as the staining agent. DNA ladder of 1kb from NEB was used to calibrate the PCR amplicon and visualization was performed on UV-trans illuminator.

3.9 Confirmation of pGPD2 vector

3.9.1 Extraction of pGPD2 from *E. coli* DH5α

Overnight culture of *E. coli* DH5α bearing pGPD2 plasmid was used for extraction of pGPD2 plasmid by Alkaline lysis method, a mini-preparation (Sambrook & Russell, 2000). Plasmid was visualized in UV-trans-illuminator after Gel-Electrophoresis in 0.8 % agarose gel. Plasmid extraction and visualization was performed according to the procedure mentioned above.

3.9.2 Preliminary screening of pGPD2 by Single digestion

Vector plasmid pGPD2 was first subjected to single digestion by two enzymes *Hin*dIII and *Xho*I separately. Reaction mixture was prepared as follows-

Table 3.5 Restriction digestion mixture (single digestion)

Components (stock concentration)	**Volume (µl) (working concentration)**	
	Reaction 1	Reaction 2
Tango buffer (5X)	2 (1X)	2 (1X)
*Hin*dIII (20 U/µl)	0.5	-
*Xho*I (20 U/µl)	-	1
Template (pGPD2)	1 (625ng)	1 (625ng)
NFW	6.5	6
Total	10	10

Reaction mixture was incubated at 37^{o}C for 4 hours to allow digestion. Reaction was stopped by incubation at -20^{o}C for 30 min. and used for 0.8% agarose gel electrophoresis followed by visualization as mentioned above.

3.9.3 Confirmation of pGPD2 by Double digestion

Confirmation of vector pGPD$_2$ was done by double digestion with *Eco*RI and *Nde*I. Digestion mixture was prepared as shown in table below and was incubated overnight at 37^{0}C to allow digestion.

Table 3.6 Restriction digestion mixture (double digestion)

Components (stock concentration)	**Volume (µl) (working concentration)**
Tango buffer (5X)	2 (1X)
*Eco*RI (10U/ µl)	0.5 (0.5U/ µl)
*Nde*I (10U/ µl)	1 (1U/ µl)
Template (pGPD2)	1 (625ng)
NFW	5.5
Total	10

3.9.4 Gel Electrophoresis of digested PGPD2

Digestion reaction was stopped by incubation at -20^{o}C for 30 minutes. Whole of the digestion mixture was used for agarose gel electrophoresis. Gel electrophoresis was performed in 0.8 % agarose gel for 90 minutes at 50 V as mentioned above and visualized in UV-transilluminator.

3.10 Preparation of Insert and Vector

Xylose isomerase (XI) gene was amplified from carrier plasmid using ORF primer set and high fidelity taq-polymerase. These primers were provided with restriction site for release of Insert from PCR product. Hence, PCR amplicon obtained by ORF primer set was subjected to double digestion using *Hin*dIII and *Xho*I. Likewise, yeast expression vector selected for the study pGPD2 was also confirmed by double digestion using *Eco*RI and *Nde*I. Confirmed pGPD2 was later subjected to double digestion by enzymes *Hin*dIII and *Xho*I similar to insert to make it compatible for ligation.

3.10.1 Preparation of Xylose Isomerase (XI) gene Insert

3.10.1.1 PCR amplification of XI gene

Confirmed carrier plasmid (pUC57+XI) with the target gene was used as template for PCR amplification of target gene using Q5 Taq polymerase (A high fidelity Taq polymerase) and full length primer set for target gene i.e. Xylose Isomerase gene. Q5 Taq polymerase is a high fidelity DNA polymerase meaning that it has less error/mutation rate during amplification of gene. PCR amplification of insert was performed as follows-

Table 3.7 PCR reaction mixture

Components	Volume (µl)
Q5 Buffer (5X)	5 (1X)
DNTPs	0.5
Forward primer	1
Reverse primer	1
Q5 Taq-polymerase*	1
NFW	15.5
Template plasmid	1 (125ng)

Total	25

* Added at the last during preparation of reaction mixture.

PCR condition used was as shown in Table no. 3.4

3.10.1.2 Gel Electrophoresis of PCR product

PCR amplicon/product was subjected to 0.8% agarose gel electrophoresis prepared in 1X TAE and ran at 50 V for 90 minutes. Visualization was done on UV-trans illuminator and EtBr was used for DNA staining. Calibration of PCR amplicon with approximate size of 1.3 kb was aided by 1 kb DNA ladder from FERMENTAS.

3.10.1.3 Restriction digestion of PCR product

PCR primers were designed with restriction site in it to aid for directional cloning. Hence the products were digested with two restriction enzymes *Hin*dIII and *Xho*I to release required target gene of approximate size 1.3 kb for directed cloning. Reaction mixture for restriction digestion was prepared as follows.

Table 3.8 Restriction digestion mixture

Components (stock concentration)	Volume (µl) (working concentration)
Buffer with BSA (5X)	5 (1X)
*Hin*dIII (10U/µl.)	2
*Xho*I (10U/µl)	2
Template/PCR product	10 (625ng)
NFW	6
Total	25

The digestion mixture prepared in fresh Eppendorf tube was incubated at 37^{o}C overnight for digestion. After overnight incubation, tube was kept at -20^{o}C for 30 mins. to stop the digestion reaction and directly used for gel electrophoresis.

3.10.1.4 Gel Electrophoresis of restriction digestion product (On low melting agarose 0.8%)

0.8% low melting agarose was prepared by melting 0.4 gm of agarose in 50ml 1X TAE. Digestion product was subjected to gel electrophoresis using 1kb DNA ladder from FERMENTAS. The gel run was performed for 90 minutes at 50 V and visualized in UV-trans illuminator. The bands seen on 1.3 kb size were cut out and stored in fresh Eppendorf tube for further processing.

3.10.1.5 Gel Elution for Insert purification from low melting agarose gel (Wizard® SV Gel and PCR Clean-Up System, Promega)

Band seen on required size was cut out and placed in fresh Eppendorf tube. Weight of the gel piece was taken and it was used for elution of insert using the Kit.

The weight of gel piece was calculated as-

Weight of gel piece = final weight (tube + gel) – initial weight (empty tube)

PCR product purification by Gel Elution

PCR product was purified by Gel elution method using Kit from Wizard® SV Gel and PCR Clean Up System, Promega as follows-

Dissolving-

To the gel piece 379 mg, 1 µl/mg membrane binding solution 379 µl was added. Gel was dissolved by incubation at 65^{o}C for 10 minutes with intermittent vortexing.

Binding of DNA-

SV mini-column was placed in collection tube and dissolved gel mixture was transferred to SV mini-column tube. It was incubated at room temperature for 1 minute. After incubation, column was centrifuged at 16000 rcf for 1 min. and flow-through was discarded.

Washing-

700 µl ethanol added membrane wash solution was pipetted to SV mini-column and centrifuged at 16000 rcf for 1 min. The flow-through was discarded and again 500 µl membrane wash solution was added to SV mini-column followed by centrifugation at 16000 rcf for 5 minutes. The flow-through was discarded and column was centrifuged again for 1 minute at 16000 rcf to dry the column.

Elution/Recovery of PCR product-

The SV mini-column was transferred to fresh epi-tube. Then 50 µl NFW was pipetted in SV mini-column and incubated at RT for 5 minutes. The assembly was then centrifuged at 16000 rcf for 1 minute. The flow-through now contained in Eppendorf tube consisted of DNA/gene.

3.10.1.6 Gel Electrophoresis for Quantification of insert

Eluted gene insert was then visualized in 0.8% agarose gel as mentioned above along with 1kb DNA ladder from FERMENTAS for calibration and quantification. Quantification is based on the intensity of the band with respect to the ladder bands. Likewise, gene insert size of 1.3 kb was also determined on the basis of relative migration of insert band with respect to the standard bands on DNA ladder.

3.10.2 Preparation of vector pGPD2 fragment for ligation

3.10.2.1 Double digestion with *Hin*dIII and *Xho*I

Confirmed pGPD2 plasmid was digested with restriction endonucleases *HindIII* and *Xho*I as that of insert to make it suitable for ligation. Digestion mixture was prepared in a fresh chilled Eppendorf's tube as shown in a table below and incubated overnight at 37^{o}C for digestion.

Table 3.9 Restriction digestion mixture

Components (stock concentration)	Volume (µl) (working concentration)
Tango buffer (5X)	5 (1X)
HindIII (10U/ µl)	1 (1U/ µl)
XhoI (10U/ µl)	1 (1U/ µl)
Template (pGPD2) plasmid	3 (1250ng)
NFW	15
Total	25

3.10.2.2 Gel Electrophoresis in low melting agarose

Post digestion whole mixture was used for gel electrophoresis. The band seen on approxiately at 5.7 kb was then extracted and purified using Wizard® SV Gel and PCR Clean-Up System, Promega Kit. On double digestion with *Hin*dIII and *Xho*I, 45 bp fragment will be removed from 5772 bp vector leaving 5727 bp vector backbone.

3.10.2.3 Gel Elution and purification

Gel elution was performed using Wizard® SV Gel and PCR Clean-Up System, Promega as per the manufacturer's instructions. Purified plasmid after elution was then visualized and quantified by agarose gel electrophoresis.

3.10.2.4 Quantification of Vector by gel electrophoresis

Purified pGPD2 vector was subjected to gel electrophoresis in 1 % agarose along 1 kb DNA ladder from FERMENTAS as standard. The concentration of the digested vector was estimated based on the intensity of band observed.

3.11 Ligation

3.11.1 Ligation mixture preparation

DNA insert and plasmid vector i.e. XI gene and pGPD2 vector prepared from double digestion were subjected to the ligation mediated by T4 DNA Ligase enzyme. The enzyme in this step forms the phosphodiester bond, a covalent linkage between insert and vector similar to the phosphate backbone of DNA. Ligation mixture was prepared as follows-

Table 3.10 Ligation Reaction Composition

Components	Volume
T4 ligation buffer (10X)	2 µl (1X)
Insert (XI) (15 ng/µl)	7 µl (105ng)
Vector (pGPD2) (125 ng/µl)	2 µl (250ng)
T4 DNA ligase (400 U/µl)	1 µl
NFW	9 µl
Total	20 µl

The ligation mixture was incubated overnight at 4^{o}C. After overnight incubation whole of the ligation mixture was used for transformation into competent *E. coli* DH5α host by heat shock method.

3.11.2 Confirmation of XI gene ligation into pGPD$_2$ vector post transformation in colonies by PCR

For confirmation of transformation harboring plasmid with target gene, full length PCR (polymerase chain reaction) was performed using the primer set TUXIF and TUXIR using plasmid extracted from Transformant colony as template.

3.11.3 Confirmation of Transformed colonies (pGPD$_2$ + XI) by Restriction Digestion

Possible candidate plasmids from PCR confirmation were further used for confirmation by restriction digestion with enzyme *Hin*dIII.

The restriction digestion mixture was then incubated at 37^{o}C for 4 hours to allow digestion. Whole of the reaction mixture was then used for 1% agarose gel electrophoresis.

3.12 Transformation of Yeast Expression Plasmid construct "B2" (pGPD2+XI) into *Saccharomyces cerevisiae* MKY09:

Confirmed construct of pGPD2+XI, now designated as 'B2' was transformed into *S. cerevisiae* MKY09 strain by electroporation. Transformants were selected on the basis of Uracil auxotrophy of the host MKY09 on YNB agar plate. Selected candidate colony were sub-cultured on YNB URA^{-ve} broth and used for plasmid extraction by glass bead method. Plasmid extracted is preferentially contaminated by Genomic DNA and other impurities, hence presence of plasmid was confirmed by PCR amplification using ORF primer set TUXIF and TUXIR followed by re-amplification of amplicon using same set of primers.

3.12.1 Transformation of Saccharomyces *cerevisiae* MKY09 by electroporation (Sambrook & Russell, 2000)

Transformation of yeast have been practiced with several methods including physical and chemical, among which highly efficient method of gene transfer in the *Saccharomyces cerevisiae* is electroporation. Electroporation with 1M sorbitol has been reported to yield 3x 10^5 Transformants/µg of DNA (Becker and Guarente, 1991).

3.12.1.1 Preparation of electro-competent cells

Electro-competent cell was prepared by inoculating 1 ml of overnight MKY09 culture broth in 200 ml of YEPD medium and overnight incubation at 28^{o}C and 240 rpm. The overnight culture was incubated in ice for 15 minutes and aliquoted in 4 falcon tubes of 50ml. The cells were harvested by centrifugation at 4100 rpm for 10 minutes at 4^{o}C and the supernatant was completely decanted carefully leaving cell pellet behind. The cell pellet was re-suspended in 50 ml of ice cold distilled autoclaved water and recovered by centrifugation at 4100 rpm for 10 min at 4^{o}C. The washing process was repeated with 40 ml autoclaved chilled distilled water. Thus obtained cells pellets were collected in single falcon tube and final volume was maintained to 20 ml by adding the 1M sterile ice cold sorbitol buffer. The cells were again pelleted by centrifuging the cell at 4100 rpm at 4^{o}C for 10 minutes. The supernatant was discarded carefully, and the cell pellets were suspended in 250 µl of 1M sterile ice cold sorbitol buffer. From cell suspension, 80 µl electro-competent cells were aliquoted in chilled sterile 1.5 ml micro-centrifuge tube and kept in ice and immediately subjected to the electroporation.

3.12.1.2 Electroporation

The 5 µl plasmid of 12 ng was added to 80 µl of electro-competent cells and mixed gently by tapping. Mixture was incubated in ice for 15 minutes. The micropulser device an electroporator was set to Sc2 and mixture of electro-competent cells and plasmid was transferred into chilled 0.2 cm Cuvettes. The cuvette was kept in chamber slide and pushed into chamber between the electrodes. Electroporation was done by one pulse at 1.5 kV for 6 millisecond. The cuvette was immediately removed from slide chamber and 1 ml of ice cold 1M sorbitol buffer was added to stabilize the cells. Then electroporated cells were transferred into ice cold sterile micro-centrifuge tube and kept on ice for 10 minutes. It was then incubated at 28^{o}C for 1 hour. Thus incubated cells were concentrated by centrifuging at 5000 rpm for 1 minute at room temperature and directly plated onto YNB URA^{-ve} plate. It was then incubated at 28^{o}C for 36 hours.

3.12.1.3 Selection of Transformants

The selection of Transformants was performed on the basis of uracil auxotrophic selection procedure. The selection was carried out on YNB URA^{-ve} plate. Only those transformant MKY09 carrying pGPD2 plasmid with URA gene will grow on selective media while MKY09 lacking URA gene won't survive in YNB URA^{-ve} plate.

3.13 Isolation of plasmid from MKY09 (Xiao, 2006)

The isolation of plasmid from yeast is difficult due to their cell wall composition. Two of the methods are in use for yeast cell disruption i.e. enzymatic method which uses enzymes like zymolase or lyticase to make the spheroplast of yeast cell followed by complete disruption of cell wall and mechanical method using sonication, glass-bead method etc. which rely on physical energy of cell disruption.

The MKY09 carrying plasmids were grown for 36 hours in YNB selective media at 28^{o}C and 220 rpm. The cultures were poured in 1.5 ml micro centrifuge tube and cells were harvested by centrifuging culture at 12000 rpm for 1 minute at room temperature. The harvesting process was repeated once to collect sufficient cells. Then supernatant was discarded and pellet was resuspended in 230 µl of DNA lysis buffer by vigorous pipetting. To the suspension, 0.4 gm of acid washed dry glass beads and 200 µl of phenol: chloroform: Isoamylalcohol were added and caped the tube. It was then vortexed at maximum speed for 2 minutes and centrifuged at 12000 rpm for 5 minutes, supernatant was transferred to fresh sterile micro centrifuge tube. To the supernatant, 600 µl of isopropanol was added and briefly vortexed at maximum speed and it was incubated at -20^{o}C for 30 minutes. The DNA was pelleted by

centrifugation at 12000 rpm for 12 minutes. The supernatant was discarded and pellet was washed with 70% ethanol. Again supernatant was decanted and pellet was allowed to air dry. Thus air dried plasmid DNA pellet was dissolve in TE buffer and stored at -20^{o}C till further processing.

3.14 Confirmation of recombinant plasmid in Yeast

The confirmation of transformants was performed by PCR, Southern blot and fluorescent microscopy.

3.14.1 Confirmation by PCR

The plasmids were primarily confirmed by PCR amplification of whole gene by ORF primer set.

3.14.2 Confirmation by Southern Hybridization

3.14.2.1 Probe Preparation

The biotin labeled probe of size 1369bp was prepared by PCR method using XI ORF primers TUXIF and TUXIR. For probe preparation, the pUC57+XI plasmid from *E. coli* DH5α was used as template and dNTPs (10mM each) was replaced by biotin labeled Uracil cocktail dNTPs (1 mM dGTP, 1 mM dATP, 1 mM dCTP, 0.65 mM dTTP, 0.35 mM Biotin-11-dUTP) of Biotin Chromogenic Detection Kit #K0661, FERMENTAS/Thermoscientific. PCR reaction mixture and PCR condition was set as mentioned in appendix. Simultaneously, another PCR reaction mixture was prepared using same template with 10mM unlabeled dNTPs as a control. Visualization was done after 1% agarose gel electrophoresis in UV trans-illumination with EtBr stained gel.

3.14.2.2 Determination of labelling efficiency of probe

The concentration of biotin labeled probe was estimated by using standard DNA marker. The different dilution of probe was prepared as 10^{0}, 10^{-2}, 10^{-4}, 10^{-6} and10^{-8} to find out optimum concentration of probe for detection. For this purpose, 1µl of each dilution of probe was poured into nylon membrane and air dried. Then probe was cross-linked with positively charged nylon membrane by keeping probe spotted membrane at 254nm for 2 minutes and was detected according biotin chromogenic detection kit #K0661 Thermoscientific.

3.14.2.3 Gel electrophoresis and blotting of DNA

10µl of 10ng plasmid was ran in 0.7% agarose gel for 2 hours and it was visualized under UV trans-illuminator. After that gel was gently transferred to container containing 300ml of denaturation solution and it was incubated in room temperature for 30 min. Thus denatured

gel was then washed with deionized water and immersed in 300ml of neutralization buffer. The neutralization procedure was repeated with fresh neutralization buffer. The gel was then transferred to southern blot assembly apparatus containing 300ml of 20X SSC buffer in upside down position over Whatman™ 3 MM filter paper. Small triangular portion of the nylon membrane from edge was cut off to mark DNA band for further analysis. The positively charged nylon paper was cut to fit the gel and was placed over gel making sure that air bubble does not come between gel and nylon membrane. A piece of Whatman™ 3 MM filter paper was kept over the nylon membrane followed by stack of absorbent blotting paper. The gel was finally pressed using 500 gm weight and it was incubated for 18 hours at RT to allow upward capillary transfer of DNA. After 18 hours incubation, DNA bonded nylon membrane was washed with 2X SSC buffer to avoid the residual gel. Membrane was then allowed to air dry at RT and the DNA was cross-linked to membrane by UV irradiation of membrane at 254nm for 2 min.

3.14.2.4 Pre-hybridization

10 mg/ml pBEVYU plasmid DNA was denatured by boiling at 100^{o}C for 5 minutes and it was immediately chilled on ice. Thus denatured DNA was then added to pre-hybridization buffer so as to maintain DNA concentration 50-100µg/ml in total 30ml volume. Then DNA cross-linked membrane was then immersed in 30ml pre-hybridization buffer containing denatured DNA and allowed for pre-hybrization for 2 hours at 42°C with constant shaking in a hybridization chamber.

3.14.2.5 Hybridization

The biotin labeled probe was denatured by boiling it at 100^{o}C for 5 min. and mixed with pre-hybridization solution at a concentration of 25-100ng/ml in 30ml final volume. The membrane was then kept in a hybridization bag/container along with 30ml of hybridization buffer and it was incubated at 42^{o}C for 18 hours with constant gentle shaking. After that, it was washed twice with 0.1X SSC + 0.1% SDS and subsequently dried by keeping it between Whatman™ 3 MM filter paper. It was then directly used for detection of biotin labeled probe that hybridized with DNA.

3.14.2.6 Detection

The biotin labeled probe was then detected by using biotin chromogenic detection kit #K0661 Thermo scientific. After hybridization, membrane was washed with 30ml of 1X blocking/washing buffer at room temperature for 5 min. with moderate shaking. The membrane was then immersed in 30 ml of 1% blocking reagent solution with moderate shaking at room temperature for 30 min. The solution was then replaced by 20 ml of Streptavidin-Alkaline phosphatase conjugate and incubated at room temperature for 30 min. with moderate shaking. The membrane was then washed twice with moderate shaking at room temperature by 60 ml of 1X blocking/washing buffer for 15 min. followed by 10 min.

incubation with 20 ml of detection buffer. Finally, enzymatic reaction was performed by overnight incubation of membrane in freshly prepared 10 ml substrate solution in dark. The membrane was then washed (with deionized water), dried and blue precipitate was observed.

3.15 Visualization of recombinant plasmid and genomic DNA of transformed *Saccharomyces cerevisiae* by Fluorescent Microscopy (Meluh Lab, 2005)

Yeast cells (wild type MKY09 and recombinant) were allowed to grow for 24 hours in YNB media. Then 1 ml of culture was taken in a micro centrifuge tube and 100 µl of 36% formaldehyde was added as a fixative agent, which prevents displacement of nucleus as well as disruption of actin cytoskeleton. Cells were then allowed to fix at 23^{o}C for 1.5 hours. Fixed cells were then pelleted by centrifuging cells at 12000 rpm for 1 minute. Supernatant was discarded and cells were washed twice with phosphate buffer. The pellet was re-suspended in 70% ethanol and allowed to stand in room temperature for 40 minutes. Cells were pelleted and re-suspended in 100 µl of PBS. Thus prepared cells were permeablized by sonication for 10 sec. Then 20 µl cells were kept on slide with 20 µl of DPX mounting media and 20 µl DAPI (200 ng/ml, 4,6-diamidino-2-phenylindole) and mixed well by pipetting. Finally, coverslip was placed and observed under fluorescent microscope.

3.16 Functional Expression Analysis

Functional Expression analysis was determined in terms of growth rate on YNB media, Glucose uptake in YNB, Glucose and Xylose uptake or Extracellular Glucose and Xylose depletion in YNBX media and Ethanol Production by Control strain MKY09D2 and Recombinant strain MKY09B2 on YNBX media.

3.16.1 Growth Kinetics of Transformant MKY09 carrying pGPD2 and pGPD2+XI recombinant construct (Palmqvist & Hahn-Hägerdal, 2000a, 2000b)

The MKY09 harboring plasmid construct with Xylose isomerase gene and control MKY09 carrying pGPD2 plasmid (designated as MKY09B2 and MKY09D2 respectively) were grown overnight in each 15 ml falcon tubes containing 5 ml of YNB media at 28^{o}C and 220 rpm. It was used as inoculum for further experiments.

Each inoculum was inoculated in 100 ml YNB and YNB + 2% xylose (YNBX) separately in such a way that final OD600 was maintained 0.005. Culture was then allowed to grow at 28^{o}C and 220 rpm. The optical density at 600nm was taken for upto 36 hours in intervals of 2 hours. The specific growth rate of MKY09 at exponential phase was determined by using formula (Palmqvist & Hahn-Hägerdal, 2000a).

$\mu = [\ln(t_1A600))-(\ln(t_0A600)]/\Delta t$

Where;

t_1A600 = Absorbance measured during the final time point (peak of log phase) at 600nm

t_0A600 = Absorbance measured at initial time point at 600nm

Δt = change in time between t_0 and t_1.

3.16.2 Extracellular Glucose and Xylose estimation

The control *S. cerevisiae* and the transformed strain (expressing an episomal plasmid construct) were grown in YNB broth with glucose 20 g/l only, and with xylose 20 g/l at 28˚C and 220 rpm. Samples were taken at 0 hour, 12 hours, 24 hours and 48 hours. Xylose concentration was measured by phloroglucinol assay and glucose concentration was measured by DNS method. Xylose or glucose uptake was calculated by subtracting the initial concentration of xylose or glucose and xylose or glucose concentration present in time interval. The difference between the substrate present in the initial time and time interval is determined as the uptake of the sugar inside the cells which are assumed to be the carbon source for growth of organism.

3.16.2.1 Determination of glucose depletion

One milliliter of sample was taken in a test tube to which 2ml of distilled water and 3 ml of DNS reagent was added. The samples were then incubated in a boiling water bath for 15 minutes. After incubation, the samples were adjusted to the final volume of 10ml by adding distilled water, homogenized and cooled to room temperature in water. Finally, absorbance were measured at 595 nm (Miller *et al*., 1959).

3.16.2.2 Standard curve of glucose

Stock solution of 2 mg/ml was prepared by dissolving glucose in distilled water. Different concentrations of glucose were prepared from the stock solution, i.e., 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, 1.8 and 2 mg/ ml were prepared. Absorbance was measured at

575nm. The standard curve was then drawn by plotting the absorbance against the concentration.

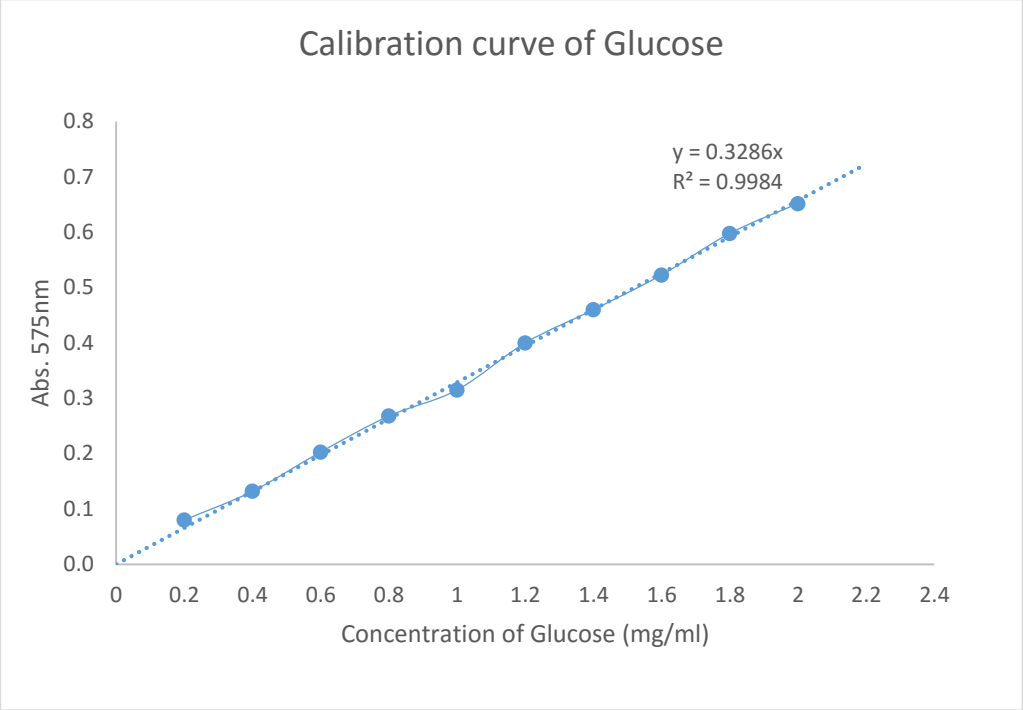


Figure 3.3: Calibration curve of Glucose

3.16.2.3 Xylose estimation by Phloroglucinol Assay (Eberts et al., 1979)

For xylose estimation, 200 µl of sample/stock was taken in a test tube to which 5 ml of phloroglucinol reagent was added. The test tubes were then incubated in a boiling water bath for 4 mins, rapidly cooled to room temperature in water and the absorbance was taken at 554 nm.

3.16.2.4 Standard curve of xylose (Eberts et al., 1979)

Stock solution of 1mg/ml was prepared by dissolving D-xylose in saturated benzoic acid solution. Different concentrations of xylose were prepared from the stock solution i.e., 0, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160 and 170 mg/lt. Standard curve was drawn by plotting the absorbance at 554nm versus the concentration.

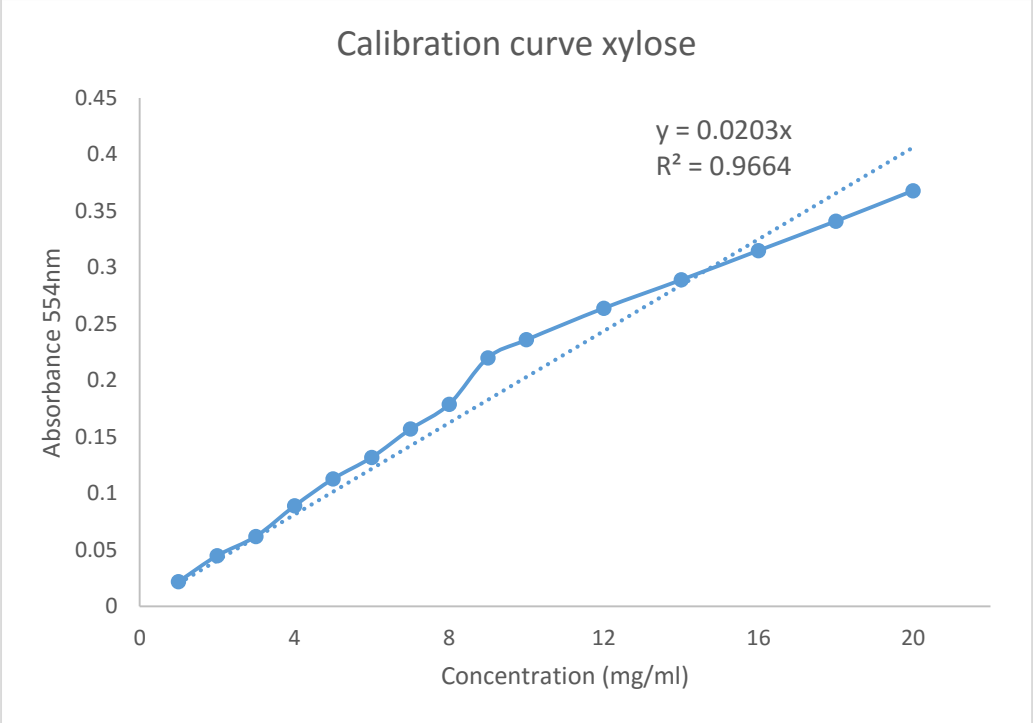


Figure 3.4: Calibration curve of Xylose.

3.17 Quantitative estimation of ethanol by Solvent Extraction and Dichromate oxidation method (Seo et al., 2009)

Stock solution of 20 mg/ml was prepared by dissolving 2.537ml of 99% ethanol in 97.4626 ml of double distilled water and different concentrations of ethanol i.e. 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 mg/ml were prepared by using stock solution.

For ethanol estimation, 1 ml of Tri-n-butyl phosphate (TBP) and 1 ml of each concentration of ethanol were mixed in a 2 ml Eppendorf tube and then vortexed vigorously using a vortex mixer for 12 minutes and it was subjected to centrifugation at 12000 rpm for 2 minutes. After phase separation, 750 µl of the solvent upper phase was transferred to a new Eppendorf tube to which 750 µl of dichromate reagent was added and vortexed vigorously for 12 minutes. It was then centrifuged at 12000 rpm for 2 min. and upper phase was completely discarded as far as possible using disposable pipette tips. After this, 500 µl of oxidized ethanol (chromium ions come to +3 state from its oxidation state +6; oxidized ethanol is green in colour) containing lower phase was pipetted out and optical density was measured at 595 nm.

The standard calibration curve was then constructed using optical density of oxidised dichromate by ethanol at 595 with respect to concentration. The concentrations of the unknown samples were then calculated by using standard curve.

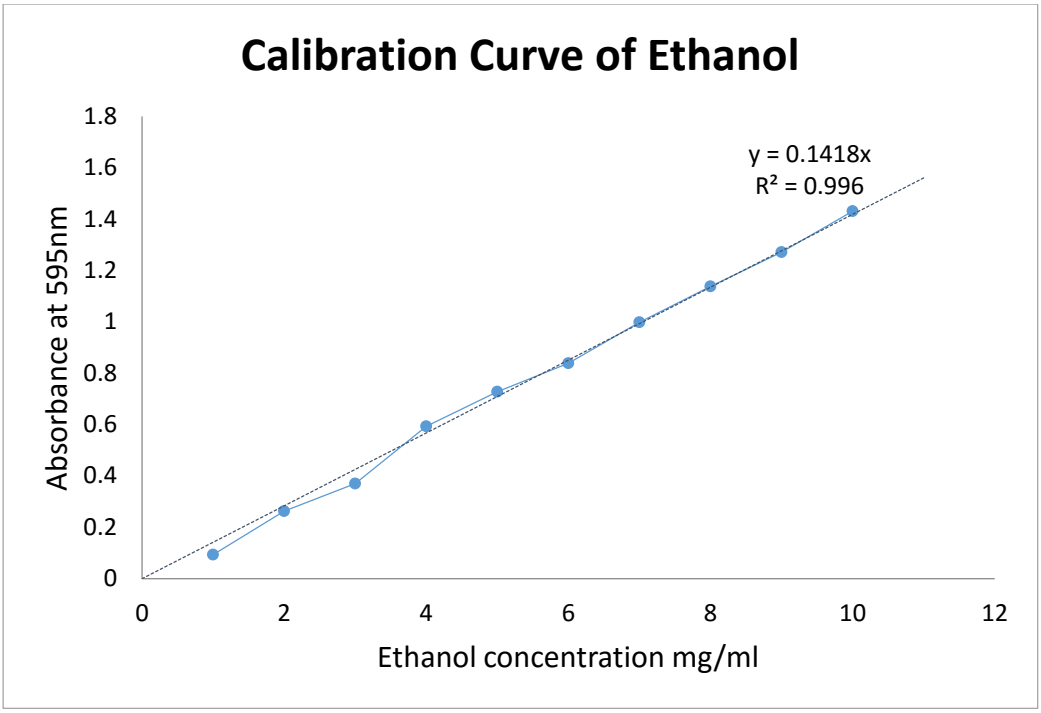


Figure 3.5 Calibration curve of ethanol.

3.18 Ethanol production by control and recombinant MKY09

The control and recombinant MKY09 was grown so as to maintain OD at 600 of 1 and 0.5ml of control and recombinant MKY09 was inoculated in a 10 ml YNB. Production of ethanol was then estimated by sampling in 2 days.

CHAPTER IV

RESULTS

4.1 Enrichment of pUC57XI

Briefly, cloning vector pUC57 containing *xyl*A gene encoding xylose isomerase enzyme was transformed into the host *E. coli* DH5α by heat-shock method. Plasmid was confirmed by restriction digestion with single endonuclease and Polymerase chain reaction.

4.1.1 Transformation of pUC57XI in DH5α

Synthetic codon optimized xylose isomerase *xylA* (XI) originally from *C. phytofermentans* was obtained in cloning vector pUC57 as pUC57XI from Genescript for transforming into *S. cerevisiae* eventually after cloning in pGPD2. Codon optimization is done to overcome the codon bias when genes are heterologously expressed. Codon bias is due to the unequal expression of aminoacid codes due to degeneracy of aminoacid codons (see APPENDIX 6). It was transformed into cloning host *E. coli* DH5α by heat shock method in chemically prepared competent *E. coli* DH5α cells as described in materials and method. Selection of transformed colonies was done on the basis of Kanamycin resistance marker that expressed enzyme from cloning vector by plating on LBA Kan (50µg/ml) plates after overnight incubation at 37^{o}C. Due to the presence of the antibiotic Kanamycin, only those transformed colonies expressing kanamycin resistance gene tends to grow on the plate.

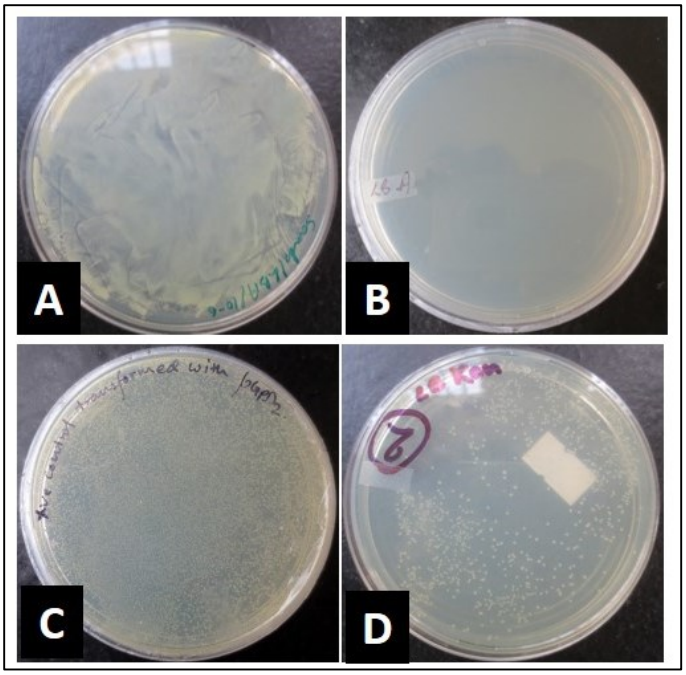


Figure 4.1: Transformation Plates, E. coli DH5α transformed with plasmids. **A-** Viability test, **B-**Negative control, **C-** Positive control (pGPD2) and **D-** Transformed colonies with pUC57XI.

4.1.2 Plasmid isolation from transformants and preliminary confirmation

Colony grown selectively on LBA Kan plate were randomly selected and sub-cultured on 10 ml LB broth supplemented with 50µg/ml Kanamycin at 37°C with shaking at 200 rpm. Plasmid isolation was performed by alkaline lysis method (mini-preparation) and visualized in 1% agarose gel electrophoresis. Thus isolated pUC57XI plasmid was subjected to restriction digestion by the enzyme *Hin*dIII at 37°C for 4 hr. incubation. Digestion product was ran along with undigested plasmid during gel electrophoresis. Single band seen on digestion product approximately at 4 kb (Fig: 4.2, L2) gives preliminary confirmation for the presence of the plasmid of interest.

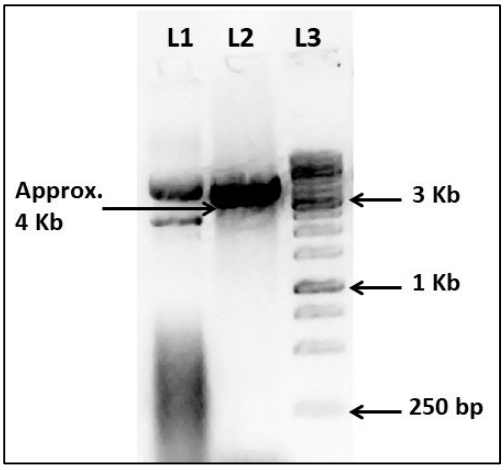


Figure 4.2: Agarose Gel (1%) Electrophoresis of digested and undigested pUC57 XI plasmid. L1- Undigested pUC57 XI plasmid, **L2-** *Hin*dIII digested pUC57 XI (4 kb) and **L3-** 1 Kb DNA ladder FERMENTAS.

4.1.3 Confirmation of true transformants by PCR

Isolated and restriction digestion confirmed plasmids were used as template for polymerase chain reaction for amplification of *XI* gene (*xyl*A) using ORF primer set TUXIF and TUXIR. PCR amplicon was subjected to 1% agarose gel electrophoresis along 1kb ladder from FERMENTAS and visualized under UV light. The amplicon band seen approximately at 1.3 kb (Fig: 4.3, L2 and L3) confirms for the presence of pUC57XI plasmid in transformed *E. coli* DH5α.

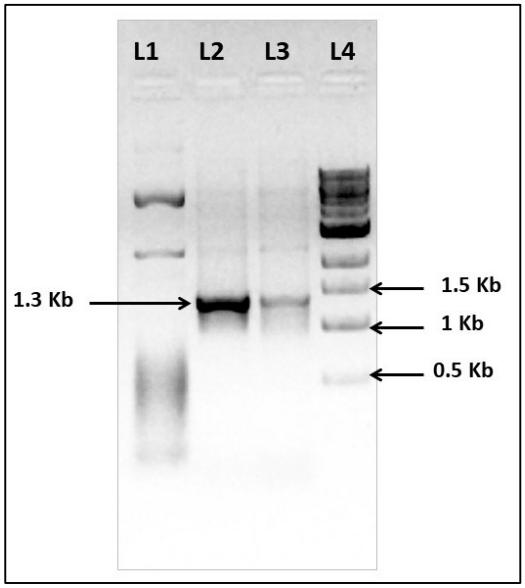


Figure 4.3: Gel Electrophoresis of PCR product and plasmid extracted from transformed colonies (1% Agarose). L1- template pUC 57 plasmid with XI, **L2** & **L3-** PCR product of ORF XI (1.3 kb) and L4- 1 Kb DNA ladder NEB

4.2 Enrichment, Extraction and Confirmation of Cloning Vector

Vector selected for the study was pGPD2 plasmid which is bacterial and yeast expression vector provided with constitutive GPD promoter. Vector was transformed and enriched in host *E. coli* DH5α by overnight incubation at 37°C in LB-Amp (100µg/ml) broth. Plasmid isolation was done by alkaline lysis method. Isolated plasmid was separately digested in single and double digestions for confirmation and compatibility of restriction enzyme as used in insert preparation.

4.2.1 Confirmation of pGPD2 by digestion

Isolated plasmid was subjected to 4 hours single digestion at 37°C using restriction enzymes *Hin*dIII and *Xho*I in different reaction mixture and double digestion using endonucleases set of *Eco*RI and *Nde*I to release two fragments of 1.8 kb and 3.9 kb. Single band seen in single digestion product approximately at 5.7 kb (Fig: 4.4 A, L2 and L3) when ran with 1 kb ladder from FERMENTAS and two bands seen at size of approx 3.9 kb and 1.8 kb (Fig: 4.4 B, L3) when ran with 1 kb ladder from NEB in 1% agarose gel gives confirmation for vector pGPD2.

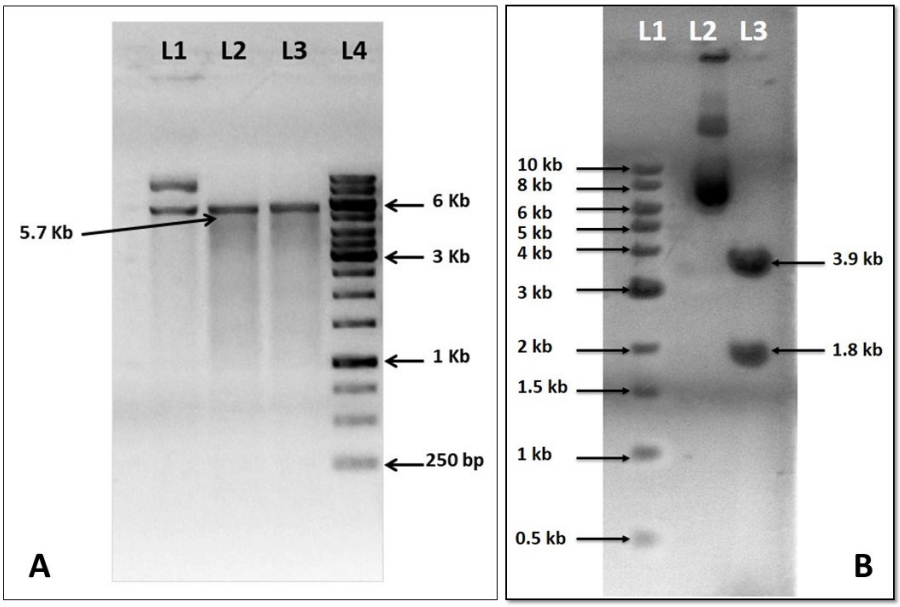


Figure 4.4 Gel Electrophoresis of digested pGPD2 (1% Agarose). A; Single digestion **L1-** Undigested pGPD2, **L2-** *Hin*dIII digested pGPD2, **L3-** *Xho*I digested pGPD2 and **L4-** 1 Kb ladder FERMENTAS. **B;** Double digestion. **L1**- 1 kb DNA ladder NEB, **L2-** Undigested pGPD2 and **L3-** Double digested pGPD2 with *Eco*RI and *Nde*I.

4.3 Preparation of Insert and Vector

PCR amplified gene and vector pGPD2 were digested with same sets of endonucleases *Hin*dIII and *Xho*I. Digested products were purified and subjected to ligation. Ligation mixture was directly used for transformation and confirmed after plasmid extraction by polymerase chain reaction and restriction digestion.

4.3.1 Gel Purification for enzyme digested PCR product and pGPD2 vector

Undigested confirmed insert and cloning vector were subjected to overnight double digestion with restriction enzymes *Hin*dIII and *Xho*I at 37^{o}C. Digested products were subjected to agarose gel electrophoresis in 0.8% low melting agarose supplemented with 0.5 µg/ml EtBr for visualization in parallel with 1 kb ladder FERMENTAS. Bands of insert and vector seen at respective sizes of 1.3 kb and 5.7 kb respectively (Figure 4.6; A and B) were excised with clean knife and stored in fresh Eppendorf tube.

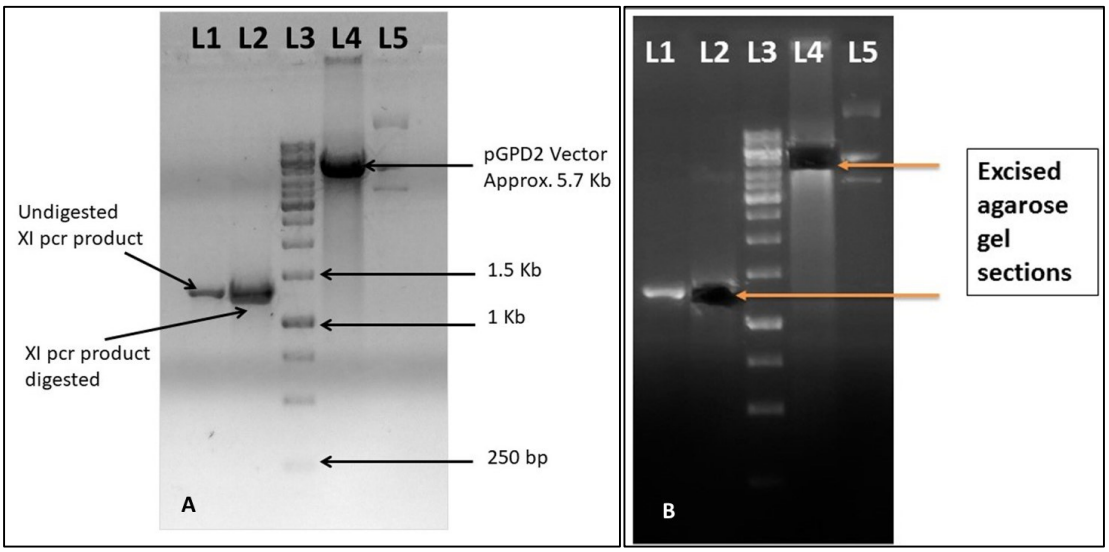


Figure 4.5 Gel Electrophoresis of double digested (with *Hin*dIII and *Xho*I enzyme) XI insert and pGPD2 vector (0.8% Low Melting Agarose). L1- Undigested XI pcr product, **L2-** Digested XI pcr product, **L3-** DNA Ladder 1 Kb FERMENTAS, **L4-** Digested pGPD2 and **L5-** Undigested pGPD2. **A;** Gel picture before excision and **B;** after excision of gel section.

Double digested insert and vector excised from low melting agarose gel were purified by gel elution method using Wizard SV Gel and PCR Clean-Up System. This purification step is done to prevent the effect of ligation reaction between insert and vector due to presence of unused dNTPs, metal ions in buffer, excess primers used in PCR, restriction enzymes used in restriction digestion etc. The respective DNA samples were then quantified by gel electrophoresis using 1 kb ladder FERMENTAS as standard. The concentration of the insert

and vector were estimated to be 15ng/µl and 125ng/µl respectively based on band intensity of the ladder used.

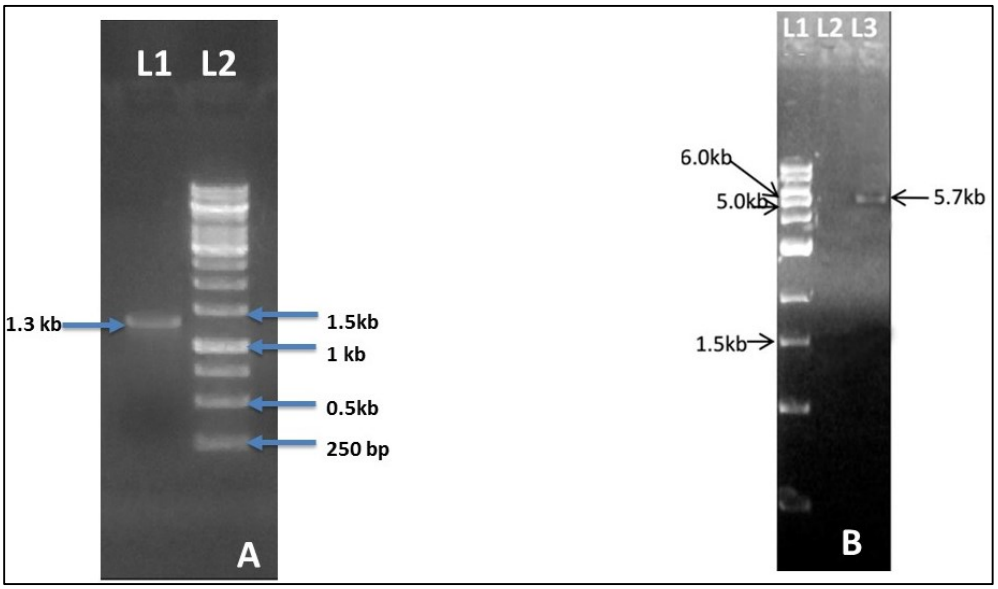


Figure 4.6 Agarose Gel Electrophoresis. A; Insert preparation, **L1;** Purified PCR product Insert of size 1.3 kb approx., **L2;** 1 kb DNA ladder FERMENTAS. **B;** Vector preparation, **L1;** 1 kb DNA ladder NEB, **L2;** pGPD2 vector prepared of size 5.7 kb purified by gel elution.

4.4 Ligation and transformation

Insert and vector were ligated by T4 DNA ligase enzyme. In ligation mixture, 6:1 molar ratio of insert and vector was maintained. Ligation mixture was incubated at 4^{o}C for 16 hours and was directly subjected to transformation into chemically prepared *E. coli* DH5α competent cell by heat shock method. Transformants selection was done by plating on LBA-Amp (100µg/ml) plate as cloned plasmid will have ampicillin resisitant gene intact as the marker for Ampicillin resistant colonies.

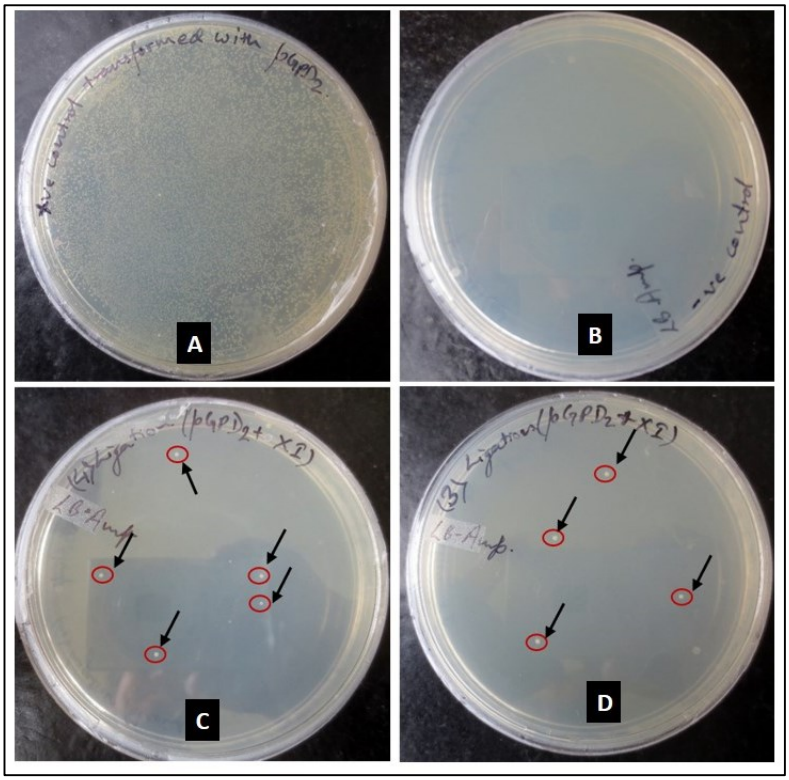


Figure 4.7 Transformation of *E. coli* DH5α competent cells. A; positive control (pGPD2), **B;** Negative control, **C** and **D;** with ligation mixture (pGPD2+XI) as experiment. Transformed colonies are enclosed in circle.

4.5 Screening of transformants

4.5.1 Plasmid extraction from transformants

The transformant colonies were subcultured overnight on 10 ml LB broth containing 100 µg/ml Ampicillin at 37°C with shaking at 150 rpm. The broth was used for isolation of plasmid by alkaline lysis method as mentioned above. Extracted plasmid was run in 1% agarose gel electrophoresis and visualized under UV illumination.

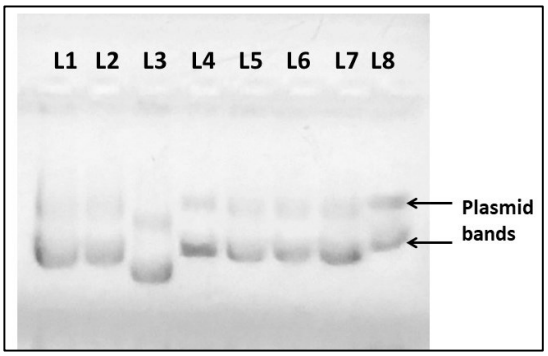


Figure 4.8 Gel Electrophoresis of plasmid extracted from transformants. L1, L2 & **L3** are plasmid from Transformant colonies **A1, A2** & **A3** respectively, **L4, L5, L6, L7** & **L8** are plasmid from Transformant colonies **B1**, **B2, B3, B4** and **B5** respectively.

4.5.2 Preliminary Screening by PCR

Plasmids isolated from putative transformed colonies were used for PCR amplification of XI gene using ORF primers. This PCR gives preliminary screening of transformation with ligated construct.

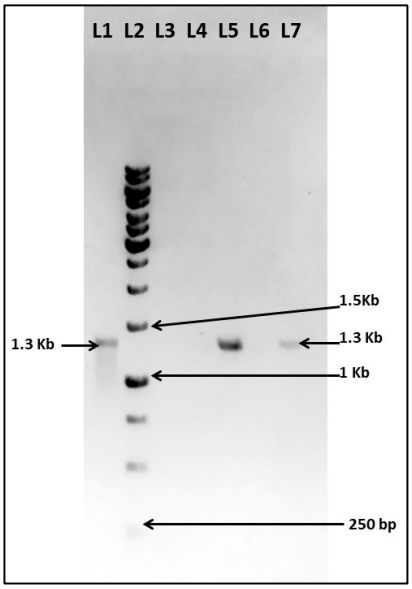


Figure 4.9 PCR confirmation of ligation and transformation using ORF-primer. L1- Positive control, **L2-** 1 kb DNA ladder FERMENTAS, **L3-** PCR product from A1 plasmid, **L4-** PCR product form A2 plasmid, **L5-** PCR product from A3 plasmid, **L6-** PCR product form B1 plasmid and **L7-** PCR product form B2 plasmid.

4.5.3 Screening by Restriction digestion

Plasmids that gave amplicon size of 1.3 kb were further subjected to screening by restriction digestion. An endonuclease used in this step was *Hin*dIII that would yield the digestion product of size approximately 7.1 kb. Digested plasmids were visualized in 1% Agarose gel electrophoresis. Among two prospective cloned plasmid constructs from PCR amplification, only one of the construct gave expected digestion product size and hence it was comfirmed to be our required construct and it was named as B2 (pGPD2+XI) onwards.

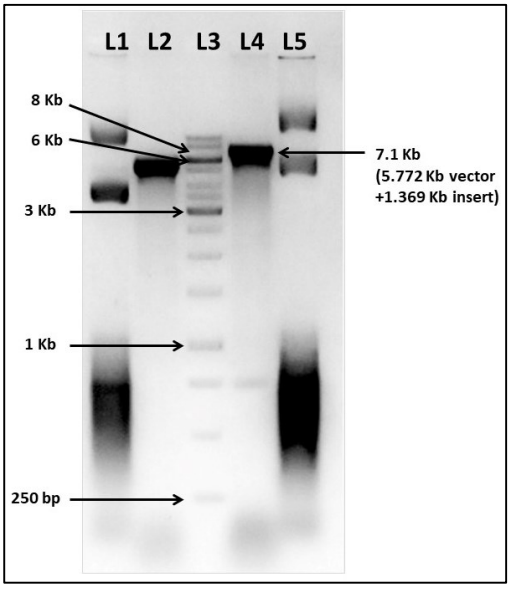


Figure 4.10 Agarose gel electrophoresis (1%) of Single digested plasmid by *Hin*dIII. L1- Undigested A3 plasmid, **L2-** Digested A3 plasmid, **L3-** 1 kb DNA Ladder FERMENTAS, **L4-** Digested B2 plasmid and **L5-** Undigested B2 plasmid.

4.6 Transformation of pGPD2+XI construct (B2) into *Saccharomyces cerevisiae* MKY09

The construct prepared and confirmed by PCR and Restriction digestion was transformed into yeast *Saccharomyces cerevisiae* MKY09 by Electroporation. Electoporation method was selected for the work, due to its high transformant yield efficiency of 3x 10^5 Transformants/µg of DNA (Becker & Guarente, 1991) when used with 1M sorbitol.

Electro-competent *Saccharomyces cerevisiae* MKY09 cells were prepared from overnight culture at 28°C with shaking at 220rpm. Cells were subjected to short electric pulse in micropulsar Electroporator (BIO RAD) set using 0.2cm electroporation cuvette in presence of 1M sorbitol buffer to stabilize competent cells and to preserve intactness of cell integrity. Transformed cells by Electroporation were selected by plating in YNB URA^{-ve} plate supplemented with 1% Glucose as carbon source over 48 hours incubation at 28°C. Transformants are able to grow on the plate by ability of uracil synthesis induced by URA3 gene present on vector.

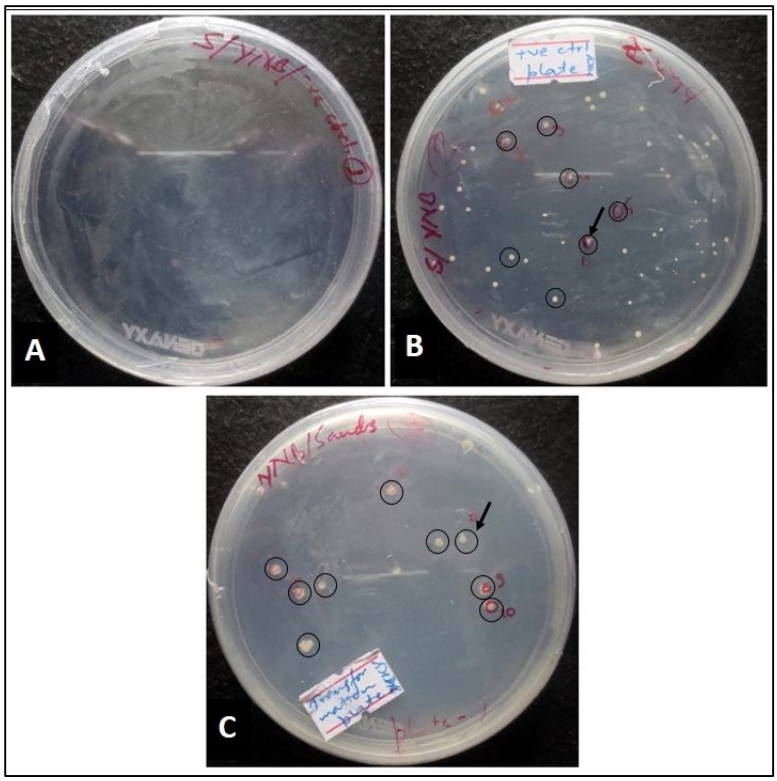


Figure 4.11 Transformation of MKY09. A; Negative control, **B;** Positive control (with pGPD2 only) and **C;** with B2 (pGPD2+XI construct). Transformed colonies grown are enclosed in circle.

4.7 Screening of Transformed Yeast

4.7.1 Plasmid Extraction from Transformants and Screening by PCR

Isolated Transformed colonies harboring positive control (MKY09+pGPD2) and recombinant construct (MKY09+PGPD2XI) were randomly selected and sub-cultured over 48 hours in YNB broth supplemented with 1% Glucose as carbon source at 28^{o}C with shaking at 220rpm. These broth cultures were used for isolation of plasmid from control and recombinant yeast by physical method of cell lysis using glass beads. Extracted plasmid were visualized in EtBr stained 1% agarose gel. The extracts were predominantly contaminated with genomic DNA and RNA fractions. Even though there was contamination of gDNA and RNA, plasmid extracts were used for PCR for preliminary screening using ORF primer set of XI. PCR products used for re-amplification and were visualized in 1% agarose gel stained with 0.5µg/ml EtBr by electrophoresis and UV-illumination.

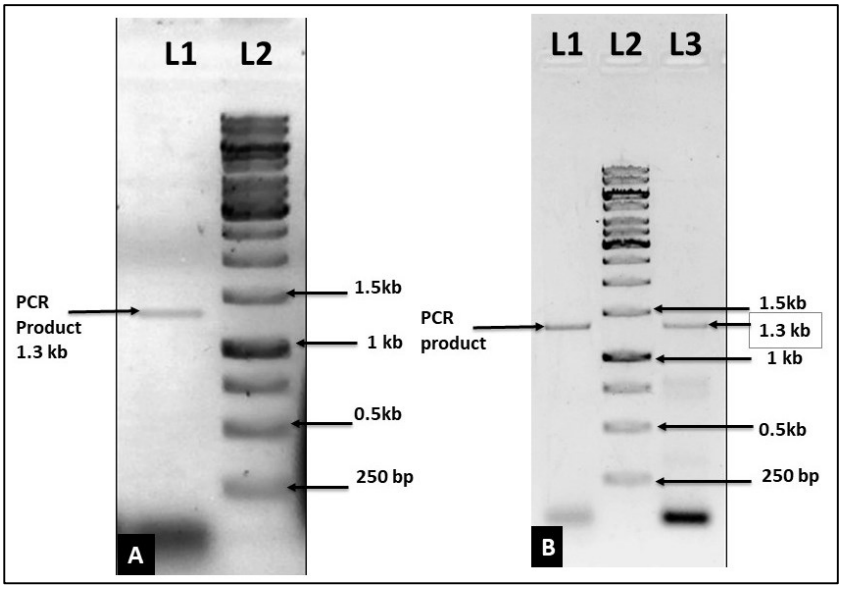


Figure 4.12: 1% Agarose Gel Electrophoresis. A- L1; PCR product of size 1.3 kb approximately using plasmid construct extracted from recombinant yeast, **L2;** 1 kb DNA ladder FERMENTAS. **B- L1;** PCR product of size 1.3 kb approximately using plasmid construct extracted from recombinant yeast, **L2;** 1 kb DNA ladder FERMENTAS, L3; Re-amplification of PCR amplicons in L1.

4.8 Confirmation of *Saccharomyces cerevisiae* MKY09 strain with pGPD2 + Xylose Isomerase plasmid (B2) by Southern Blotting

Finally, the presence of plasmid construct (pGPD2 carrying Xylose isomerase) inside the MKY09 was verified by Southern blotting. The southern blot is a hybridization process of transferring nucleic acids onto the positively charged membranes and it is widely used technique in gene manipulation and analysis for detection of a specific DNA sequence in DNA samples (Primrose and Twyman, 2006). In this study, transformed colony of *Saccharomyces*

cerevisiae MKY09 with B2 construct (pGPD2+XI gene) was confirmed using biotin labeled 1.3 kb DNA probe prepared by PCR using XI gene specific ORF primers.

4.8.1 Probe preparation

The biotin labeled probe was prepared by PCR using XI gene specific ORF primer. Two sets of PCR mixture were prepared; one with biotin labeled Uracil cocktail dNTPs (1 mM dGTP, 1 mM dATP, 1 mM dCTP, 0.65 mM dTTP, 0.35 mM Biotin-11-dUTP) and another with 200 µM of unlabeled dNTPs. The pUC57 + XI vector from bacteria was used as template for PCR amplification. Thus amplified PCR products were run in 1% agarose gel electrophoresis with DNA marker (Fig. 4.14). The biotin labeled PCR product ran slightly slower (Fig. 4.14, L2) in comparison to unlabeled PCR product (Fig. 4.14, L3).

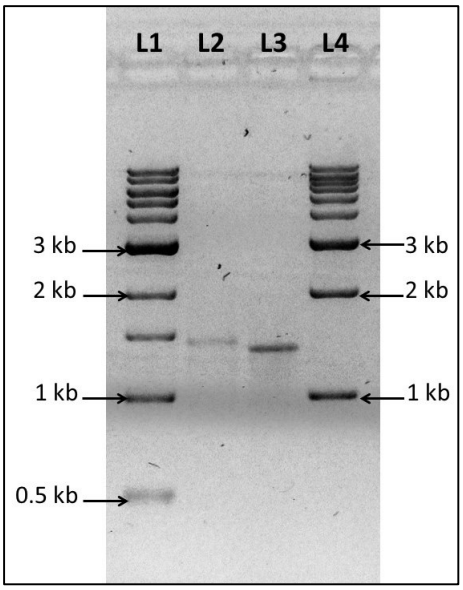


Figure 4.13 Electrophoresis for confirmation of Biotin labeled probe to be used for Southern Blotting. L1- 1 kb DNA Ladder NEB, **L2-** Biotin labelled probe, **L3-** Positive PCR product of XI and **L4-** 1 kb DNA Ladder GeNei.

4.8.2 Determination of labelling efficiency of probe by dot blotting

The concentration of biotin labeled probe was estimated by using standard biotin labeled DNA (5ng/µl) Thermo scientific USA. Different dilution of probe and standard biotin labeled DNA were prepared as Control, 10^{-2}, 10^{-4} and 10^{-6} to find out concentration of biotin labeled probe which was estimated to be 2.5 ng/µl. Minimum of 250 fg/µl of thus prepared probe was detected after overnight incubation which was close to acceptable value of 100 fg/µl. Thus prepared probe was then subsequently used for hybridization.

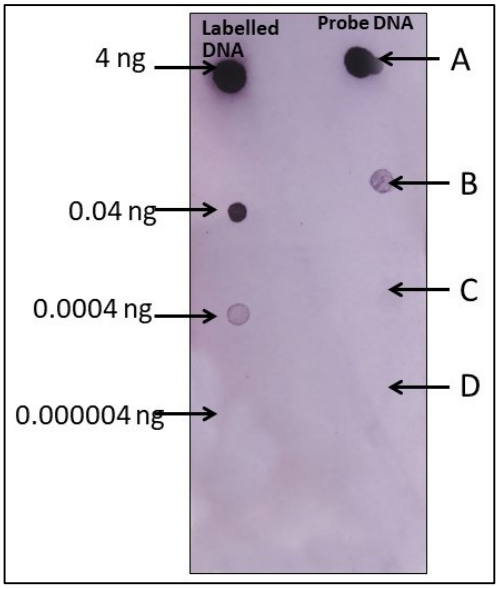


Figure 4.14 Dot blot for estimation of labeling efficiency of probe. A – 10^{0} dilution. **B –** 10^{-2} dilution. **C –** 10^{-4} dilution. **D –** 10^{-6} dilution.

4.8.3 Detection of biotin labeled probe bound with Xylose Isomerase gene

Biotin labeled probe bound Xylose Isomerase gene from MKY09 was detected using biotin chromogenic kit K0661. The blue precipitation band of hybridized XI gene from recombinant MKY09 with gene specific probe was observed in corresponding well of plasmid loaded with pGPD2+XI construct from MKY09. On the contrary, no band was observed in membrane containing plasmid band from MKY09 harboring pGPD2 plasmid only. Hence, this final experiment proved that the chosen MKY09 colony contained the construct pGPD2 + XI in its cytosol.

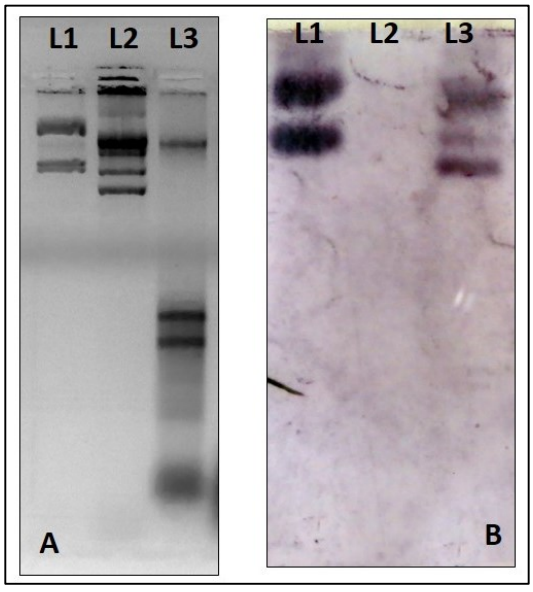


Figure 4.15 Southern Blot analysis of the construct pGPD2+XI. (A) 0.8% Agarose gel electrophoresis for separation of plasmid DNA. **L1** – Positive control (pGPD2 + Xylose isomerase plasmid from *E. coli*). **L2** – pGPD2 Plasmid without Xylose isomerase. **L3** – Plasmid from the recombinant yeast colony. **(B)** Probe bound pGPD2 Xylose isomerase construct on the nylon membrane. **L1** – Plasmid from the recombinant yeast

colony labeled with the probe. **L2** – Unlabeled Plasmid that is pGPD2 without xylose isomerase. **L3** – Positive control (pGPD2 + Xylose isomerase plasmid from *E. coli*) hybridized with labeled probe.

4.9 Visualization of Plasmid construct in Transformant yeast *S. cerevisiae* MKY09 by Fluorescent Microscopy (Meluh Lab, 2005)

Permeablized and chemically fixed cells were stained with DAPI and observed under fluorescent microscope to investigate the presence of recombinant construct plasmid in MKY09 cells. The construct plasmids fluoresced within the cytoplasmic region of recombinant MKY09 along the nuclear DNA but not in control MKY09 where only nuclear region gave the fluorescence.

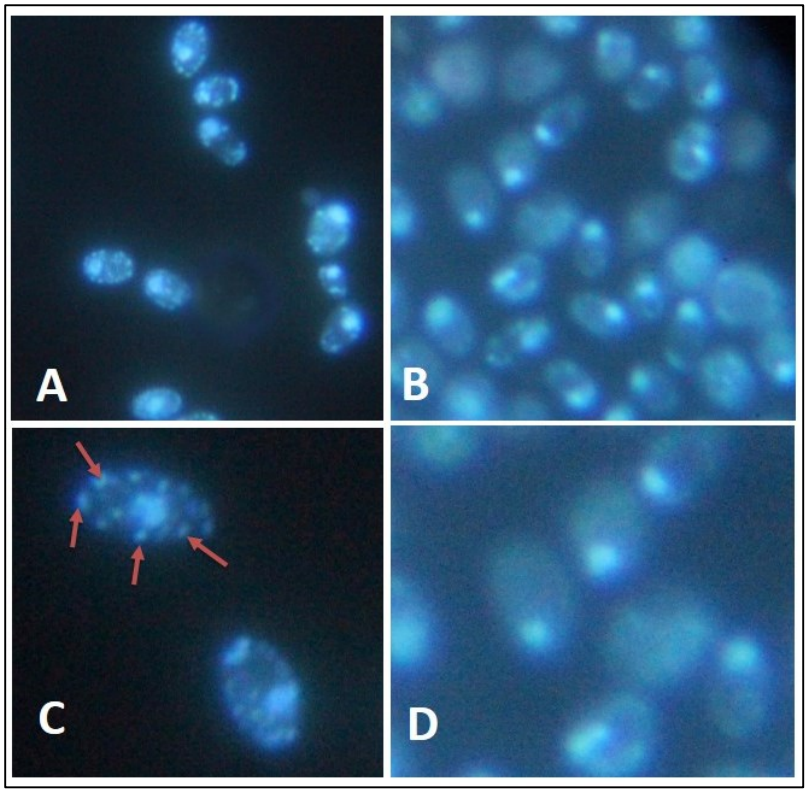


Figure 4.16 DAPI staining of *S. cerevisiae* MKY09 under fluorescent microscope. A- *S. cerevisiae* MKY09 with episomal plasmid construct (pGPD2+XI), **C-** enlarged view of A (arrowheads are pointing to episomal plasmids), **B-** *S. cerevisiae* MKY09 without plasmid and **D**- enlarged view of B (only nucleus is fluorescent).

4.10 Functional expression analysis

4.10.1 Growth kinetic/rate of Control MKY09 and Recombinat MKY09 (MKY09D2 & MKY09B2)

Growth rates of control (MKY09 transformed with pGPD2) and recombinant MKY09 (MKY09 transformed with pGPD2+XI) designated as MKY09D2 and MKY09B2 respectively were observed in YNB and YNB + 2% xylose. There were no significant differences among control and recombinant in terms of growth rates in YNB broth (as shown in Figure: 4.18). On the other hand, growth of recombinant strain (MKY09 carrying xylose isomerase gene) was slightly lesser in comparison to control MKY09 carrying pGPD2 only (Figure: 4.19) when grown in media supplemented with 2% xylose.

The growth rate of control and experimental MKY09 were found to be 0.160 hr^{-1} and 0.195 hr^{-1} respectively in YNB broth. Similarly, growth rates on YNB with 2% xylose were determined to be 0.163hr^{-1} and 0.155 hr^{-1} respectively for control and experimental MKY09 strains.

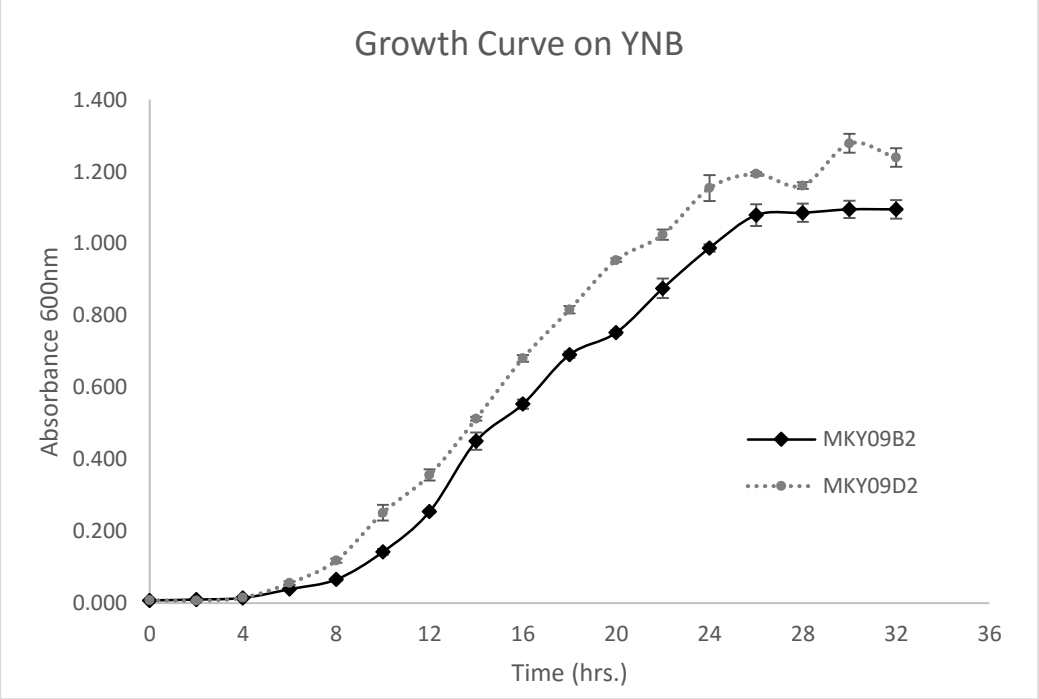


Figure 4.17 Growth curve of Control and Recombinant yeast MKY09 in YNB URA- media. (Values in graphs are mean ±SE where n=3).

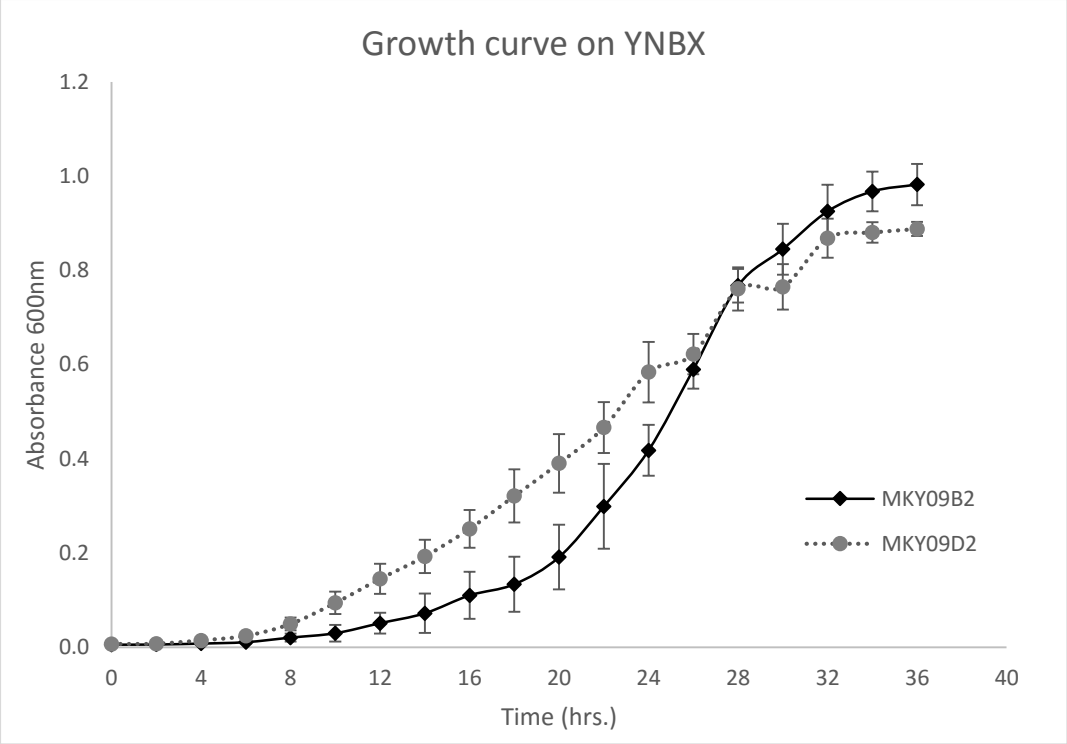


Figure 4.18 Growth curve of Control and Recombinant yeast MKY09 in YNBX (YNB URA- broth with 2% xylose). (Values in graphs are mean ±SE where n=3).

4.10.2 Extracellular Glucose and Xylose Concentration

The control *S. cerevisiae* and the transformed strain (expressing an episomal plasmid construct) were grown in YNB with 2% glucose and YNBX broth with glucose 20g/l and xylose 20g/l at 28˚C with shaking at 220 rpm. Glucose depletion was estimated in YNB upto 36 hours. Samples from YNBX were taken at 0 hour, 12 hours, 24 hours, 48 hours, 72 hours and 96 hours. Xylose concentration was measured by phloroglucinol assay and glucose concentration was measured by DNS method. Glucose uptake was calculated upto 72 hours while xylose utilization was determined upto 96 hours. Xylose and glucose uptake was calculated by subtracting the initial concentration of xylose or glucose and xylose and glucose concentration present in time interval. The difference between the sugar concentration present in the initial time and time interval is determined as the uptake of the sugar inside the cells which are assumed to be the reduced carbon source for growth of organism.

4.10.2.1 Extracellular Glucose depletion in YNB

Extracellular glucose was measured on culture broth by DNS method. Measure of decrease in glucose content of broth corresponds to the glucose imported into the cell. In this study, glucose content declined to 8.09 ± 0.207 mg/ml and 8.67 ± 0.161 mg/ml over 36 hours in MKY09B2 and MKY09D2 strains respectively, indicating that amount of glucose used by respective strains were 11.91± 0.207 mg and 11.33 ± 0.161 mg.

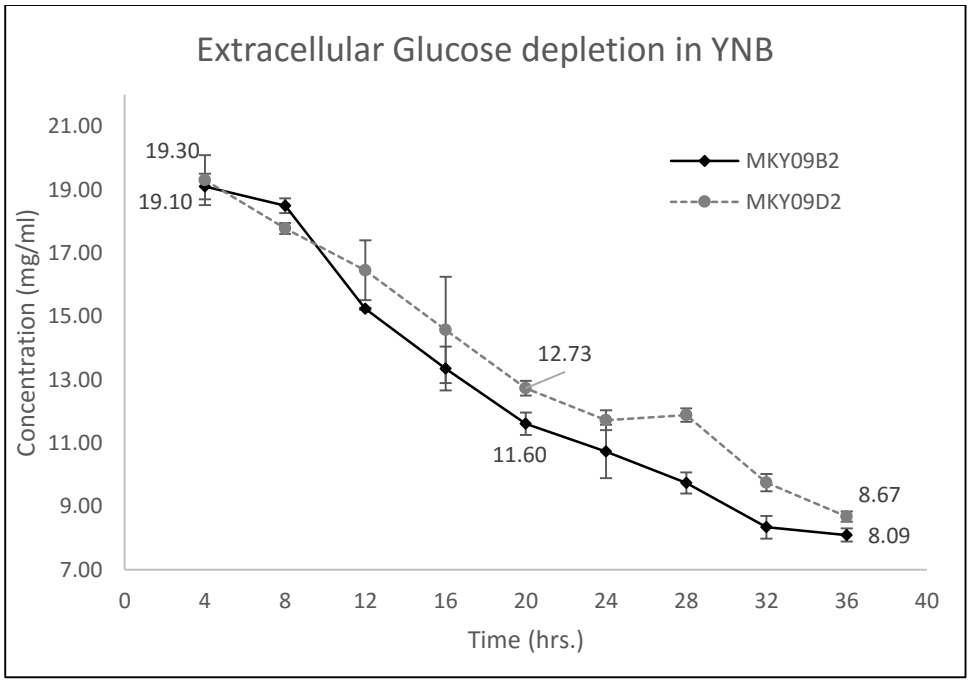
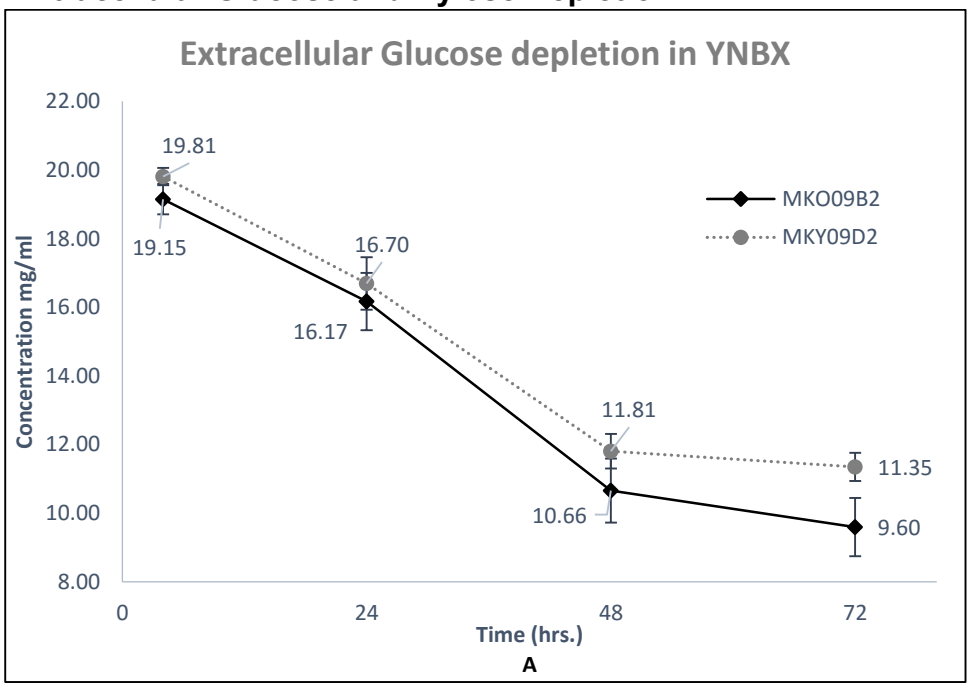


Figure 4.19 Graph showing the extra-cellular glucose depletion on YNB media by MKY09D2 and MKY09B2. (Values in graphs are mean ±SE where n=3).

In YNB broth, both control and recombinant strain utilized nearly equal amount of glucose.

4.10.2.2 Extracellular Glucose and Xylose Depletion



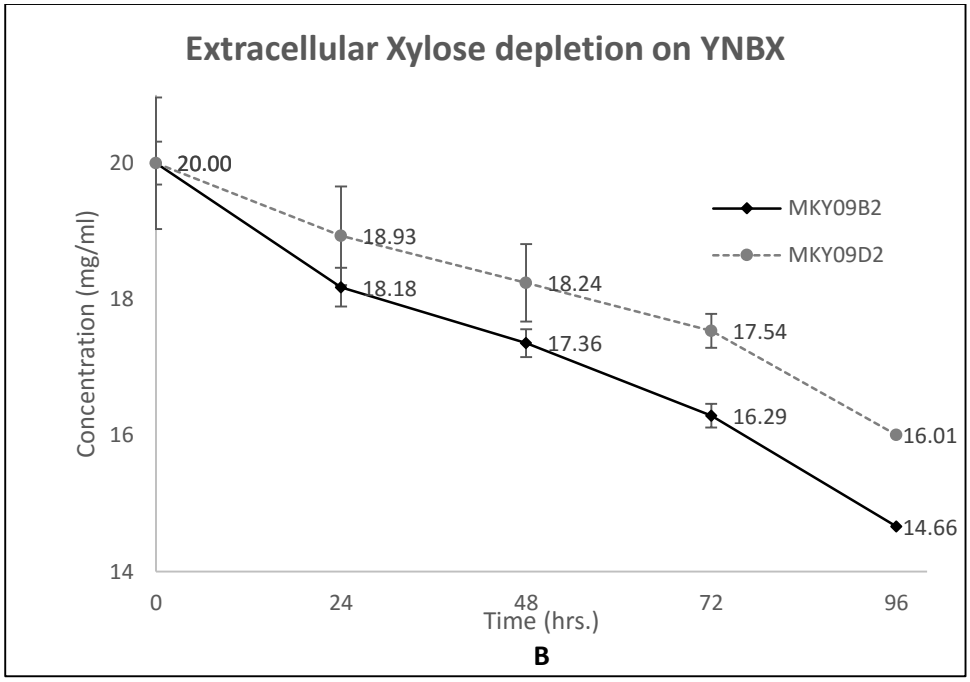


Figure 4.20 Extracellular depletion of fermentable sugars from YNBX media. A- Glucose depletion and B- Xylose depletion. (Values in graphs are mean ±SE where n=3).

Glucose depletion in YNBX media was calculated upto 72 hours. Glucose concentration in media declined from 20 mg/ml to 9.6 mg/ml and 11.35 mg/ml over 72 hours in cultures of MKY09B2 and MKY09D2 respectively (Figure 4.21 A) indicating amount of glucose used to be 8.65 mg and 10.4 mg respectively. On the other hand, Xylose depletion was observed upto 96 hours where xylose concentration declined from 20 mg/ml to 16.29 mg/ml and 17.54

mg/ml over 72 hours in culture of MKY09B2 and MKY09 D2 respectively (Figure 4.21 B) which further declined to 14.66 mg/ml and 16.01 mg/ml respectively over 96 hours. Xylose used over 72 hours was thus 2.46 mg and 3.71 mg over 72 hrs by MKY09D2 and MKY09B2 respectively. While over 96 hours xylose consumed was 3.99 mg and 5.34 mg by control and recombinant strains. Decline in glucose and xylose concentration in media corresponds to the sugar intake by cells. This decrease in xylose along with glucose would lead us to conclude the utilization of glucose transporters (HXTs) for the uptake of xylose as well from the medium but has to be further validated. Amount of sugars imported in both strains are listed as follows-

Table 4.1: Amount of sugar imported into the cells.

Strain	Sugar used over 72 hrs.	
	Glucose (mg)	Xylose (mg)
Recombinant MKY09B2	10.40	3.71
Control MKY09D2	8.65	2.46

Strain MKY09B2 used 20.2 % more glucose and 50.8% more xylose from the media than that of strain MKY09D2 over 72 hours incubation. From this we can conclude for the increased ability of engineered strain MKY09B2 for elevated sugar utilization including xylose along the glucose.

4.10.3 Ethanol Estimation

Ethanol productivity of both control and recombinant strains were estimated in YNBX media over different time interval by solvent extraction and dichromate oxidation. Ethanol production by MKY09D2 and MKY09B2 was found to gradually increase with time of incubation (Figure: 4.22). Production of ethanol in YNBX media by recombinant strain MKY09B2 was found to be higher than that produced by control strain MKY09D2. On the basis of results observed, the production of ethanol could have had effect of diauxic growth, however it was not confirmed in the present study.

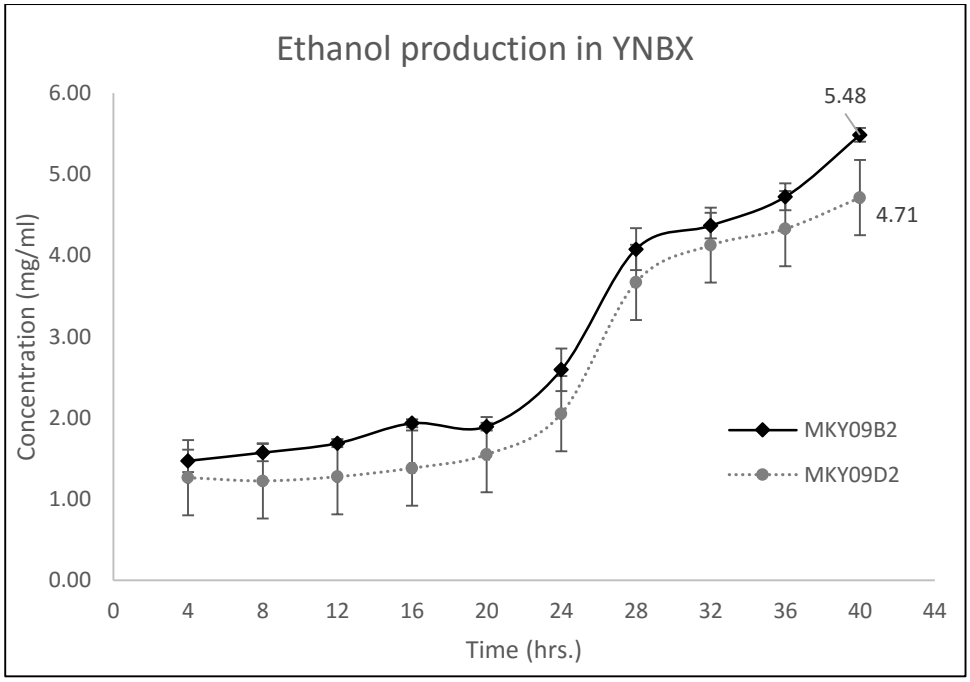


Figure 4.21 Ethanol production trend of yeast strains on YNBX media. (Values in graphs are mean ±SE where n=3).

In another experiment for maximum Ethanol production over long fermentation period by control and recombinant MKY09, maximum production was observed between 96 to 120 hours incubation where maximum ethanol produced was found to be 5.87 mg/ml and 5.84 mg/ml respectively by MKY09B2 (Figure: 4.23) recombinant strain.

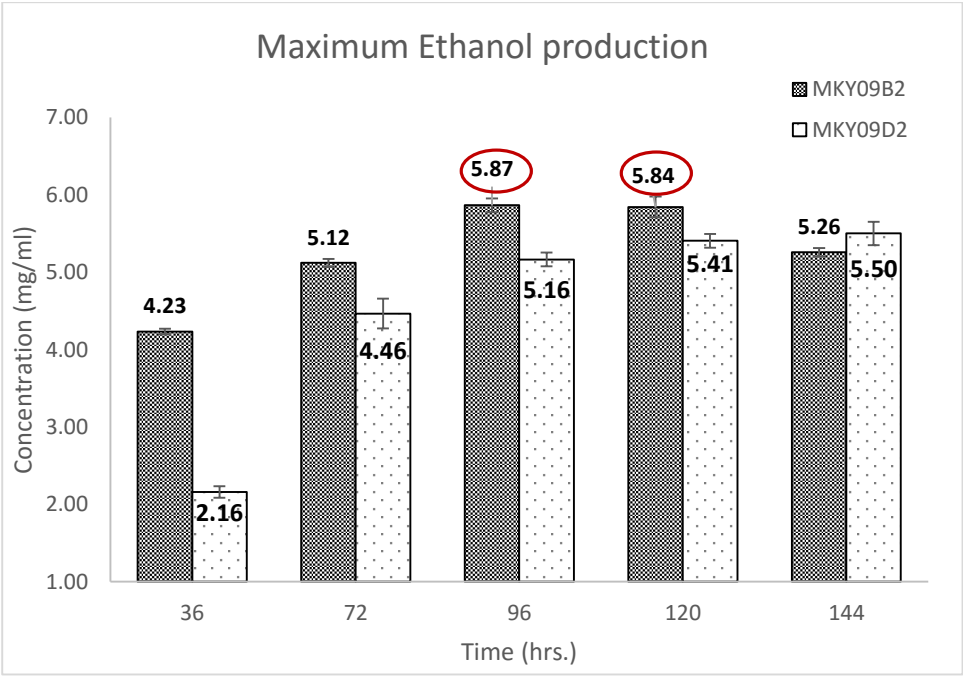


Figure 4.22 Maximum ethanol yield on YNBX by Control (MKY09D2) and Recombinant (MKY09B2) strains of *S. cerevisiae*. (Values in graphs are mean ±SE where n=3).

4.10.4 Ethanol yield on YNB

Ethanol yield by both the strains on YNB media over the 36 hours incubation was also determined

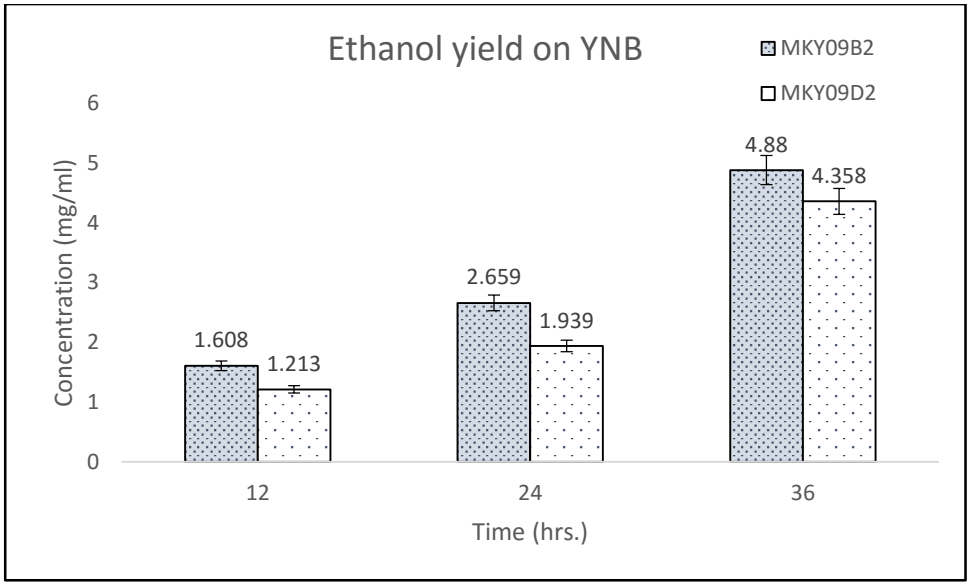


Figure 4.23 Ethanol yield on YNB media. (Values in graphs are mean ±SE where n=3).

As observed from the graph, the initial stages when the recombinant yeast would be utilizing both glucose and xylose when compared to the control yeast that would have the ability to consume only glucose, the amount of ethanol production is significantly higher hence supporting our hypothesis that expression of xylose isomerase in the yeast would enable it to produce higher quantity of ethanol when compared to the yeast that does not possess this ability.

CHAPTER V
DISCUSSION

5.1 Construction of episomal XI yeast expression system

Codon Optimized Xylose Isomerase (XI) was obtained in pUC57 shuttle vector from Gene-script. Codon optimization is done to overcome the codon bias between the source of the gene and host organism. Codon bias is also an inherent characteristic due to the preference shown to certain genetic code by the cell during translation (see appendix 6). It can create problem during heterologous expression due to less availability of tRNA for that codon, hence less translation (Wiedemann & Boles, 2008). The plasmid with the XI gene, designated as pUC57XI was transformed into DH5α. Confirmation of true transformants was done by restriction digestion and PCR using gene specific primers TUXIF and TUXIR for XI. Upon restriction digestion by *Hin*d III enzyme, single band was observed at approximate size of 4 kb equivalent to sum of native pUC57 (2.7kb) and XI gene (1.3 kb). While on PCR using gene specific ORF primer set, amplicon of size approximately 1.3 kb was observed on gel. Gene insert of XI was amplified by PCR using high fidelity Q5 Taq polymerase with proof reading activity that reduces chances of mutation due to addition of incorrect nucleotide during amplification. Thus amplified product was digested with *Hin*dIII and *Xho*I, as primers for gene amplification were designed with insertion of the restriction enzymes recognition site to make amplicon compatible for digestion and subsequent ligation into the yeast expression vector pGPD2, which too was cleaved with the same set of enzymes. Selection of enzyme was made in such a way that gene of interest will be inserted in between GPD promoter and CYC1 terminator of pGPD2 vector. Insertion of heterologous gene downstream to the constitutive promoter provides with continuous expression of the gene in host.

Ju-Yong et al. in 2013, enhanced expression of XI gene from *C. phytofermentans* into *S. cerevisiae* MBL-JY by supplementation of galactose under GAL promoter. Likewise, Leandro et al., (2006; 2007), expressed transporters such as GXS1/GXF1 from *C. intermedia*, At5g59250/At5g17010 from *A. thaliana*, *Xyl*E from *E. coli* and XUT from *P. stipitis* under GPD promoter in p425-GPD plasmid. Similarly, Tanino et al. (2010) reported integration of over-expression cassette of XI and XKS in genome of *S. cerevisiae* MT18-1 strain under GAPDH (Glyceraldehyde 3- phosphate dehydrogenase) promoter by delta integration method as described by Madhavan et al., (2009a) and Yamada et al., (2010). In the same way, Kuyper et al. in 2003 cloned *Xyl*A gene from *Piromyces* sp. E2 isolated by PCR from cDNA library into pYES2 and pPICZα under GAL1 promoter and trasformed into *S. cerevisiae.*

Purified insert and vector was then subjected to the ligation reaction mediated by T4 DNA Ligase enzyme. The enzyme in this step forms the phospho-di-ester bond, a covalent linkage between insert and vector similar to the phosphate backbone of DNA. The ligation was carried out overnight at 4^{o}C. The DNA ligase enzyme has optimal activity at 25°C so the ligation reaction is carried out at a temperature that is a trade-off between the optimal temperatures for bringing the DNA ends together (1°C) and the enzymatic reaction (25°C). Normally 1 hr. at 16°C is fine but since bringing the DNA ends together is the least efficient part of the reaction, by lowering the temperature to 4°C, the process can be made more efficient. However, the enzyme will work very slowly at this temperature so a long (e.g. overnight) incubation time is required. After overnight incubation whole of the ligation mixture was used for transformation into competent *E. coli* DH5α host by heat shock method. Confirmation of ligation was performed by restriction digestion of plasmid isolated from transformant colony and PCR using ORF primer set. It is now well practiced to confirm the ligation and transformation by sequencing, but we confirmed the ligation by PCR and restriction digestion due to lack of resources.

5.2 Transformation of XI into *Saccharomyces cerevisiae* MKY09 strain

In our study, *S. cerevisiae* MKY09 was successfully transformed by electroporation with codon optimized XI gene from *C. phytofermentans* in an episomal expression system. Choice of the electroporation for transformation is based on report of high efficiency yeast transformation made by Becker & Guarente, 1991. Xylose isomerase gene was constructed to express in yeast under constitutive GPD promoter and CYC1 terminator in pGPD2 vector. Transformants were selected on the basis of Uracil metabolism auxotrophy induced by URA3 gene in pGPD2 plasmid that enables MKY09 to grow in Uracil deficit media YNBUra^{-ve}. Transformation was confirmed by PCR and southern blotting. In earlier works done as M. Sc. dissertation at Central Department of Biotechnology, Tribhuvan University, by Nepal N. (2016) and Regmi P. (2016), transporter genes GXS1 and GXF1 were separately transformed into the *Saccharomyces cerevisiae* DTY165 by electroporation with successful expression.

The transformation in the study was confirmed by PCR using gene specific primers for XI gene. In addition to PCR, Southern Blotting was also performed to visualize transformed plasmid into the yeast. The reason for using these methods to confirm presence of plasmid in transformed yeast lies in the fact that, plasmid isolated from recombinant yeast is actually in very low concentration to be visualized in agarose gel electrophoresis and/or is being dominantly contaminated by genomic material.

Earlier few attempts with heterologous expression of bacterial Xylose isomerase gene *XYL*A from *E. coli* (Sarthy et al., 1987), *Clostridium thermosulfurogenes* (Moes et al., 1996), *Bacillus subtilis* (Amore et al., 1989), *Thermus thermophilus* (Walfridsson et al., 1996) were reported to be unsuccessful in terms of functional expression by Hang Zhou et al., 2012. Successful attempt of XI transformation with functional expression was reported by Kuyper et al., in 2003, where XI gene was taken from anaerobic fungus *Piromyces* sp.. Possibility of high level expression of heterologous XI gene was also reported by Madhavan et al. in 2009 when gene was taken from *Orpinomyces* sp.. Likewise, Brat et al., in 2009 also expressed bacterial XI from *Clostridium phytofermentans* in *S. cerevisiae*, which was also less susceptible to inhibition by xylitol, an intermediate during xylose metabolism in comparison to XI from *Piromyces* strain. Expression of XI gene used for cloning and heterologous expression by Brat et al., (2009) was further improved by codon optimization for *S. cerevisiae* which motivated us to use codon optimized synthetic xylose isomerase gene for the study. First successful expression of bacterial *xyl*A gene coding for XI in *S. cerevisiae* was reported by Kaisa Karhumaa et al., in 2005. Study involved expression of *C. phytofermentans* XI and *Piromyces* sp. XI through YEp plasmid constructs (YEp-Opt.XI-Clos and YEp-Opt.XI-Piro) based on p426H7 (URA3) vectors into MKY09. Transformants were selected on the basis of Uracil auxotrophy induced by URA3 gene in plasmid constructs. Plasmid YEp-Opt.XIClos consisting of XI from *Clostridium phytofermentans* enabled growth of recombinant *S. cerevisiae* MKY09 on xylose media. These results formed the basis for our own work.

5.3 Functional Expression Analysis

5.3.1 Growth on media with both Xylose and Glucose as carbon source

The growth rates of control and recombinant strains were found to be 0.160 hr^{-1} and 0.195 hr^{-1} respectively in YNB broth while on YNBX (YNB with 2% xylose) were determined to be 0.163hr^{-1} and 0.155 hr^{-1} respectively for control and experimental MKY09 strains.

When we observe the growth curve irrespective of the growth rate, growth of control strain MKY09D2 (MKY09 with pGPD2 only) was slightly higher in YNB media in comparison to recombinant strain MKY09B2 (MKY09 with yeast expression construct of XI). Likewise, growth of MKY09D2 was found to increase earlier than that of MKY09B2 in YNBX media (YNB with 2% glucose and 2% xylose), whose growth continued beyond MKY09D2 when cultured up to 36 hours.

Although having slower growth when compared to the control strain, the transformed strain had a higher biomass yield. The long lag phase followed by steep log phase could be

accounted with time needed for yeast to acclimatize in new media and additional expression of XI gene which enabled co-consumption of both glucose and xylose from media by recombinant strain. Comparatively high growth of MKY09D2 in YNB media may be attributed to the additional burden borne on recombinant strain due to addition of foreign gene. Likewise, delayed but higher/increased growth of recombinant strain in YNBX media may be due to the ability of recombinant strain to use xylose also as sugar source when glucose runs out in the growth media. The control strain despite growing faster reached its stationary phase relatively earlier. The additional ability to metabolize xylose must have helped our recombinant strain to continue growth in log phase for a longer period. The growth also helps us to conclude the efficacy of the expression of xylose isomerase in metabolizing xylose present in medium.

5.3.2 Growth on media with Xylose as sole carbon source

Growth of both MKY09D2 and MKY09B2 were either absent or under detectable level in the media with xylose as sole source of carbon. This may be due to inability of *S. cerevisiae* to recognize xylose as a strictly fermentable carbon source (Jin et al., 2004) and lack of xylose specific transporter in yeast (Jeffries & Jin, 2004). This is in favour as the study of Brat et al., 2009 where recombinant strain could not grow on media with xylose as sole carbon source but growth was enabled by repeated shake flask cultures till 28 days on synthetic media (20gm/lit xylose, 1gm/lit yeast extract and 2 gm/lit peptone). This restoration for growth in xylose media may be attributed to change in Xylose transporter expression which does occur in *S. cerevisiae* through hexose transporters at significantly lower rates than glucose (Hamachar et al., 2002). *Saccharomyces cerevisiae* strain are also reported to undergo adaptive mutation under selection pressure (Chu & Lee, 2007). Growth on xylose was not reported in strains in which PPP genes (*TAL*, *TKL*, *RKI* and *RPE*) were not over-expressed (Karhumaa et al., 2005). When compared with the previous experiments where in the presence of glucose, the recombinant yeast was able to uptake xylose from media with the help of hexose transporters. However, when xylose alone is kept in the media as a carbon source, the yeast is barred from consuming the xylose as the transporters are not activated at all.

5.3.3 Glucose and Xylose consumption

Glucose utilization by both control MKY09D2 and recombinant MKY09B2 were similar in both YNB and YNBX media, slightly higher amount being used by MKY09B2. This may be due to higher energy demand needed by recombinant yeast. On the other hand, xylose utilization by recombinant MKY09B2 was found to be higher than by MKY09D2 in YNBX media indicating

for xylose utilization ability due to introduced XI gene. Better utilization of XI introduced recombinant yeast was also reported by Kuyper et al., 2003; Tanino et al., and Brat et al., 2009.

Xylose was consumed only in presence of glucose suggesting for co-consumption of glucose and xylose, although in a relatively lesser amount. Xylose consumed by MKY09D2 and MKY09B2 over 72 hours was 2.46 mg and 3.71 mg respectively which increased to 3.99 mg and 5.34 mg in 96 hours. Xylose consumption and ethanol production rates were 0.07 gm/hr and 0.03 gm ethanol/hr respectively while ethanol yield was 0.43 gm ethanol/gm D-xylose consumed (Brat et al., 2009). The amount of xylose consumed was increased when glucose concentration in media declined. Lesser amount of xylose consumption may be attributed to absence of xylose specific high affinity transporters and hence it is only transported in small amount through hexose transporters. Km value for xylose (100-190mM) transport through hexose transporters are significantly higher than for glucose (Km = 1-20 mM) (Ozean and Johnston, 1999) indicating that high concentration of xylose is required for transport. Work done by Nepal N. (2016) on GXS1 and Regmi P. (2016) on GXF1 have shown to increase xylose transport into the yeast cell. Hence, trasformation of such transporter in yeast expressing XI gene may further increase xylose consumption.

Furthermore, lesser amount of xylose assimilation may be due to absence of overexpression of other down-streaming enzymes such as Xylulokinase and enzymes of non-oxidative pentose phosphate pathway that regulates xylose metabolism. Recombinant yeast strains able to assimilate xylose by heterologous expression of XI gene are accompanied by complementary expression of PPP genes (*TAL*, *TKL*, *RKI* and *RPE*) by Karhumaa et al., 2005 for efficient utilization of xylose.

Poor growth rate of recombinant strain on xylose media and co-metabolism of xylose and glucose was as expected is similar to the work performed by Brat et al., 2009. Poor growth (or even absence in some cases) may be attributed to poor affinity of native/endogenous sugar transporters towards xylose. Xylose that enters then can be utilized into PPP only after it is acted upon by endogenous *XKS*1 (Xylulokinase enzyme) into Xylulose 5- phosphate.

5.3.4 Ethanol Production

Ethanol yield by recombinant strain expressing *Piromyces Xyl*A gene was reported to be significantly higher in glucose media by Kuyper et al., 2003. This is similar to our result on ethanol yield by recombinant yeast expressing *C. phytofermentans* XI on both YNB and YNBX media.

Ethanol production by MKY09D2 and MKY09B2 was found to gradually increase with time of incubation in both media (YNB and YNBX). The trend of ethanol production was found to be

similar in both strains but comparatively production of ethanol in YNBX media by recombinant strain MKY09B2 was found to be higher than that produced by control strain MKY09D2. The additional production of ethanol by the recombinant yeast would surmise the possibility of efficient fuel production as well as utilization of agricultural wastes which would lead to reduction of wastes as well as dependence on the fossil fuel. Although a long way from perfection, this study should be a stepping stone for generation of genetically modified ideal yeast. Theoretically, this yeast would be able to degrade the entire content of the agricultural wastes as well as forest produce leading to cleaner and greener environment free from the smog of burnt fuel and greenhouse gases, known to be responsible for depletion of environment quality as well as the health standard of all living beings.

Ethanol production over long fermentation period by control and recombinant MKY09 was observed between 96 to 120 hours incubation where maximum ethanol produced was found to be 5.87 mg/ml and 5.84 mg/ml respectively by MKY09B2 by recombinant strain. The additional quantity of ethanol produced by the recombinant yeast strain could be attributed to continuous utilization of xylose as well as glucose present in the medium, hence a synergistic effect. Over a long period of incubation, with glucose being rapidly metabolized, xylose might have acted as a backup energy source which further supported the yeast to grow. This added biomass in turn would help in supplementary production of ethanol from the residual glucose in the medium.

The Ethanol yield in YNBX medium by MKY09B2 is found to be higher in earlier phase reaching upto 5.87 mg/ml and 5.84 mg/ml on 96 hrs and 120 hrs respectively while ethanol yields by control strain MKY09D2 were 5.16 mg/ml and 5.41 mg/ml respectively. The yield later decreased to 5.26 mg/ml on 144 hrs. in case of MKY09B2. The decrease in ethanol concentration could be attributed to decline in the concentration of substrate as well as may be due to ethanol itself being used as carbon source by yeast (Orlandi et al., 2013).

5.3.5 Functional Expression of XI and xylose consumption for ethanol production

Based on results from functional expression analysis, there is successful expression of codon optimized XI originally from *C. phytofermentans* in *S. cerevisiae* as reported by Kuyper et al. in 2003 for successful expression of *Piromyces Xyl*A gene in *S. cerevisiae*. Successful expression suggests for similar and optimal protein folding in native strain as well as host strain after transformation.

The challenge of anaerobic growth of engineered *S. cerevisiae* strain on xylose as sole carbon source still remains, which is the ultimate target yet to meet. However, in presence of hexose (Glucose), co-consumption of xylose is reported by Hector et al., 2008 as seen in our

observation. Xylose consumption and ethanol production is not as high as expected which can be further enhanced by overexpression of *XKS* and enzymes of PPP as suggested by study of Kuyper et al., 2003,Tanino et al., 2010 and Zhau et al., 2012.

It is strongly recommended to combine heterologous expression of XI gene along with introduction of heterologous high affinity xylose transportes such as GXS1 and GXF1 from *Candida intermedia*. These genes GXS1 and GXF1 have been separately transformed in yeast *S. cerevisiae* DTY195 as episomal yeast expression system with successful expression in previous studies by Nepal N. (2016) and Regmi P. (2016) during their M. Sc. dissertation at CDBT, TU.

The additional quantity of ethanol produced by the recombinant yeast strain could be attributed to the continuous utilization of the xylose as well as glucose present in the medium, hence a synergistic effect. Over a long period of incubation, with glucose being rapidly metabolized, xylose might have acted as a backup energy source which further supported the yeast to growth.

CHAPTER VI

SUMMARY

Most of the bioethanol presently produced are based on the energy rich crop hydrolysates such as corn, wheat and sugarcane (Saini et al., 2015) and raised ethical issues known as food vs. fuel. As an alternative, use of abundant lignocellulosic biomass hydrolysates as substrate is possible which can significantly reduce overall GHG emission due to renewable nature. Major roadblock on this approach for current technology is the reluctance of fermenting organism to use pentoses such as xylose and arabinose that makes significant portion of sugar content following glucose in lignocellulose. Inability of fermenting yeasts to utilize xylose is due to lack of efficient xylose transport system into the cell and enzyme system to drive xylose into the central metabolic pathway. Ethanol fermenting yeasts *S. cerevisiae* have been introduced with xylose specific transporter which showed significant accumulation of xylose in cell due to lack of enzyme systems that drives xylose into metabolism. Hence, current study involves expression of synthetic codon optimized Xylose isomerase gene from *Clostridium phytofermentans* in *Saccharomyces cerevisiae* using episomal plasmid construct (GPD-XI-CYC1) by sub-cloning codon optimized and synthetic Xylose isomerase gene under GPD constitutive promoter. Insert and vector were digested using same set of Restriction endonucleases *Hin*dIII and *Xho*I and directional ligation was carried out using T4 DNA ligase. Ligation was enriched by Transformation in *E. coli* DH5α cells. Yeast episomal expression construct GPD-XI-CYC1 system was transformed in *S. cerevisiae* MKY09 by electroporation and confirmed by restriction digestion, PCR, southern blot and fluorescence microscopy. Functional expression analysis was done by Phloroglucinol assay for xylose, DNS for glucose likewise Solvent extraction and dichromate oxidation for ethanol estimation. Similarly, growth rates of recombinant and control strains MKY09B2 and MKY09D2 respectively were also determined on YNB and YNBX media. The growth rate of control MKY09D2 and experimental MKY09B2 were found to be 0.160 hr^{-1} and 0.195 hr^{-1} respectively in YNB broth while on YNBX it was determined to be 0.163hr^{-1} and 0.155 hr^{-1} respectively for MKY09D2 and MKY09B2 strains. Both strains showed approximately similar amount of glucose consumption in media irrespective of sugar supplied but were unable to grow in media with xylose as sole source of carbon. On the other hand, xylose consumed was 3.71 mg and 2.46 mg by MKY09B2 and MKY09D2 respectively in YNBX suggesting that recombinant strain was enabled with slightly high xylose metabolism by co-consumption with glucose. Ethanol production by strain MKY09B2 was found always higher than that produced by MKY09D2 no matter the composition of media in terms of sugar content keeping all other growth condition constant. This suggests that introduction of bacterial xylose isomerase into the *Saccharomyces cerevisiae* does enable it in xylose metabolism which may have contributed ethanol production in excess.

CHAPTER VII

CONCLUSION

Current study involved the successful construction of a yeast expression plasmid construct pGPD2+XI by sub-cloning synthetic codon optimized xylose isomerase gene originally from *Clostridium phytofermentans*. Xylose isomerase gene was inserted in MCS of pGPD2 under the constitutive promoter GPD and flanked downward by CYC1. The final construct thus prepared was GPD-XI-CYC1, by inserting XI gene in between *Hin*dIII and *Xho*I restriction sites by T4 DNA ligase on pGPD2 backbone. Construction of the yeast expression plasmid was performed by using standard molecular techniques.

Thus prepared construct was first enriched in bacterial host *E. coli* DH5α, accumulated and then transformed into *Saccharomyces cerevisiae* MKY09 by electroporation. Positive transformation was selected on the basis of Uracil auxotrophy mediated by plasmid construct on YNB Ura –ve plate. Introduction of construct into yeast was confirmed by PCR, southern blotting and fluorescence microscopy.

Recombinant strain was designated as MKY09B2 and control strain carrying only pGPD2 plasmid was named MKY09D2. Both of these strains were then subjected to the expression analysis in terms of growth rate determination, ability to use glucose and xylose and ethanol production.

RECOMMENDATIONS

Since the gene is only expressed in episomal form it may get lost due to plasmid curation in absence of selection pressure, hence it is recommended to integrate the gene into genome of yeast.

Expression of Xylose Isomerase gene has induced the ability to metabolize xylose but rate of xylose consumption can be increased only after introduction of xylose specific transporters such as GXS1/GXF1. Hence, expression of both Xylose Isomerase and GXS1/GXF1 in same strain is recommended for better ethanol yield.

It is also recommended to over-express the enzymes involved in Pentose Phosphate Pathway and Xylulokinase (XKS) along the Xylose Isomerase gene for better performance.

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APPENDICES

APPENDIX 1. Web server used for Primer designing

Following server were used to design the Xylose Isomerse ORF primer.

1. http://eu.idtdna.com/analyzer/applications/oligoanalyzer/defult.aspx

2. http://mfold.rna.albany.edu/?q=mfold/dna-folding-form

3. http://ncbi.nlm.nih.gov

APPENDIX 2. Media used during study

Composition of LB per 100ml

Yeast extracts	0.5gm (0.5%)
Enzyme Casein hydrolase	1 gm (1%)
Sodium Chloride	1 gm (1%)
Agar*	1.5 gm for solid media/agar plate (1.5%)

Composition of YEPD per 100 ml

Yeast extract	1gm (1%)
Peptone	2 gm (2%)
Dextrose	2 gm (2%)
Agar*	1.5 gm for solid media/agar plate (1.5%)

YNB preparation.

Dissolve 1.7 grams Yeast Nitrogen Base (YNB) in 100 ml autoclaved distilled water. Add 5 grams Ammonium Sulphate. Sterilized by syringe filter and stored at 4ºC. 5ml of 10X YNB should be suspended in 50ml distilled water and following AA ingredients should be added individually for 45 ml of autoclaved 1% glucose.

S.N.	Component	Weight For 10 mg/ml stock	Volume to be added (50ml media)
1	L-histidine,	10 mg	10 microlitre
2	DL-methionine	20 mg	10 microlitre
3	Leucine	60mg	60 microlitre
4	Lysine		
*	Method of Sterilization	Autoclave	
5	DL-tryptophan	20 mg	20 microlitre
*	Method of sterilization	Filter through 0.2 Micrometer	
6	Glucose	1%	Autoclave
7	Uracil[#]		
8	Agar (for plate)	1.5 %	Autoclave

only for Complete YNB Media in order to grow wild type MKY09 *S. cerevisiae*

APPENDIX 3. Chemical composition of different reagents

Alkaline Lysis Solution I

50 mM Glucose

25 mM Tris-Cl (pH 8.0)

10 mM EDTA (pH 8.0)

The solution was sterilized by autoclaving and stored at 4^{o}C.

Alkaline Lysis Solution II (Freshly prepared)

0.2 N NaOH

1 % (w/v) SDS

Alkaline Lysis Solution III (per 100 ml)

5 M Potassium Acetate	60.0 ml (Final concentration in 100 ml is 3 M)
Glacial Acetic Acid	11.5 ml
Autoclaved Distilled Water	28.5 ml

DNA lysis buffer

Titron X-100	2%
Sodium Dodecyl Sulphate (SDS)	2%
Sodium chloride (NaCl)	0.1M
Ethylene-diamine-tetra-acetic acid (EDTA)	1mM
Tris – HCl pH 8	10mM

Tris EDTA buffer

Tris pH 8	1mM
EDTA pH 8	10mM

50 x Tris Acetate – EDTA (TAE) buffer

Tris base	24.2 gram
Glacial acetic acid	5.7 ml
0.5 M EDTA (pH 8.0)	10 ml

Final volume 100 ml to be made with Distilled Water

APPENDIX 4. Reagents for Southern Blotting*

Depurination Buffer

0.2 M HCl (6.62 ml in 300ml)

Denaturation Solution (350 ml)

1.5 M NaCl + 0.5 M NaOH (30.681 gm NaCl + 7 gm NaOH in 350 ml)

Neutralization Solution (500ml)

1.5 M NaCl + 0.5 M Tris-HCl (pH7.2) + 1 mM EDTA

{43.83 gm NaCl + 30.28 gm Tris-HCl + 1 ml EDTA (0.5 M stock) in 500 ml}

100X Denhardt's Solution (10 ml)

2% w/v BSA + 2% w/v Ficoll + 2% w/v PVP

(0.2 gm BSA + 0.2 gm Ficoll + 0.2 gm PVP in 10 ml)

20X SSC (pH7.2) / Blotting Buffer (100 ml)

3M NaCl + 0.3 M Sodium Citrate

Component	**100 ml**	**300 ml**
NaCl	17.532 gm	52.596 gm
Sodium Citrate	8.823 gm	26.532 gm

Pre-hybridization Solution 30 ml (prepared prior to use)

6X SSC	9 ml from 20X
5X Denhardt's solution	1.5 ml from 100X
50% Formamide	15 ml
0.5% SDS	0.15 gm D
Autoclaved Distilled water	4.35 ml

Denatured Salmon sperm DNA

* Reagents are prepared in autoclaved distilled water

Detection Solution/Buffers

Blocking/Washing Buffer 200 ml

20 ml of 10X buffer + 180 ml Autoclaved distilled water

Blocking Solution 50 ml

0.5 gm of Blocking reagent in 50 ml of 1X Blocking/Washing Buffer

Streptavidin-Alkaline Phosphatase conjugate 20 ml (Freshly prepared)

Dilute Streptavidin-AP conjugate by 5000 fold in Blocking solution i.e. 4 µl S-AP conjugate in 20 ml Blocking solution.

Detection Buffer 30 ml

3 ml detection Buffer + 27 ml Autoclaved Distilled Water

Substrate Solution 10 ml

200 µl BCIP + 9.8 ml Detection Buffer

APPENDIX 5. PCR Condition for Amplification of XI gene (ORF)

Stage	Temperature (°C)	Time (minutes)	Cycle
I	95	5	1
II	95	0.5	5
	50	0.5	
	72	1.5	
III	95	0.5	25
	63	0.5	
	72	1.5	
IV	72	5	1
V	4		

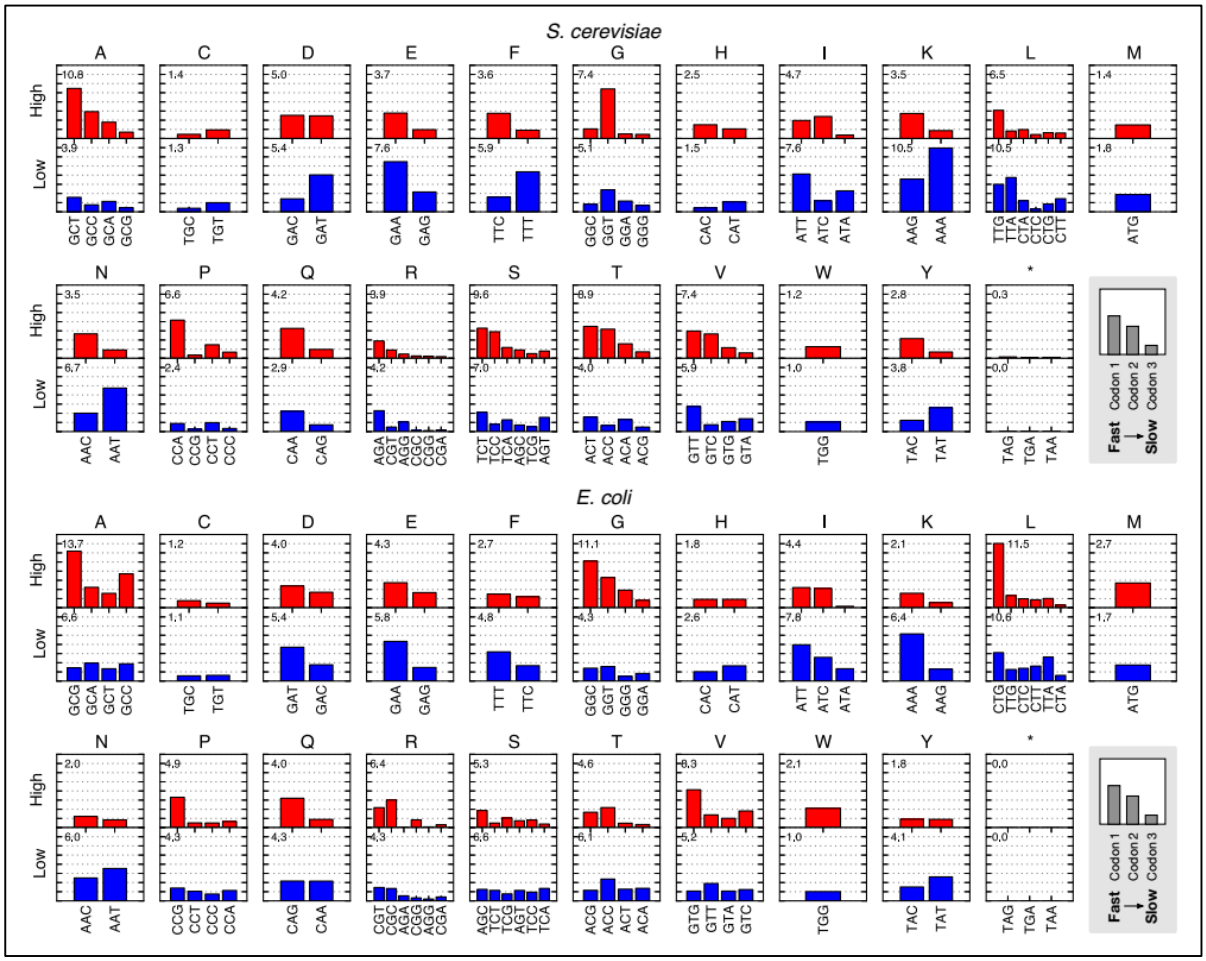
PCR Condition for Probe preparation of Southern Blotting (XI ORF amplification using Biotin labelled Uracil dNTPs cocktail)

Stage	Temperature (°C)	Time (minutes)	Cycle
I	95	5	1
II	95	0.5	5
	50	0.5	
	72	1.5	
III	95	0.5	25
	61	0.5	
	72	1.5	
IV	72	5	1
V	4		

APPENDIX 6. Amino acid Codon Table

UUU UUC phenyl alanine UUA UUG leucine	UCU UCC UCA UCG serine	UAU UAC tyrosine UAA UAG stop	UGU UGC cysteine UGA stop UGG tryptophan
CUU CUC CUA CUG leucine	CCU CCC CCA CCG proline	CAU CAC histidine CAA CAG glutamine	CGU CGC CGA CGG arginine
AUU AUC AUA isoleucine AUG methionine	ACU ACC ACA ACG threonine	AAU AAC asparagine AAA AAG lysine	AGU AGC serine AGA AGG arginine
GUU GUC GUA GUG valine	GCU GCC GCA GCG alanine	GAU GAC aspartic acid GAA GAG glutamic acid	GGU GGC GGA GGG glycine

APPENDIX 7. Amino acid Codon Preference among *S. cerevisiae* and *E. coli*



(Adapted from Gorochowski et al., 2015)

APPENDIX 8. Representative schematic of the yeast expression construct.

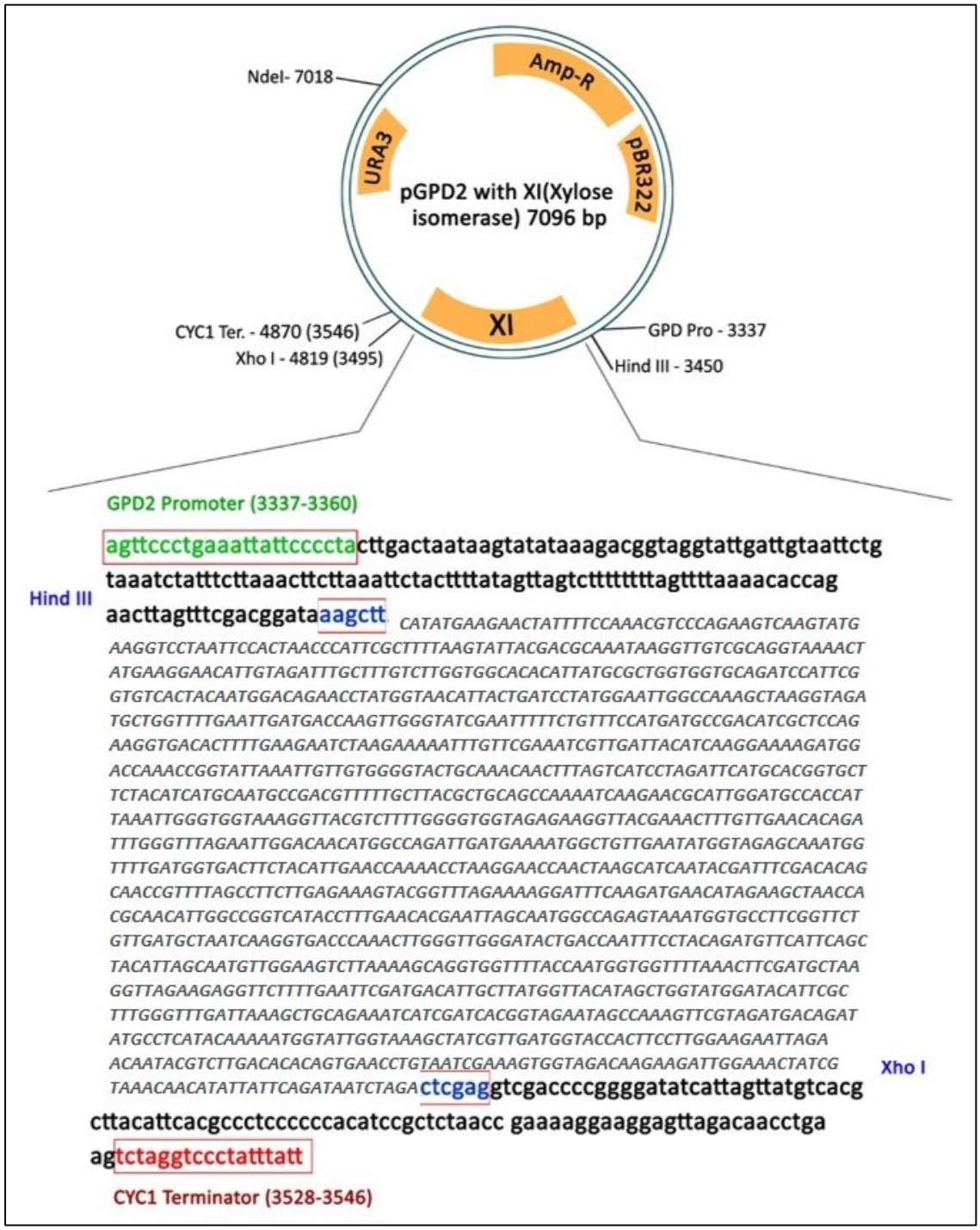


Figure: Yeast expression construct prepared in the study.