TRANSFORMATION OF GLUCOSE/XYLOSE SYMPORTER(pGXS1) IN *SACCHAROMYCES CEREVESIAE* FOR XYLOSE TRANSPORTATION

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ABSTRACT

The increasing demand and the high carbon emission of the gasoline fuel, led the bioethanol as the best alternative renewable sources. The second generation biofuel production has also mitigated the use of feedstock as the substrate for ethanol production. The high amount of the pentose sugars present in the lignocellulosic hydrolysate and the present of the less efficient organism to convert pentose to ethanol has led to the molecular approaches to come in action for the production of biofuel. But the molecular transport of the pentose sugar limits the process of pentose fermentation. GXS1 is the glucose/xylose symporter present in the *Candida intermedia*.

In the present study the heterologous expression of the GXS1 gene in the *S. cerevesiae* was performed. The yeast expression plasmid YEplac195-HXT7-GXS1-PGK1 was transformed into *S. cerevesiae* by electroporation and confirmed by the southern blot. Functional activity of the cloned gene was done by the spectrophotometric quantification of glucose by DNS method, xylose by phloroglucinol assay, ethanol by dichromate oxidation method.

The specific growth rate of the transformed and the wild *S. cerevesiae* at the glucose(20g/l) was found to be 0.0820±0.00065/h and 0.092±0.00071/h respectively. In the xylose at the concentration of the 20g/l the specific growth rate of the transformed and the wild *S. cerevesiae* was found to be 0.049±0.0033/hr and 0.0065±0.001860/hr respectively. The xylose uptake rate of the host harbouring the GXS1 gene was found to be 0.2200±0.04543g/l/h during 48 hr interval, whereas the control *S. cerevesiae* have only the 0.11±0.03291g/l/h during 48hr interval. The pH increased from the 5.00 to 5.26±0.0001942 in the host harbouring GXS1 gene whereas only slight increase i.e,5.00 to 5.012±0.0003906 depicting the symport activity. The X/G preference ratio of the transformed *S. cerevesiae* was found to be 0.415176 where, 69.9% of total sugar was consumed.Whereas in wild *S. cerevesiae* , X/G preference ratio was found to be 0.206 and 56.86% of total sugar was consumed The ethanol production by the transformant from the xylose(20g/l) was found to be 0.00794±0.0002603 mg/ml whereas the control *S. cerevesiae* produced 0.007621±0.0085mg/ml. Similarly the transformant *S. cerevesiae* produced 0.35284±0.0010 mg/ml ethanol, the control strain produced 0.3259±0.0085 mg/ml ethanol from 20g/l of glucose. The loxp-kan^r was cloned in the integrative plasmid by the link lock method, which was confirmed by the restriction digestion.

The increase in the growth rate, increase in the extracellular xylose uptake show that the transport of the xylose in the *S. cerevesiae* is the rate limiting process for the growth of *S. cerevesiae* in xylose. The increase in the pH indicates the symport activity of the transporter. But the growth rate was less than compared to that from glucose and the ethanol production from xylose was only slightly increased. This is due to the cofactor imbalance and the xylitol production which led to the cellular and metabolic stress ultimately killed the cell. Hence the deletion of the GRE3 gene and the introduction of the xylose isomerase may enhance the ethanol production.

Keywords: Electroporation, Symport, GRE3, Xylose isomerase, cofactor

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ACRONYMS

µg	Microgram
µl	Microliter
µM	Micromolar
ATP	Adenosine triphosphate
BLAST	Basic local alignment tool
Bp	Base pair
CAI	Codon adaptation index
DNA	Deoxyribonuleic acid
dNTP	Deoxyribonucleotide triphosphate
EDTA	Ethylene Diamine Tetra Acetic Acid
EtBr	Ethidium Bromide
g/l	Gram per liter
Hr	Hour
IDT	Integrated DNA Technology
Kb	Kilo base pairs
K^r	Kanamycin resistance
L	Liter
LB	Luria Bertani
m	milli
M	Molar
Mg	Microgram
Min	Minute
NADH	Nicotinamide Adenosine Diphosphate

NADPH	Nicotinamide Adenosine Diphosphate(H)
NCBI	National center for Biotechnology Information
NEB	New England’s Biolab
ng	nanogram
OD_{600}	Optical Density at 600nm
Orf	Open reading frame
PCR	Polymerase Chain Reaction
RNA	Ribonucleic Acid
RNase	Ribonuclease
Rpm	Revolution per minute
RT	Room Temparature
TAE	Tris- Acetate EDTA
TE	Tris EDTA
T_m	Melting temperature
Tris	Tris-(Hydroxymethyl)-aminmethane
UV	Ultravoilet light
YEp	Yeast expression plasmid
YEPD	Yeast Extract peptone dextrose
YEPX	Yeast Extract peptone xylose
YNB	Yeast Nitrogen Base
YIp	Yeast integrative plasmid

CHAPTER I

INTRODUCTION

1.1 BACKGROUND

Ethanol has been described as one of the most exotic synthetic oxygen-containing organic chemicals because of its unique combination of properties as a solvent, a germicide, an antifreeze, a fuel, a depressant and especially of its versatility as a chemical intermediate for other organic chemicals. Since the energy crisis of the 1970, the development of low-cost, sustainable and renewable energy sources such as ethanol has been a major focus in scientific research (Favela *et al*, 1986; Ingledew, 1999;Pramanik, 2003; Pramanik, 2005). In the EU, transport is responsible for an estimated 21% of all greenhouse gas (GHG) emissions. More than 90% of the total transport emissions are due to road transport. A range of actions is being taken to reduce emissions from transport by the production of biofuels which include bioethanol, biobutanol, biodiesel, vegetable oils, biomethanol, pyrolysis oils, biogas, and biohydrogen. There are two global biomass based liquid transportation fuels that might replace gasoline and diesel fuel. These are bioethanol and biodiesel. World production of biofuel was about 68 billion L in 2007. Bioethanol is a gasoline additive/substitute, widely used biofuel for transportation worldwide (Demirbas A. 2007).The production of bioethanol from biomass is one way to reduce both consumption of crude oil and environmental pollution (Balat M.2011). It is possible that wood, straw, and even household wastes may be economically converted to bioethanol. It is derived from alcoholic fermentation of sucrose or simple sugars, which are produced from biomass by hydrolysis process (Demirbas 2008). Traditional solid fuels account for more than 90% of the energy supply for 3 billion people in developing countries. However, liquid biofuels (e.g., ethanol) are perceived as an important alternative to fossil fuel. Global crop residue production is estimated at about 4 billion Mg for all crops and 3 billion Mg per annum for lignocellulosic residues of cereals. One Mg of corn stover can produce 280 L of ethanol, compared with 400 L from 1 Mg of corn grains; 1 Mg of biomass is also equivalent to 18.5 GJ of energy. Thus, 3 billion Mg of residues are equivalent to 840 billion L of ethanol or 56 × 109 GJ of energy. However, removal of crop residues exacerbates soil degradation, increases net emission of CO_2, and aggravates food insecurity. Increasing the SOC pool by 1 Mg C ha−1 yr−1 through residue retention on soil can increase world food grain production by 24 –40 million Mg yr−1, and root/tuber production by 6–11 million Mg yr−1. Thus, identifying alternate sources of biofuel feedstock (e.g., biofuel plantations, animal waste, municipal sold waste) is a high priority. Establishing biofuel plantations on agriculturally marginal or degraded lands can off -set 3.5–4 Pg C yr−1 (R. lal 2008) Modern bioenergy technologies have been recognized as one of the potential energy sources in Nepal to address the current energy crisis and contribute to the rural development . The Nepalese foresters perceived positively the importance of bioenergy development in Nepal for employment and rural income generation, and improving the current energy situation by supplying locally produced and affordable energy sources. The respondents held community participation in the future bioenergy projects as a key to its successful implementation in Nepal. However, there is a strong need, as perceived by the respondents, to formulate a separate bioenergy policy for Nepal. Among various policy interventions formulation of a new bioenergy policy, mobilizing community participation in bioenergy projects, developingbioenergy markets, and implementing new bioenergy projects were considered as urgent for Nepal (Baral. Y *et al* 2012). The scarcity of known petroleum reserves will make renewable energy resources more attractive. The most feasible way to meet this growing demand is by utilizing alternative fuels. Biodiesel is the best candidate for diesel fuels in diesel engines. The biggest advantage that biodiesel has over gasoline and petroleum diesel is its environmental friendliness. Biodiesel burns similar to petroleum diesel as it concerns regulated pollutants. On the other hand, biodiesel probably has better efficiency than gasoline. One such fuel for compression-ignition engines that exhibit great potential. The major economic factor to consider for input costs of biodiesel production is the feedstock, which is about 80% of the total operating cost. The high price of biodiesel is in large part due to the high price of the feedstock. Economic benefits of a biodiesel industry would include value added to the feedstock, an increased number of rural manufacturing jobs, an increased income taxes and investments in plant and equipment. The production and utilization of biodiesel is facilitated firstly through the agricultural policy of subsidizing the cultivation of non-food crops. The European Union accounted for nearly 89% of all biodiesel production worldwide in 2005. By 2010, the United States is expected to become the world's largest single biodiesel market, accounting for roughly 18% of world biodiesel consumption, followed by Germany (Demirbas A 2007). The cost-effective production of biofuels from renewable materials will begin to address energy security and climate change concerns. Ethanol, naturally produced by microorganisms, is currently the major biofuel in the transportation sector. However, its low energy content and incompatibility with existing fuel distribution and storage infrastructure limits its economic use in the future. Study is concentrated on the metabolic engineering of genetically tractable organisms such as *Escherichia coli* and *S cerevisiae* for the production of ethanol. *S cerevesiae*, also known as brewers yeast, is the most widely used fermentation microbe because of the baking and beer brewing industries (Gunasekaran and Chandra,1999; Michilka, 2007) Many of the sugar crops that would be suitable for industrial fermentation include sugarcane, sugar beets, fruits, sweet potato, sweet sorghum, Jerusalem artichokes and agricultural wastes used for ethanol production (Atiyeh and Duvnjak, 2002; Hang et al., 1981;) *S cerevisiae*, as the most efficient glucose fermenter and capable of withstanding both high ethanol concentrations and the presence of toxic compounds in hemicellulose hydrolysates, would also be used to convert the pentose fraction in these hydrolysates into ethanol. However, *S. cerevisiae* does not utilize xylose, which is the predominant pentose in hemicellulose, as a carbon and energy source (Leonardo *et al*.2006).

1.1.1 Source Diversity of microbial metabolism

First-generation biofuels, mainly from corn and other food-based crops are being used as a direct substitute for fossil fuels in transport. However, they are available in limited volumes that do not make them serious replacements for petroleum. Second- generation biofuels from forest and crop residues, energy crops and municipal and construction waste, will arguably reduce net carbon emission, increase energy efficiency and reduce energy dependency, potentially solving the limitations of first -generation biofuels. Nevertheless, implementation of second-generation biofuels technology will require a sustainable management of energy, or development of local bioenergy systems. Locally produced second-generation biofuels will exploit local biomass to optimize their production and consumption (Blanca and juan 2008). First-generation biofuels derived from corn, sugarcane and oilseed are currently available and are seen as an intermediate step to reduce GHG emissions and to diversify transport energy sources among other alternatives. However, they are available in limited volumes that do not make them serious replacements for petroleum. The goal of second-generation biofuels is to extend the amount of biofuel that can be produced sustainably by using biomass comprised of the residual non-food parts of current crops, as well as other crops that are not used for food purposes and also municipal, industrial and construction waste (Blanca and Juan 2008). The commercialization of second-generation bioethanol has not been realized due to several factors, including poor biomass utilization and high production cost. It is generally accepted that the most important parameters in reducing the production cost are the ethanol yield and the ethanol concentration in the fermentation broth. Agricultural residues contain large amounts of hemicellulose, and the utilization of xylose is thus a plausible way to improve the concentration and yield of ethanol during fermentation. Most naturally occurring ethanol-fermenting microorganisms do not utilize xylose, but a yeast strain, TMB3400,produced from the random mutagenesis has the ability to co-ferment glucose and xylose. However, the xylose uptake rate is only enhanced when the glucose concentration is low (Barbara *et al*. 2012). Xylose fermentation is therefore very important for converting the lignosellulosic biomass sugars into ethanol, to increase the concentration in the fermentation broth. However, wild-type *S* cerevisiae, which is the most commonly used yeast in ethanol fermentation due to its attractive properties, such as high yield of ethanol, high specific rate of fermentation (Verduyn C. *et al*1990) and its high tolerance to the end product, ethanol (Casey W. and Ingledew W 1986), is not able to metabolize xylose.

Energy crops	Agriculture and wood residues	Organic waste	Traditional breeding and genetically modified crops	Vegetable oils
Amaranth Bamboo Energy maize Eucalyptus Grasss Mischanthus Oilseed rape Poplar Salix Sugarbeet Sweet sorghum Switch grass Willow Winter wheat	Barn Citrus waste Corn stover Green waste Industrial waste Sugarcane bagasse Sawdust Wheat straw Waste ricestraw Wood Woodchips	Animal fats Food waste Municipal solid waste Olive pulp Recycled cooking oil Wastewater from paper and pulp industry Wastewater from tofu and sugar factory	Mischanthus Switch grass Mellow Etc.	Callophyllum inophyllum Corn oil Castor bean Cotton seed Jatropha Palm Pogania pinnata Rapeseed Soybean Sunflower

Table 1.1 Second generation biomass feedstock (Blanca and juan 2008)

There many rate limiting step in pentose fermentation by *S. cerevisiae* and therefore, strain engineering for enhanced flux from substrate to ethanol remains a challenge. There is the route dependent problem associated for conversion of D-xylose and L- arabinose into D-xylulose, the great task for organism is to maintain of a balanced ratio for oxidized and reduced forms of NADP and NAD $^{+}$. Generally, NADPH was used for reduction whereas NAD $^{+}$ is used for oxidation results in a poor recycling of redox cofactors in the xylulose formation step of pentose metabolism which in turn leads to a hindering the distribution of fermentation products in which by-products i.e, xylitol are formed in excess

(Krahule S. *et al*. 2009).

1.2CURRENT STUDIES

In the current studies characterization of GXS1 gene from *C intermedia* and heterologous expression of plasmid consisting GXS1 gene into the haploid *S cerevesiae* was done. The confirmation of the transformants and the functional expression studies was done for the transport and the funneling of the xylose to the pentose phosphate pathway and the production of the bioethanol. The detail information of the gene GXS1 was given below:

1.2.1 Gxs1 belongs to a family of fungal monosaccharide symporters

The Gxs1 protein was predicted to have 12 transmembrane domains (TMHMM Server v.2.0,http://www.cbs.dtu.dk/services/TMHMM-2.0/) and shows the highest similarity with uncharacterized putative sugar transporters from the yeasts *Debaryomyces hansenii, Yarrowia lipolytica* and *Candida albicans* [NCBI (National Center for Biotechnology Information) protein accession numbers CAG86664, CAG81819 and EAL01541 respectively]. It is also quite similar to the MSTA protein of *Aspergillus niger*, which was recently shown to be a monosaccharide–H^+ symporter with a broad substrate specificity. This MSTA protein, in turn, bears high resemblance to the as-yet- uncharacterized transporters in the ascomycetous filamentous fungi *Neurospora crassa and Magnaporthe grisea* (Vankuyk P *et al* 2004).

http://www.ncbi.nlm.nih.gov/nuccore/85057134,GenBank: AJ875406.1

1.2.2 Kinetics of sugar transport by GXS1

The Vmax values for glucose and xylose transport in *Candida intermedia* strain [0.26±0.02 and 0.39±0.09 mmol•h−1•(g of dry mass)−1 respectively] were one order of magnitude lower when compared with the Vmax determined in strain MJY1 expressing the glucose/xylose facilitator GXF1. Nevertheless, the estimated Km values for xylose transport were analogous to those determined in *C. intermedia* (0.4±0.1 mM) (Leonardo *et al* , 2006).

1.3 REASEARCH HYPOTHESIS

Molecuar transport is the vital rate limiting step for the pentose fermentation in *S cerevesiae*. The pGXS1 will be transform into the *S cerevesiae* under the control of the constitutive promoter HXT7 and the terminator CYC1. It can be hypothesized that transformed *S cerevesiae* may expressed the GXS1 symporter and help to grow in the xylose containing medium.

The xylose may be transport intracellularly in the *S cerevesiae*. Hence the first rate limiting step of the fermentation of xylose will be overcome. And it can be hypothesized that xylose present in the lignocellulosic biomass can be utilized and transported inside the cell.

The recombinant *S. cerevesiae* may be able to ferment the intracellular xylose to produce the ethanol utilizing the oxidoreductase pathway. Cloning of the Loxp flanking exciseable kanamycin resistance gene in the integrative plasmid aids in a manner that transporters and other enzymes can be cloned into the wild type diploid strain of *S cerevesiae*. Kanamycin resistance gene is the marker for the selection of the transformant *S cerevesiae*. The marker can be readily excised from the transformants by transforming the Cre recombinase containing plasmid in the yeast by homologous recombination.

1.4 OBJECTIVES

1.4.1 General objective

Transformation and functional expression analysis of pGXS1 (xylose/glucose symporter) in the *S cerevesiae* for xylose transportation and cloning of exciseable marker gene.

1.4.2 Specific objectives

1) Transformation of the pGXS1 in the *S cereveisiae*

2) Screening of positive transformants by PCR assay

3) Functional expression analysis of glucose uptake , xylose uptake , ethanol production and determination of xylose to glucose preference ratio..

4) Cloning of the loxp flanking kanamycin resistance gene in the integrative plasmid and conformation of transformants.

1.5 RATIONALE AND SCOPE

Agricultural residues contain large amounts of hemicellulose, and the utilization of hemicellulose is thus a plausible way to improve the concentration and yield of ethanol during fermentation. Most naturally occurring ethanol-fermenting microorganisms do not utilize xylose, but a genetically modified yeast strain, has the ability to co-ferment glucose and xylose. However, the xylose uptake rate is only enhanced when the glucose concentration is low (Barbara *et al*.2012). Primarily the first generation biomass was mainly composed of the food and the feedstocks such as sugarcane, grain crops etc. As the use of the food and the feed stock such as sucrose, starch as a substrate for bioethanol production leads to the food scarcity problems in the world. The population of the world goes on rising each year so limited supply of the food limits the use of the food and feedstock .So this work is aiming the step ahead for the production of bioethanol fom such agricultural wastes or second generation biomass. This work aims to introduce the glucose/xylose symporters in the yeast and enhance the xylose fermentation in yeast. In future, work can be done on the introduction of the xylose metabolism gene in this transformed yeast such as xylose isomerase gene to convert the intracellular xylose into the xylulose5 phosphate and which further enter into the pentose phosphate pathway and produce ethanol. Carbon catabolite repression by the glucose can be reduced further by producing the HX2 null mutants. The cofactor imbalance is the major problem, i.e, the conversion of the xylose to the xylulose because of the aldose reductase and the xylitol dehydrogenase. The accumulation of toxic xylitol is also a major problem for fermenetation of xylose. So the introduction of the new aldose reductase which is specific for the NADH or the deletion of the endogeneous aldose reductase (GRE3) and the introduction of the bacterial pathway for conversion of xylose to xylulose may mitigate this problem.

CHAPTER II

LITERATURE REVIEW

2.1 Role of bioethanol

The beneficiary aspect of the modern fossil fuels has impacting the daily life and the global economy. Liquid transportation fuels, petrochemical feed stocks, solvents, process oils, lubricants, and other specialty products are boon for us. But the rising demand of these products lead to the concluding its reserve. (Lee H *et al*. 2008). Fossil fuels account for over 80.3% of the primary energy consumed in the world, and 57.7% of that amount is used in the transport sector. (IEA,2006)

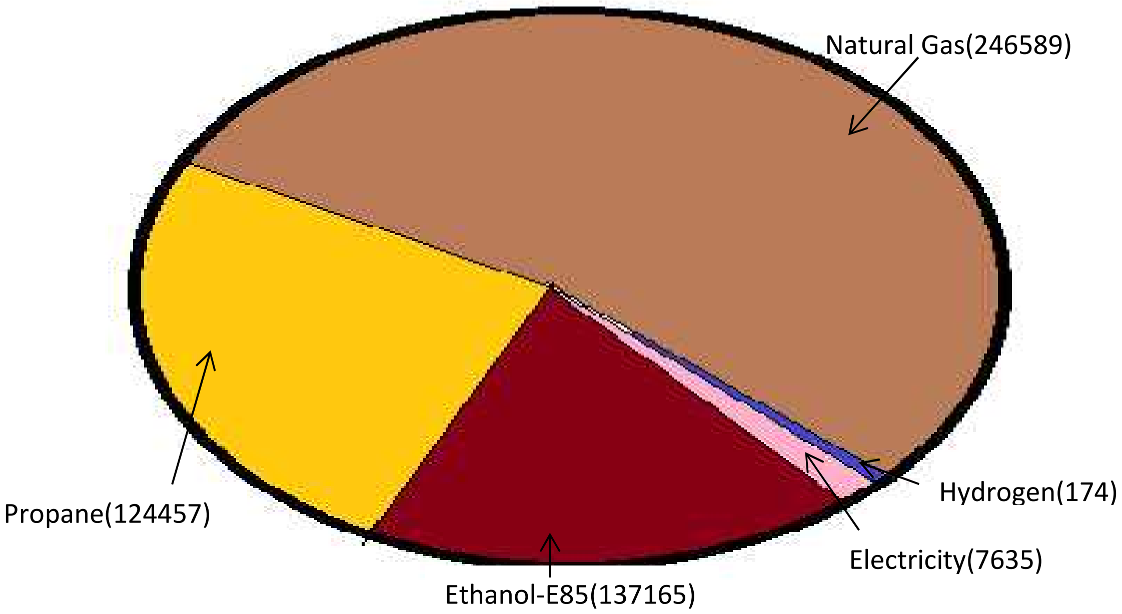

Figure 2.1 Consumption of alternative fuels in vehicles in 2011 in thousand gasoline.(US energy information administration)

The fossil fuel have lower octane rating than ethanol and was much more toxic and emits the harmful air pollutants. Fossil fuel was much more physically and chemically diverse than ethanol, which requires the complex refining procedures for ensuring the manufacture of consistent gasoline product. Ethanol was first suggested as an automotive fuel in USA in the 1930s, but was widely used only after 1970. Nowadays, ethanol is used as fuel, mainly in Brazil, and as a gasoline additive for octane number enhancement and improved combustion it is also used in USA, Canada and India. The increase in the gasoline prices increase and environmental protection become more stringent, attention was diverted towards ethanol as a renewable fuel or gasoline additive. In all these countries ethanol as a biofuel was produced commercially from cereal food and sugarcane (Biomass Bioenergy 2005 and Calle R. *et al* 1998). So alcohol is an alternative of transportation fuel because off its high octane number than gasoline, also cheap production from the locally grown crops even agricultural wastes alcohol, burns cleaner than gasoline and produce less carbon monooxide, hydrocarbon and oxides of nitrogen and contribrute on reducing the global warming process (Stump *et al*, 1996). Climatic changes, caused by greenhouse gases, mainly carbon dioxide produced during the burning of fossil fuels, have been devastating the ecosystems and leading to nearly 150,000 additional mortality every year (Teske S *et al*. 2007). Lignocellulosic biomass is the cheap and renewable source for the microbes to produce industrially relevant chemicals such as ethanol, succinic acid, lactic acid, butanol and acetone (Hong *et al* 2012). Due to the high-octane premium fuel, ethanol permits the fuel to be burned at a higher compression ratio which results in higher power output, efficiency, and consequently amiable fuel economy (RT *et al* 2007).

2.2 Ethanol production

2.2.1 Ethanol production from glucose

The high moles of ATP production by *Saccharomyces cerevesiae* during the fermentation of carbohydrates shows it's promising behaviour for fermentation (Laundry et al, 2006). Under anaerobic conditions, pyruvate converts into ethanol and carbon dioxide by maintaining the redox balance by reoxidizing the NADH providing the sufficient energy for fermentation. During fermentation, pyruvate is first converted into acetaldehyde, a reaction catalysed by the enzyme pyruvate decarboxylase. The acetaldehyde acts as an electron acceptor for NADH reoxidation. The reaction is catalysed by alcohol dehydrogenase. An alternative route for the regeneration of NAD^+ is the conversion of dihydroxyacetone phosphate to glycerol (Neuberg, 1919).

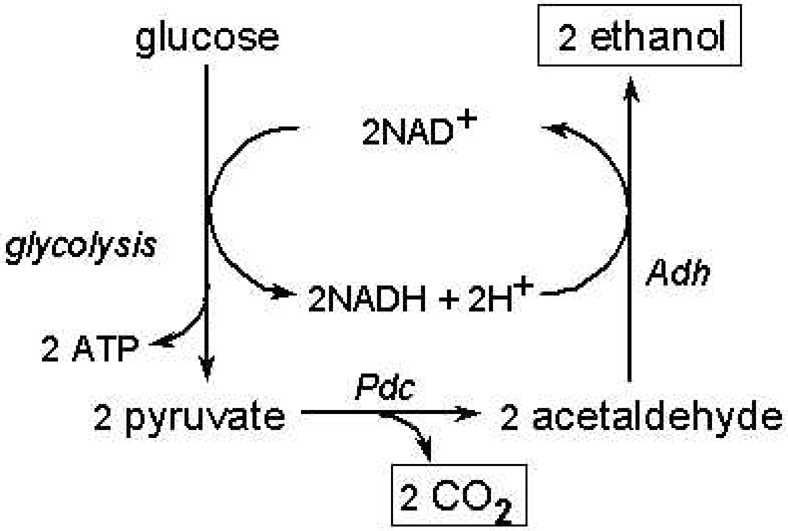

Figure 2.2. Reoxidation of NADH via the alcoholic fermentation pathway in *Saccharomyces cerevisiae*. Pdc, pyruvate decarboxylase; Adh, alcohol dyhdrogenase.

2.2.2 Ethanol production from xylose

Different species of bacteria, yeast and filamentous fungi are capable of fermenting xylose to ethanol (Hahn Hagedral and B.2007).There are two pathways used by the microorganism to ferment xylose to ethanol .Yeast and most fungi converts xylose to xylitol using xylitol reductase(XR) and xylitol to xylulose by using the xylitol dehydrogenase(XDH). In bacteria, isomerization of xylose to xylulose is catalyzed by the enzyme by xylose isomerase(XI). The xylulose is then enter into the pentose phosphate pathway and undergoes iso-ketal reaction steps and ultimately enter into the EMP pathway to produce ethanol (Hagedral *et* al 2007).There are several xylose fermenting yeasts such as *Pichia stipites* and *Candida shahatae* but they require a tight control of oxygen but less tolerance to inhibitory compound generated during most pretreatment process limits its use (P.J. *et* al 1990, Olsson *et al* 1996).The genes *pdc* and *adh* from *Z. mobilis* that encode pyruvate decarboxylase and alcohol dehydrogenase, key enzymes for ethanol production, have been genetically introduced in the *E.coli* KO11 (Ingram *et al* 1997). *E. coli* is less tolerant to lignocellulose-derived inhibitors so they are not capable of produce large quantity of ethanol. The pH is acidic during the saccharification of lignocellulosic waste which is unfavorable for the bacteria, requiring neutral pH, making simultaneous saccharification and fermentation (SSF) problematic *S. cerevisiae*, industrially proven ethanologen, the major disadvantage to using it for hydrolysate fermentation is its inability to ferment the pentose sugars such as xylose and arabinose to ethanol (Hahn-Hagedral *et al* 2007).

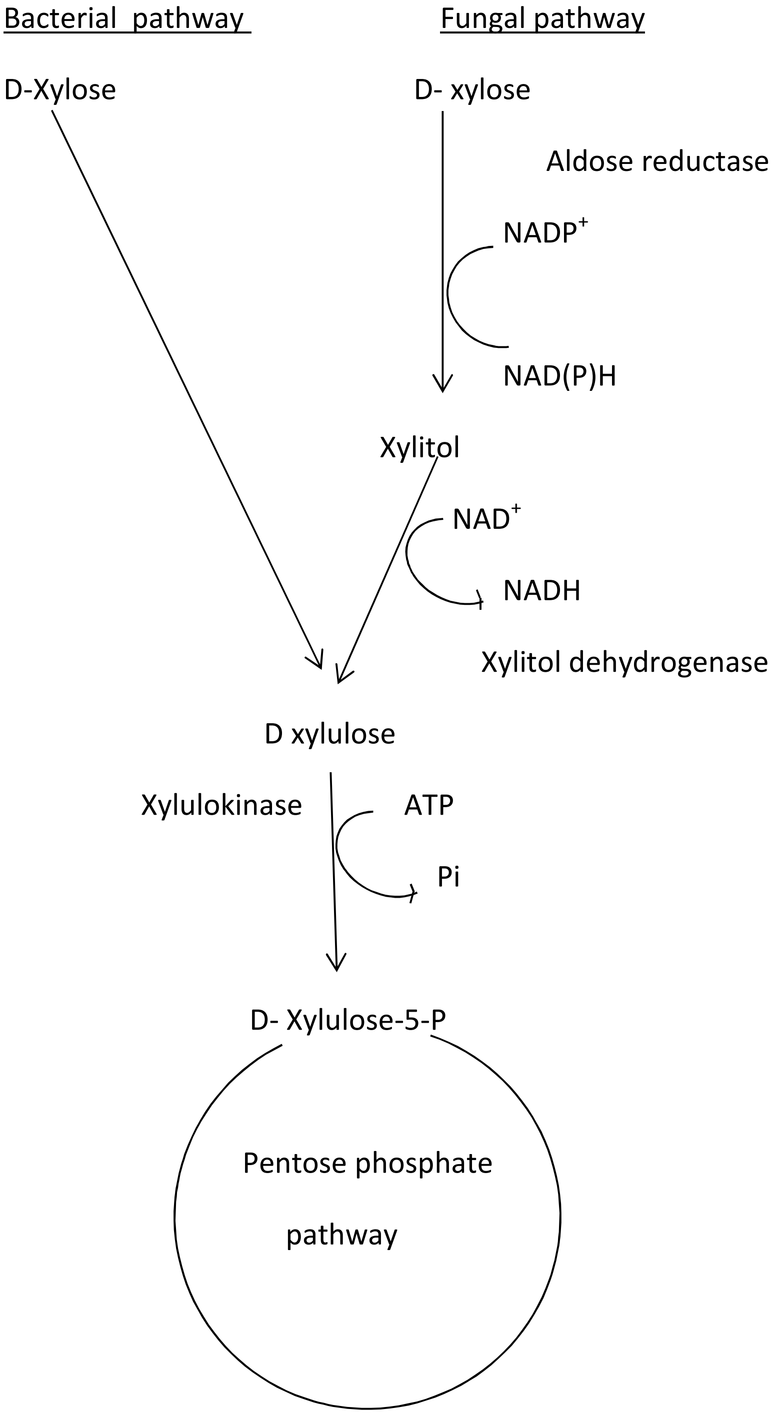

Figure 2.3 Overview of fermentation pathway of xylose in bacteria and fungi (Hagedral *et al* 2007)

Since, *S. cerevisiae* is capable of fermenting xylulose, a ketal isomer of xylose, to ethanol (Maleszekha and H. salander 1982,Gong *et al* 1981, Chandrakant *et al* 2000,Chiang *et al* 1981). The most abundant pentose sugar, D -xylose, was a target for fermentation through a combination of heterologous pathway engineering and native pentose phosphate pathway (PPP) optimization (Hahn Hagedral *et al* 2007). The two different strategies used for the fermentation of xylose, One approach involves the introduction of xylose reductase (XR) and xylitol dehydrogenase (XDH) genes from other yeasts such as *P. stipites*. However, cofactor imbalances between XR and XDH result in xylitol accumulation at the expense of ethanol yield (Kyuper *et al* 2005,Karuhama *et al* 2005). Site directed mutagenesis is another strategy used to obtain the mutants for better xylose fermentation. Multiple site directed mutagenesis of the NAD^+ dependent XDH from *Pichia stipititis* and introduction of the structural Zinc atom for the complete reversal of coenzyme specificity. The selected mutants were found to exhibit significant thermostability and enhanced catalytic activity with $NADP^+$ (Watanabe *et al* 2005). The second approach incorporates xylose isomerase (XI) genes from bacteria and fungi(Karhumaa *et al* 2007,Kyuper *et al* 2007,Sarthy *et al* 1987), with the deletion of an unspecific aldose reductase gene GRE3 in *Saccharomyces cerevesiae* (Kyuper *et al* 2005,Karuhama *et al* 2006, Karuhama *et al* 2007) to block xylitol formation. This approach bypass the XR/XDH redox reaction, avoiding the co-factor imbalance. But the XI expression was strongly inhibited by xylitol (Brat *et al* 2009). The rate of D-xylose utilization in XI expressing strains was found to be inferior to that in strains harboring the XR/XDH pathway (Bettiga *et al* 2008). The active expression and low throughput is the imitation of the XI pathway. Further overexpression and complementation of the native PPP enzymes xylulokinase (XKS) , transaldolase (TAL) ,and transketolase (TKT) improved xylose catabolic rates (Eliasson *et al* 2000,Jin *et al* 2005,Karuhama *et al* 2007,Walfridsson *et al* 1995). Together, these metabolic engineering efforts have led to increased xylose catabolic flux and improved ethanol yields. However, independently of the pathway used, xylose flux in recombinant yeast has been shown to be limited by it's transport (Saloheimo *et al* 2007,Gardonyi *et al* 2003). Different metabolic and evolutionary engineering strategies have been used successfully to improve D-xylose utilization in a yeast strain expressing *Piromyces* xylose isomerase. Overexpression of genes encoding xylulokinase and enzymes of the non-oxidative part of the pentose phosphate pathway, combined with deletion of GRE3 to reduce xylitol formation, considerably improved the D -xylose consumption rate (Kyuper *et.al* 2005). However, All these engineered strains were still restrain to a haploid laboratory yeast strain background, displaying in general suboptimal fermentation performance and poor robustness and stress tolerance, which makes them unsuitable for use in industrial fermentations. Since work has been shown that *S. cerevesiae* expressing XI displayed higher yield of ethanol per consumed D-xylose compared to that harboring the XR/XDH pathway (Bettiga M *et al* 2008).

2.3 *Saccharomyces cerevesia*

Saccharomyces cerevisiae, single-celled Eukaryotic budding yeast belonging to the Ascomycetes, measuring approximately 8 micrometers in diameter, and displaying a round to ovoid morphology. *S. cerevisiae* is an important model organism as it was the first Eukaryote to have its genome fully sequenced. Despite the lack of oxygen, *S. cerevisiae* can also metabolize and reproduce, resulting in CO2 and alcohol.(Madigan *et al*, 2009). *S. cerevisiae* ferments sugars into carbon dioxide and alcohol during the brewing and baking processes. Because of these behaviour, *S. cerevisiae* is commonly depicted as “Baker’s yeast” or “Brewer’s yeast”. In addition to brewing and bread making, *Saccharomyces cerevisiae* has many pharmaceutical functions. The genome can be engineered to overproduce certain metabolites and commercially important compounds for the use in pharmaceutical agents. Examples of drugs that *S. cerevisiae* can express compounds for include naringenin, coumarate, artemisinin, taxol, amorphadiene and vitamin C (Huang *et al*, 2008)

The *S. cerevisiae* genome consists of 16 chromosomes (8 pairs) in its haploid vegetative state. In nature, two of these haploid cells will fuse to form a diploid cell with 32 chromosomes (16 pairs). The haploid stage can exist in two forms, α and a, and two cells of the opposite form will fuse to form the diploid cell. *S. cerevisiae* haploid cells have the ability to switch between the two haploid mating types. The MAT, *aka* mating type, operon on the *S. cerevisiae* genome determines which mating type will be expressed. This mating process produce the great genetic variability in successive generation (Madigan *et al*, 2009).

2.4 Lignocellulosic raw material for bioethanol production

Lignocellulosic biomass are the cheapest , widely found and the largest source of the renewable feedstock. It can be obtained from agricultural and forestry residues, waste obtained from the food and beverage industry, pulp and paper industry, municipal wastes and dedicated energy crops (Bashir and Lee 1994). Utilization of the biomass polymers such as cellulose, hemicellulose, lignin is beneficial in social aspects also i.e, in terms of reducing solid waste, carbon- dioxide and green house gases (Hatti *et al a*nd Saha 2003). The composition of lignocellulosic biomass is the integrating form of the complex structure of cellulose (40–50%), hemicellulose (25–35%), and lignin(15-20%). A complex polymer of hexosans and pentosans is known as henicellulose and it is closely associated with lignin giving a structural rigidity to higher plant tissues.Mainly, the structure of hemicellulose contains D•xylose as a backbone with the side chains of arabinose, hexoses and modified carbons. Roughly 30% of the dry mass of lignocellulosic biomass composed of the pentose sugar containing polymer hemicellulose. 30% of hemicelluloses sugars present in hardwood trees is D-xylose a number which can vary upto 70% in many types of straw, sugarcane bagasse and soybean stalks (Lin and Tanaka 2006).

Feedstock	Cellulose	Hemicellulose	Lignin	Reference
Corn stover	36.4	22.6	16.6	DOE, 2006
Corn cob	42.0	39.0	14.0	Kuhad and Singh 1993
Rice straw	32.0	24.0	13.0	Kuhad and Singh 1993
Wheat straw	30.0	24.0	18.0	Kuhad and Singh 1993
Switch grass	31.0	24.4	17.6	DOE, 2006
Saw dust	55.0	21.0	21.0	Olson and Hagredral, 1996
Poplar	49.9	20.4	18.1	DOE, 2006

Table 2.1 : Cellulose, Hemicellulose and lignin content in various sources of biomass

2.5 Problem on lignocellulosic metabolism by *Sachharomyces cerevesiae*

Efficient utilization of raw material due to the presence of diverse range of biomass feedstock and complex polymers mixture, lack of effective pretreatment method for solving the problem of carryover of furfural and hydroxyl methyl furfural , high cost of the hydrolytic enzymes used for hydrolysis, availability of enzyme , requiring the complex enzyme mixture has limit the fermentation of lignocellulosic mass to ethanol (Simmons and B.A. 2010, Dadi *et* al.2007).As well as, xylose fermentation causes an inefficient metabolic state. It is because of carbon/energy starvation and reduced glycolytic flux in recombinant industrial *Saccharomyces cerevisiae* (Matsushika *et* al 2013).

Diverse range of species such as bacteria, yeast and filamentous fungi are capable of fermenting xylose to ethanol (Hahn Hagedral and B.2007). There are two major pathways utilize by the microorganism to ferment xylose to ethanol. Yeast and most fungi converts xylose to xylitol using xylitol reductase(XR) and xylitol to xylulose by using the xylitol dehydrogenase(XDH). Whereas in bacteria xylose is isomerized to xylulose by the enzyme xylose isomerase(XI). Further the xylulose can enters into the pentose phosphate pathway and undergo several reaction steps and ultimately enter into the EMP pathway to produce ethanol (Hagedral *et* al 2007). The xylose fementing yeasts includes *Pichia stipites* and *Candida shahatae* but limitation is due to the, strict control of oxygen and less tolerance to inhibitory compound generated during most pretreatment process (P.J. *et* al 1990, Olsson *et al* 1996).The genes pdc and adh from *Z. mobilis* that encode pyruvate decarboxylase and alcohol dehydrogenase, are the fundamental enzymes for ethanol production, have been genetically introduced in the *E.coli* KO11 (Ingram *et al* 1997). Bacteria is less tolerant to lignocellulose-derived inhibitors. The acidic pH during the saccharification of lignocellulosic waste impose threat to the bacteria during xylose utilization due to it's neutral pH requirement, making simultaneous saccharification and fermentation (SSF) problematic. *S. cerevisiae* is an industrial proven ethanologen. The major disadvantage to using it for hydrolysate fermentation is its inability to ferment the pentose sugars such as xylose and arabinose to ethanol.(Hahn-Hagedral *et al* 2007). Since *S. cerevisiae* is capable of fermenting xylulose, a ketal isomer of xylose, to ethanol.(Maleszekha and H. salander 1982,Gong *et al* 1981, Chandrakant *et al* 2000,Chiang *et al* 1981). The introduction of the heterologous enzyme xylose reductase (XR) and xylitol dehydrogenase (XDH) genes from yeasts such as *P. stipites*. However, cofactor imbalances between XR and XDH result in xylitol accumulation at the expense of ethanol yield (Kyuper *et al* 2005,Karuhama *et al* 2005). The second approach incorporates xylose isomerase (XI) genes from bacteria and fungi (Karhumaa *et al* 2007,Kyuper *et al* 2007,Sarthy *et al* 1987), with deletion of an unspecific aldose reductase gene GRE3 (Kyuper *et al* 2005,Karuhama *et al* 2006, Karuhama *et al* 2007) to block xylitol formation. This approach does not employ a redox reaction, avoiding the co-factor imbalance. Xylose flux in recombinant yeast has been shown to be limited by it's molecular transport (Saloheimo *et al* 2007,Gardonyi *et al* 2003). The first recombinant *S. cerevisiae* strain demonstrates high activity of prokaryotic XI , using codon-optimized XylA gene from *Clostridium phytofermentans*. the rate of D-xylose consumption and ethanol production by this recombinant strain was not satisfactory (Kyuper *et.al* 2005). All these engineered strains were still made in a haploid laboratory yeast strain background, displaying in general suboptimal fermentation performance and poor robustness and stress tolerance, which makes them unsuitable for use in industrial fermentations (Bettiga M *et al* 2008).

2.6 Role of heterologous expression of xylose transporters in *Saccharomyces cerevesiae*

Molecular transport is a key process in cellular metabolism which is often limiting step when using a nonnative carbon source.. The first molecular characterization of the pentose transporters, annotated XUT genes of *Scheffersomyces stipitis* and open reading frames from the yeasts *Yarrowia lipolytica* and *Debaryomyces hanseniihas has been done*. Phylogenetic analysis demonstrates that transporter function clusters into three distinct groups. One particular group comprised of *D. hansenii* XylHP and *S. stipites* XUT1 and XUT3 demonstrated which have moderate transport efficiency and higher xylose preferences (Yong *et al* 2011). Native pentose-utilizing organisms exist but , a lack of well-developed genetic tools and low product tolerances (Hahn Hagedral *et al* 1991) limit their utility as hosts for industrial scale lignocellulosic conversion processes. So, a important effort has focused on the introducing and modifying the pentose catabolic pathways in the yeast *S. cerevisiae* to enable xylose and arabinose fermentation (Hahn Hagedral *et al*,2007). The xylose transport in recombinant yeast is facilitated by native glucose transporters (Hamacher *et al* 2002, Leonardo *et al* 2009). The lack of a dedicated xylose transport system in recombinant *S. cerevisiae* thus limits the capacity for dual xylose and glucose fermentation as well as high xylose catabolic pathway flux (Jefferies and T.W. 2006). This limitation foregrounds the need to identify and/or engineering of efficient, heterologous xylose transport proteins in yeast. However, lesser number of proteins have been experimentally identified for xylose transportation in *Saccharomyces cerevisiae*, and all of these have been shown to favor glucose over xylose in a mixed-sugar culture. Moreover, while these proteins show affinity toward two structurally different monosaccharides (glucose and xylose). Such a characterization research would expand our knowledge regarding of molecular transporter function as well as potentially useful classes of transport proteins for the improvement of xylose utilization in yeast. A functional survey and characterization of 23 heterologous and 3 native *S. cerevisiae* yeast proteins expressed in a recombinant xylose- utilizing *S. cerevisiae* host devoid of glucose and xylose transporters (Wieczorke *et al* 1999). These proteins represent both putative and known transporters capable of xylose transport traversing the organisms *Arabidopsis thaliana, Candida intermedia,Cryptococcus neoformans,Debaryomyces hansenii,Escherichia coli, Scheffersomyces stipites* formerly *Pichia stipites* (Krutzman *et al* 2007). The *E. coli* xylE transporter functions is characterized as an exclusive xylose transporter of the heterologous transport proteins (Leonardo *et al* 2006). The research also indicates that these ORFs encode broad hexose transporters that have additional affinity for xylose for GXF1 and both xylose and ribose for GXS1. The research also depicts that *D. hansenii* xylHP, previously which was indicated as a potential candidate for heterologous expression ,is also a hexose transporter also having affinity for xylose (Leonardo *et al* 2009). The efficiency of transporters for intracellular accumulation of xylose was as follows: HXT7 > HXT5 > GAL2 > WT > HXT1 > HXT4 >>> RE700A. Furthermore, evidence suggest that the *Saccharomyces* galactose transporter system could be a highly effective xylose transporter. An attempt to isolate xylose transporters from *P. stipitis* by functional complementation yielded the low-affinity Sut1-3 transporters (Weierstall *et al*., 1999), but the isolation of a high-affinity xylose transporter from the same yeast remains elusive, despite the recent sequencing of its genome (Jeffries *et al*., 2007). Although the recombinant yeasts are currently the most effective industrial microorganisms for large-scale production of ethanol from lignocellulosic biomass, but the problem is they still utilize glucose several times faster than xylose (Ho *et al*, 1999, Sedlak *et al*, 2003). One reason might be that the *Saccharomyces* transport xylose much less efficiently than glucose (Kotter and Ciriacy, 1993). There seems to be no known xylose- specific transporters in the *Saccharomyces* yeasts. The hexose transporters, which transport glucose in the *Saccharomyces*, transport xylose. However, the hexose transporters greatly favor transporting glucose over xylose (Kotyk,1967; Lagunas *et al*, 1982; Kotter and Ciriacy,1993; Lee *et al*, 2002). Two glucose/xylose transporter genes from *Candida intermedia* were cloned and characterized: which includes GXF1, encodes a glucose/xylose facilitator; and

GXS1, which encodes a glucose/xylose proton symporter(Leonardo *et al* 2006). , Coexpression of glucose/xylose facilitators with Gxs1 promoter strongly reduced GXS1 mRNA levels, and consequently symport activity, in glucose-grown, but not in ethanol-grown, cells. The observed decrease in GXS1 transcript levels is due to the enhanced glucose influx mediated by glucose facilitator protein(s), and not to a specific interaction between GXS1 promoter and other transporters. It was found that GXS1 mRNA levels to be severely reduced as a result of glucose addition, and this effect takes place at the level of GXS1 mRNA stability, suggesting that a decrease in mRNAs depicting high-affinity/active sugar transport systems may be a widespread and conserved mechanism in yeasts, limiting expression of these proteins whenever their activity is unnecessary (Leonardo *et al* 2008). Transporters, HXT1 and HXT7 from *Saccharomyces cerevisiae*, and GXF1 and GXS1 from *Candida intermedia*, were overexpressed in xylose-metabolizing *S. cerevisiae* harboring a xylose isomerase-based pathway. Overexpression of transporter enhanced sugar consumption and ethanol production, and found that GXF1 was efficient for ethanol fermentation from both glucose and xylose.(Tanino *et al* 2012). The cloning and characterization of two sugar transporters, AraT from the yeast *Scheffersomyces stipitis* and Stp2 from the plant *Arabidopsis thaliana*, which mediate the uptake of L-arabinose but not of D-glucose into *S. cerevisiae* cells.(Boles and Subtil 2011). An expression cassette containing 13 genes including *Clostridium phytofermentans* XylA, encoding D xylose isomerase (XI), and enzymes of the pentose phosphate pathway was inserted in two copies in the genome of ethanol red *S. cerevesiae*. Subsequent EMS mutagenesis, genome shuffling and selection in D- xylose enriched lignocellulose hydrolysate, followed by multiple rounds of evolutionary engineering in complex medium with D-xylose, gradually established efficient D- xylose fermentation.(Demeke *et al* 2013). The *S. stipitis* strains resulting from this intense multigene mutagenesis strategy have possible application in industrial fuel ethanol production from lignocellulosic hydrolysates.(Hughes 2012). In *Pichia stipitis*, three D- glucose/D-xylose transporters (SUT1-3) were cloned and characterized at the molecular level. Overexpression of SUT1 has been shown to facilitate D-xylose consumption and ethanol production in recombinant D-xylose assimilating *S. cerevisiae* (Katahira et al 2008). The traditional glucose fermenting yeast, *Saccharomyces* cerevisiae, cannot ferment xylose because it lacks a functional xylose assimilation pathway. Xylose uptake by *S. cerevisiae* is facilitated by hexose transporters which are severely inhibited by glucose. In the recombinant yeast xylose utilization is however slow compared to glucose, and during anaerobic conditions addition of glucose has been necessary for cellular growth. Xylose utilizing *S. cerevisiae* strains have been constructed by expressing a reduction/oxidation pathway involving xylose reductase (XR) and xylitol dehydrogenase (XDH). or a xylose isomerase (XI) pathway. Successive cycles of metabolic engineering have improved xylose utilization in recombinant *S. cerevisiae* . However the ethanol productivity from xylose is still low when comparing with glucose (Kyuper *et al* 2005)

Glucose transport kinetics and the mRNA level of different glucose transporters were determined in *Saccharomyces cerevisiae* strains expressing different sugar kinases. During exponential growth on glucose, a HXk2 null strain exhibited high -affinity hexose transport associated with an elevated transcription of the genes HXT2 and HXT7, encoding high-affinity transporters, and a diminished expression of the HXT1 and HXT3 genes, encoding low-affinity transporters. Deletion of HXT7 revealed that the high-affinity component is mostly due to HXT7. HXT7 encode high-affinity transporters (Km 1 to 2 mM)(Reifenberger et al.1997) A *Saccharomyces cerevisiae* screening strain was engineered by combining multiple genetic modifications for improving the xylose utilization with the primary objective of enhancing growth on xylose and fermentation in xylose isomerase (XI)-expressing strains. Strain TMB 3045 was obtained by expressing the XI gene from *Thermus thermophilus* in a strain in which the GRE3 gene coding for aldose reductase was deleted, and the genes encoding xylulokinase (XK) and the enzymes of the non-oxidative pentose phosphate pathway (PPP) (transaldolase (TAL), transketolase (TKL), ribose 5-phosphate ketol-isomerase (RKI) and ribulose 5-phosphate epimerase (RPE)) were overexpressed.(Johansson and Hahn-Hagerdal 2002). Functional expression of XI in *S. cerevisiae* has been successful with the XI genes from the thermophilic bacterium *Thermus thermophilus* (Walfridsson et al., 1996) and from the fungus *Piromyces* spp. (Kuyper *et al*., 2003). Despite the low activity of *T. thermophilus* XI in yeast, anaerobic ethanol production has been demonstrated at 40C (Lonnet *et al*., 2003; Walfridsson *et al*. 1996) due to the increased activity of the thermophilic enzyme at higher temperature. Additional improvement of xylose utilization has been achieved by deletion of the endogenous GRE3 gene (Traff *et al*., 2001), which encodes an unspecific aldose reductase (AR) that reduces xylose to xylitol (Kuhn *et al*., 1995), an inhibitor of XI (Yamanaka, 1969). Similarly, the expression of the *Piromyces* spp. XI gene in *S. cerevisiae* initially resulted in very slow xylose growth (Kuyper *et al*., 2003) which has been improved by adaptation of growth in xylose in continuous culture (Kuyper et al.,2004). Fungal as well as bacterial xylose isomerases could be functionally expressed and enabled the yeast cells to ferment D-xylose efficiently. The expression of pentose converting enzymes in *S. cerevisiae* is not sufficient for optimal pentose fermentation. Overexpression of xylulokinase or genes of the non-oxidative pentose phosphate pathway turned out to be beneficial as well as overexpression of uptake systems able to transport pentoses. Nevertheless, co-consumption of pentoses with D-glucose is still limited and normally *S. cerevisiae* does not utilize pentoses before D- glucose depletion. Due to the preference of the transporters for D- glucose, pentose uptake is competitively inhibited by D-glucose. Unfortunately, up to now no specific pentose transporters could be functionally expressed in yeast which specifically mediate uptake of only pentoses into *S. cerevisiae* cells but are not able to transport hexoses or are not inhibited by D-glucose. Therefore, it is believed that co-consumption of D-glucose and pentoses is impaired due to the preference of the uptake systems for D-glucose and its competition with pentose sugars. Nevertheless, it has never been investigated whether the further steps of pentose catabolism might also be impaired by the simultaneous catabolism of D-glucose. D-glucose and pentose catabolism share all the enzymes from glycolysis starting with phosphofructokinase (Subtil R and Boles E, 2012).Heterologous expression of a high-affinity xylose transporter is expected to represent an asset for xylose-fermenting S. cerevisiae strains in which other limiting factors have already been optimized (Gardonyi *et al*, 2003; Karhumaa *et al*, 2006).

2.7 Glucose/xylose symporter(GXS1) and recombinant *Saccharomyces cerevesiae*

Naturally xylose-utilizing yeasts transport xylose by a facilitated diffusion mechanism, for which the sugar gradient is the only driving force, or through an active transport system, usually sugar/proton symports, driven by the proton motive force that allows the intracellular accumulation of substrate. They are strongly regulated by the extracellular substrate concentration. At first glance a heterologous active transport protein would be the prime choice for metabolic engineering of xylose-fermenting *S. cerevisiae*. However, this transport mechanism concomitantly consumes 1 mol of ATP for each proton co transported with the sugar to pump out the proton and provide intracellular acidification. This functions under aerobic conditions without creating major energetic constraints; however, the depletion of a significant fraction of the conserved ATP considerably reduces anaerobic growth (Tanin *et al* 2012).

Gxs1 belongs to a family of fungal monosaccharide symporters. The GXS1 protein was predicted to have 12 transmembrane domain.(TMHMM Server v.2.0,http://www.cbs.dtu.dk/services/TMHMM-2.0/) and show the highest similarity with uncharacterized putative sugar transporters from the D*ebaryomyces hansenii, Yarrowia lipolytica* and *Candida albicans* [NCBI (National Center for Biotechnology Information) protein accession numbers CAG86664, CAG81819 and EAL01541 respectively. It is also quite similar to the MSTA protein of *Aspergillus niger*, which was recently shown to be a monosaccharide–H+ symporter with a broad substrate specificity. This MSTA protein, in turn, bears high resemblance to the as-yet- uncharacterized transporters in the ascomycetous filamentous fungi *Neurospora crassa.* (Vankuyk P. et al, 2004).Genbank: AJ875406.1

The Vmax values for glucose and xylose transport in *Candida intermedia* strain (0.26±0.02 and 0.39±0.09 mmol•h−1•(g of dry mass)−1 respectively) were one order of magnitude lower when compared with the Vmax determined in strain MJY1 expressing the glucose/xylose facilitator GXF1. Nevertheless, the estimated Km values for xylose transport were analogous to those determined in *C. intermedia* (0.4±0.1 mM).(Leonardo *et al* , 2006). The increase in the growth rate of the strain in xylose was observed which harbours heterologous gene of the GXS1 in the HXT null mutants *S. cerevesiae* (Yong *et al*, 2011).

The xylose is symport along with the H^+ into the yeast cells and thus increasing the pH of the surrounding (Hagedral and Karuhama 2007). The heterologous transporter conferred an improved growth on xylose and glucose-xylose co metabolism. However, glucose still remains to be more preferred carbon source indicating the need for more specific xylose transporter (Hector *et al*, 2008).

2.8 Further problem in xylose metabolism in *Saccharomyces cerevesiae*

Preferential sugar uptake combined with the transcriptional and/or enzymatic exclusion of less preferred sugars turns out one of the major barriers in increasing the yield and productivity of fermentation process.(Vinuselvi *et al*,2012). *S. cerevisiae* has low and high affinity hexose transport systems, which accounts for 18 different transporters (Hxt1-17 and Gal2) that could transport a wide range of substrate including xylose and glucose. Hexose transporters are differentially expressed depending on the concentration of glucose. The specificity of these transporters to facilitate xylose uptake varies as a function of glucose concentration. Of the 18 transporters, Hxt4p, Hxt5p, Hxt7p and Gal2P exhibit a higher specificity towards xylose. *S. cerevisiae* expresses the xylose specific transporters, Hxt4p and Hxt7p, only at the low concentration of glucose. Hence, in the initial stages of fermentation glucose concentration will be relatively high and hence the expresssion of xylose specific hexose transporters would be inhibited. With time, glucose concentration would reduce leading to the expression of Hxt4p and Hst7p transporters and hence favor xylose uptake. Strong competition between glucose and xylose for a single transporter would also limit xylose uptake leading to CCR. (Bertilsson *et al* , 2008). In *Saccharomyces cerevisiae* the HXK2 gene, which encodes the glycolytic enzyme hexokinase II, is involved in the regulatory mechanism known as 'glucose repression'. Its deletion leads to fully respiratory growth at high glucose concentrations where the wild type ferments profusely (L Rossell *et al* 2008).

There is not a single limiting step in pentose fermentation by *S. cerevisiae* and therefore, strain engineering for enhanced flux from substrate to ethanol remains a challenge. Depending on the route explored for conversion of D-xylose a nd L- arabinose into D-xylulose, maintenance of a balanced ratio for oxidized and reduced forms of NADP and NAD^+ constitutes a fundamental issue of strain physiology during pentose fermentation. Utilization of (mainly) NADPH for reduction when NAD^+ is exclusively employed for oxidation results in a poor recycling of redox cofactors in the initial steps of pentose metabolism which in turn leads to a highly unfavourable distribution of fermentation products in which by-products like xylitol are formed in excess.(Krahule S. *et al* 2009)

In 1996, *S. cerevisiae* was the first Eukaryote to have its genome completely sequenced. (Goffeau et al 1996). The genome consists of 16 chromosomes (8 pairs) in its haploid vegetative state. In nature, two of these haploid cells will fuse to form a diploid cell with 32 chromosomes (16 pairs). The haploid stage can exist in two forms, α and a, and two cells of the opposite form will fuse to form the diploid cell. *S. cerevisiae* haploid cells have the ability to switch between the two haploid mating types. The MAT, aka mating type, operon on the *S. cerevisiae* genome determines which mating type will be expressed. This mating process can produce great genetic variability from generation to generation (Madigan *et al*, 2009).

CHAPTER III

MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Enzymes, Chemicals and Reagent Used in the Study

The sources of chemicals, restriction enzymes, DNA polymerase and other DNA modifyingenzymes employed in this study are: restriction enzymes (*PstI*-HF^{TM},*Sac*I, and *Hind*III-HF^{TM}), Phusion High-Fidelity (HF) DNA polymerase and T4- DNA ligase from Thermoscientific. USA. DNA amplifying primers were purchased from Macrogen, Korea. All the chemicals and reagents were obtained from Hi-media and Merck.

3.1.2 Bacteria and plasmid Used in the Study

Transformation experiment was done in *E. coli* -DH5α, DTY165 haploid yeast. Plasmids used in this study were pGXS1, pUG6 and YIplac211. pGXS1 was kindly gifted by Paul Gonzaleus (Leonardo *et al* 2006) pUG plasmid was kindly gifted by Bole's Lab, University of Franfurt. and YIplac211 was kindly gifted by Koch's Lab, University of Erlaryen. The description of the plasmid was given in the Appendix 2.

3.2 Isolation of bacterial plasmid

3.2.1.Bacterial culture and cell harvest

E. coli DH5-α strain was revived by streaking on Luria Bertani (LB)/ampicillin (50 µg/ml) agar plate and incubated overnight at 37°C . A single isolated colony was inoculated in 2 ml of LB/ampicillin (100 µg/ml) medium and incubated at 37°C for 12 hours at 200 rpm. Then, 1.5 ml of the overnight culture was transferred to sterilized eppendorf tube and centrifuged at 5,000 rpm for 5 minutes at 4°C. Then supernatant was removed as much as possible without disturbing the cell pellet and the cells were resuspended in 500 µl of ice cold deionized water and again centrifuged at 5,000 rpm for 5 minutes at

4°C and water was drained completely.

3.2.2 Cell Lysis: Alkaline Lysis Method

The cell pellet after washing with deionized water was resuspended in 200 µl of alkaline lysis solution I and mixed well by pipetting. To this solution, 200 µl of freshly prepared alkaline lysis solution II was added and mixed by gentle inversion of tube for five times. The bacterial solution becomes clear as the cell lysis occurs. To this mixture 200 µl of ice cold alkaline lysis solution III was added to this mixture and mixed by inverting the tube gently for several times. During this step, a cloudy precipitate becomes visible due to precipitation of proteins. The mixture was then centrifuged at 13,000 rpm for 10 minutes at 4°C. Then supernatant solution consisting plasmid DNA was transferred to a fresh eppendorf tube.

3.2.3 Plasmid Recovery

To the supernatant 25 µl of RNAse (25mg/ µl) was added , mixed well and incubated for 30 minutes at 37°C then equal volume (600 µl) of phenol :chloroform (1:1) was added and mixed by vortexing. The mixture was then centrifuged at 13,000 rpm for 2 minutes at 4°C.Then supernatant was transferred to fresh eppendroff tube carefµlly and equal volume of chloroform was added , mixed properly inverting the tubes and centrifuged at 13,000 rpm for 2 minutes at 4°C. After centrifugation supernatant was collected in fresh tube and plasmid DNA was concentrated by adding 20 µl of sodium acetate (3M, pH5.2), and double volume of Isopraponal and centrifuged at 13000rpm for 10 minutes. Then supernatant was discarded carefully and DNA pellet was washed in 1 ml 70% ethanol and pelleted by centrifugation at 13000 rpm for 2 minutes. Then supernatant as discarded, pellet was dried by blotting and short spinning and pellet was dissolved in 30 µl of autoclaved distilled water.

3.2.4 Gel Quantification of Plasmid DNA

The quantification of isolated plasmid DNA was done by gel electrophoresis of 5 µl of plasmid DNA along with 1kb plus thermoscientific DNA ladder in 1% agarose gel. The DNA concentration of super coiled plasmid DNA was approximated by comparing the band intensity of sample DNA with known concentration of standard DNA ladder bands.

Briefly, the agarose gel was prepared by boiling 0.2 gm of agarose dissolved in 20 ml of 1X TAE buffer with EtBr . The gel was casted and allowed to solidify. Then, it was placed in gel tank containing 1X TAE buffer. Plasmid DNA sample (1µl of 6X Thermoscientific DNA loading dyes + 5µl of Plasmid DNA sample) was loaded in the well and also 1 µl of 1X Thermoscientific 1kb plus DNA ladder was loaded as the marker in another well. The gel was run for 30 min at 150 V in Mini Run GE-100, *S.* Korea. After completion of electrophoresis the gel was visualized under UV transilluminator.

3.2.5 DNA Quantification

The DNA quantification and purity assessment was done by spectrophotometer. The absorbance of the sample was measured at 260 nm and 280 nm wavelength. The concentration of DNA was calculated using the following formula:

DNA concentration (µg/ml) = A_{260} X 50 X Dilution factor

A_{260} of 1 corresponds to approximately 50 µg/ml of DNA.

DNA purity = A_{260} / A_{280}

Where, A_{260} and A_{280} are Absorbance of DNA or RNA at wavelength 260 nm and protein at wavelength 280 nm, respectively. Pure DNA sample has A_{260} / A_{280} ratio equals to 1.8, the ratio above 1.8 indicates RNA contamination whereas the ratio below 1.8 suggests protein contamination in the DNA sample.

3.3 Preparation of *E. coli* DH5α Competent Cells

E. coli DH5α competent cells were prepared by Calcium Chloride method. First, the bacterial strain was revived by streaking on LB agar plate and incubated overnight at 37°C . After incubation, a single isolated colony was inoculated in 50 ml of LB medium and incubated at 37°C for 24 hours at 250 rpm. Then 1ml of overnight culture was 100 times diluted in 100 ml of LB medium and incubated at 37°C with shaking at 220 rpm for an hour or until OD_{600nm} reaches 0.4-0.6. Then pellet was collected by centrifuging at 4100 rpm for 5 minutes at 4°C. Then the cells were re-suspended into 30ml of ice cold Calcium chloride, Magnesium chloride solution(20mm $MgCl_2$ and 80mm $CaCl_2$) and incubated in ice bath for 15 minutes. Again the cells were collected by centrifuging at 4100 rpm for 5 minutes at 4°C . The supernatant was discarded and cell pellet was resuspended in 2ml of ice cold 0.1M $CaCl_2$ with 15% glycerol solution and allowed to keep in ice bath for 15 minutes. Thus prepared *E. coli* DH5α competent cells were ready for transformation or stored at -40°C until use.

3.4 Primer Designing

Two sets of primers of the GXS1 gene were designed for the detection of the transformants by the PCR. The reference sequence of GXS1 gene was GenBank: AJ875406.1 of *Candida intermedia*. For the cloning of loxp flanked kanamycin resiatance gene pUG6 template vectorGenBank: AF298793.1.To meet the general criteria's of primer designing regarding length, melting temperature (T_m), GC content, secondary structure, self and hetero dimer etc. BLAST- search (non-redundant database in NCBI) was performed for each primer to ensure that the designed primers would not prime to non-specific regions. The basic information of designed primers is presented in Appendix .

Fw- *5′* -GTA C GAGCTC AT GGG TTT GGA GGA CAA TAG AAT GGT(TUGXS1-Fw)

Rv- *5′* -GG TCTAGA T TAA ACA GAA GCT TCT TCA GAC ATA ATA GC(TUGXS1- Rv)

3.5 ELECTROPORATRION OF *SACHHAROMYCES CEREVESIAE*

3.5.1 Preparation of electrocompenent cells

Sachharomyces cerevesiae was grown overnight and was inoculated in 500ml of YPD media, It was incubated at 30˚C ,250rpm overnight. The cells were chilled in an ice for 15 min to stop growth and were decanted into five sterile 50ml falcon tube and then the cells were pelleted by centrifugation at 4100rpm for 5 min. The supernatant was then discarded, and the falcon tube with cell pellet was placed in ice,50ml of sterile ice cold water was added to each falcon tube and vortexed to resuspend the cell pellet and placed on 250 ml conical flask and the volume was bought to 250 ml. The cells pellet were obtained by centrifugation at 4100rpm for 5 min and the supernatant was discarded. The cells were washed again with a 250ml of ice cold sterile water .The cell pellet were resuspended in 20 ml of sterile ice cold 1M sorbitol and transferred to a chilled 50ml falcon tube. The cells were pelleted by centrifugation at 4100rpm for 5 min and the supernatant was discarded. The cell pellet were resuspended in 0.5ml of sterile ice cold 1M sorbitol and the cell were kept in ice .

3.5.2 Electroporation

The DNA samples (100ng in a volume of 5µl) was pipetted into a sterile 1.5ml microfuge tubes and the tubes were placed on ice. 40µl of electrocompetent cells was added to each DNA samples and mixed gently and incubated in ice for 5 min. The DNA cells samples were transferred to the 0.2cm electroporation cuvette which have been chilled in ice. The suspension was tapped to the bottom of the tubes. The cuvette was placed in a chamber slide and 1.5kV voltage and 1 pulse was used for electroporation. The cuvette was removed immediately from the chamber and 1ml of ice cold 1M sorbitol was added and gently transferred the cells into the sterile microfuge tubes. The electroporated cells was spread on the selective agar plates(YNB agar without uracil) containing 1M sorbitol. The plates were incubated for 48 hours at 30 ˚C.

3.6 Plasmid extraction from yeast (Modified- Prasad D 2012)

Yeast strains were grown on YEPD medium and yeast strains harboring plasmid vectors were grown on at 30°C till the OD600nm reached ~0.4 to 0.5. 1.5 ml of the culture was centrifuged at 4000 rpm for 2 min and washed twice with sterile water. The cells were suspended in 1/10th volume of 50 mM Tris-HCl pH 7.4 containing 10 mM EDTA, 0.3 M sucrose and 10 mM β-mercaptoethanol, and disrupted with treatment with the lyticase enzyme. Five unit of enzyme was added to the tube and was incubated at 37 with shaking at 250rpm for 45 minutes. The pellet was suspended in 200 µl in 50mM Tris-HCl pH 7.4 buffer containing 10mM EDTA and 10 mM β-mercaptoethanol. The preparations were extracted once each with phenol:chloroform: isoamyl alcohol (25:24:1,V/V) and chloroform: isoamyl alcohol (24:1,V/V). The aqueous phase was transferred to separate tubes, 1/10th volume of 0.3 M sodium acetate, pH 5.0 and two volume of 100% ethanol was added, mixed gently and kept for precipitation at –70°C for one hour. It was centrifuged at 12000 rpm for 15 min at 4°C and was washed with 70% aqueous ethanol before air-drying. Dried pellet was dissolved in 100 µl of sterile distilled water and treated with RNAse (1 μg/μl) for 30 min at 37°C. Extracted once with phenol:chloroform:isoamyl alcohol (25:24:1,V/V) followed by chloroform: isoamyl alcohol (24:1, V/V). DNA was precipitated by the addition of 1/10th volume of 0.3 M sodium acetate pH 5.0 and two volume of 100% ethanol. Washed with 70% aqueous ethanol and air-dried. The dried pellet was dissolved in 20 μl of TE (10 mM Tris-HCl pH 8.0 containing 1 mM EDTA) and used for PCR amplification and southern blotting.

3.7 Specific growth rate

Cells were grown in YEPD broth with glucose 20g/l overnight for 28˚C, 220 rpm cells were washed and cultured in YEP with different carbon sources (xylose 20g/l, glucose 20g/l),optical density was measured at 600nm from 0 to 48 hours in 3 hour interval. Specific growth rate was calculated using formula (Palmiquivist *et al*,1999).

$$\mu =(\ln(t_1A_{600}))-(\ln(t_0A_{600}))/\Delta t$$

where,

t_1A_{600} = Absorbance measured during the final time point(peak of log phase) at 600nm

t_0A_{600}= Absorbance measured at intial time point at 600nm

Δt= change in time between t_0 and t_1.

3.8 Intracellular accumulation of xylose (Yong *et al* 2011)

Single colonies of the wild *S cerevesiae* and the transformed strain (expressing a target transporter) was inoculated in a culture tube with 2 mL YEPD medium supplemented with 20g/l glucose. Seed culture was then used to inoculate a 50 mL culture in a 250 mL flask. Cell culture was harvested by centrifugation after 2 days of growth and resuspended in YEPD medium supplemented with 20g/l D- xylose to a final OD600 of 10, At 30 min, 60 min, 120 min, and 24 hours, 5 mL of culture samples were taken for measuring intracellular sugar concentrations. Culture samples were washed twice with ice-cold water and resuspended in 3 mL of deionized water. Cell suspensions were incubated at 37 °C with 250 rpm agitation for 1 day to extract intracellµlar sugars. The resµlting cell suspension was assayed for the xylose concentration by pholoroglucinol assay. The sugar uptake activity was calculated to be mg sugar extracted through osmosis per mL of cell culture at OD~10. The xylose transported out of the cells by disruption of cells is considered as intracellular xylose concentration.

3.9 EXTRACELLULAR GLUCOSE AND XYLOSE CONCENTRATION

The wild *S cerevesiae* and the transformed strain (expressing a target transporter) were grown in YEPD broth with glucose 20g/l overnight for 28˚C, 220rpm. Cells were washed with distilled water and cultured in YEP with different carbon sources (xylose 20g/l, glucose20g/l). 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 and glucose concentration present in time interval. The difference between the substrate present in the intial time and time interval is determined as the uptake of the sugar inside the cells.

3.10 pH elicitor experiment (Foensca *et al* 2007)

The wild *S cerevesiae* and the transformed strain (expressing a symporter)were cultured in 20g/l glucose overnight at 28˚C ,200rpm . ,The cells were washed twice with sterile water and OD_{640nm} of 0.7 was taken . 1.32 ml of deionized water was taken and 150µl of cell suspension was added. pH 5.0 was adjusted. 30µl of 6.7mM xylose was added and the increase in pH was noted in 5 sec time interval from 0 sec to 100 sec. The increase in the pH of the medium indicates the co consumption of the H^+ with the xylose inside the cells indicating the symporter mechanism.

3.11 X/G preference ratio (Yong *et al* 2011)

The wild *S cerevesiae* and the transformed strain (expressing a target transporter) were grown in YEPD broth with glucose 20g/l overnight for 28˚C, 220rpm. Cells were washed with distilled water and cultured in YEP with different xylose 20g/l and glucose20g/l for cofermentation. 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. The uptake of the glucose and the xylose concentration was determined. Then it was converted to the molar consumption rate of each sugar,which was calculated at lag phase to stationary phase time interval. Then the xylose molar consumption rate was divided by the glucose molar consumption rate.Which gives the metric component for the cells preference for xylose molecules to glucose molecules.

3.12 Quantitative estimation of ethanol by Solvent Extraction and Dichromate oxidation

Ethanol estimation (Seo *et al*., 2009)

1 ml of TBP and 1 ml of sample solution were mixed in a 2 ml eppendroff tube and then vortexed vigourously using a vortex mixer for few seconds. After phase separation, 750µl of the solvent phase (upper) was transferred to a new eppendroff tube to which 750 µl of dichromate reagent was added and vortexed vigourously for few seconds. After phase separation, 500µl of dichromate reagent-containing lower phase was pipetted out for optical density measurement at 595 nm.

3.13 Standard graph for ethanol

Stock solution of 1mg/ml was prepared by dissolving ethanol in distilled water. Different concentrations of ethanol were prepared from the stock solution, i.e0.0079, 0.0158, 0.0237, 0.0316, 0.0395, 0.0474, 0.0553, 0.0632, 0.0711, 0.079 and 0.0869 mg/ml. The standard graph was then plotted using the values obtained from the optical density measurement of the different ethanol concentrations. The concentrations of the unknown samples were then obtained by plotting the values on the standard curve.

3.14 Determination of glucose depletion

1 ml of sample was taken in a test tube to which 3 ml of DNS reagent was added. The samples were then incubated in a boiling water bath for 5 minutes. After incubation, the samples were immediately cooled to room temperature in water and absorbance were

measured at 595 nm (Miller *et al.*, 1959).

3.15 Standard curve of glucose

Stock solution of 1mg/ml was prepared by dissolving glucose in distilled water. Different concentrations of ethanol were prepared from the stock solution, i.e.,0.1,0.2, 0.3,0.4,0.5,0.6,0.7,0.8 and 0.9mg/ml was prepared . Absorbance was measured at 575nm directly. The standard curve was then drawn by plotting the absorbance against the concentration.

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

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 540 nm.

3.17 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.

3.18 Culture of preserved yeast strains

Wild was cultured in autoclaved YEPD broth and transformed yeast was cultured in autoclaved YEPX and incubated at 200 rpm, 28^{O}C for 24 hrs. The 24 hrs culture were then streaked on YEPD/YEPX plates and incubated at 28^{O}C for 24 hrs to check the contamination. Single colonies of the yeasts were then streaked on YEPD slants and incubated at 28^{O}C for 72 hrs. The 72 hrs yeast culture slants were then stored at 4^{O}C for further use.

3.19 Construction of YIplac211-loxp-Kan^r plasmid

3.19.1 Cloning of loxp-Kan^r in YIplac211 Plasmid to Construct Vector YIplac211-loxp-Kan^r plasmid

3.19.1.1 PCR amplification of loxp-Kan^r

A pUG6 was used as the template for PCR amplification of loxp-Kan^r using specifically designed primers. PCR amplification was carried in two step reaction conditions as mentioned above. PCR reaction mixture and specific PCR condition used for amplification is given in appendix. Then 5 µl of PCR product was subjected to 1% agarose gel electrophoresis along with 1.0 kb plus DNA ladder as described previously.

The sequence of the primer is given below:

- Fw: TCCAC AAGCTT CGTCGACAACCCTTAATATAACTTC (YTMLOXPF)
- Rv: GAGTTG CTGCAG GAGTCAGTGAGCGAGGAAG (YTMLOXPR)

The PCR reaction reaction mixture was given below:

Components	25 µl Reaction	Final concentration
Template	4 µl	15 ng
2mM dNTPs each	3.2 µl	250 µM dNTPs of each
10µM Forward primer	2.5 µl	1 µM
10µM Reverse primer	2.5 µl	1 µM
5 X Reaction Buffer	5 µl	1X
5U/µl *Pfu* DNA polymerase	0.2 µl	0.5U
Nuclease free water	Upto 25µl	

The PCR condition was given below

	Cycles	Temp	Time
Initial Denaturation	1 Cycle	95°C	5 min
Denaturation	5 Cycles	95°C	30 seconds
Annealing		**55°C**	30 seconds
Extension		72°C	**2 minutes**
Denaturation	25 Cycles	95°C	30 seconds
Annealing		**65°C**	30 seconds
Extension		72°C	**2 minutes**
Final Extension	1 Cycle	72°C	5 minutes
Hold	1 Cycle	4°C	
Amplicon size= 1700			

3.19.1.2 PCR Product Purification

After confirmation of correct amplicon size (1700bp) from gel electrophoresis, PCR product was purified by chilled phenol-chloroform method to remove Phusion DNA polymerase and buffers that might interfere subsequent step like restriction digestion and ligation.

3.19.1.3 Restriction Digestion of insert

Purified PCR product of DNA fragment containing kan^r was digested partially by *Hind*III and *PstII*I. First of all the purified PCR product was completely digested with the *PstII*I enzyme.

Restriction digestion of insert

Insert	10µl
PstI (5U/µl)	1 µl
BSA	2 µl
Buffer Tango (10X)	2 µl
Water	4 µl
Total	20 µl

The above preparation was incubated at 37°C for 1hour. After complete digestion, 72µl of water was added ,8µl of 10X Tango buffer was added for a final volume of 100µl. It was placed on ice tube labelled A. It was mixed well. The preparation was aliquoted 20µl from tube A to 3 tubes labelled B,C,D and 10µl in the tube labelled E. 1µl of *Hind*III enzyme was added to the tube labelled A.It was mixed well.10µl of solution was transferred from tube A to tube B. It was mixed well. 10µl of solution was transferred from B to C and so on from C to D and D to E by mixing well. The tubes were incubated at 37°C from 30 seconds to 4 minutes. It was immediately placed on ice. The dilution was essential for partial digestion. Restriction map of the pUG6 template vector was found in appendix 2.3.

3.19.1.4 Restriction digestion of vector

The integrative plasmid YIplac211 was digested with the *PstI* and *Hind*III for the ligation of the insert. The digestion mixture was given below :

Restriction digestion of vector

Insert	10 µl
PstI (5U/µl)	1 µl
HindIII(5U/ µl)	1 µl
BSA	2 µl
Buffer Tango (10X)	2 µl
Water	4µl
Total	20 µl

3.19.1.5 Gel Electrophoresis

1% agarose gel was prepared by boiling 0.3 gm of low melting agarose (HiMedia) dissolved in 30 ml 1X TAE buffer. Once the gel was cooled to about 50° C- 60° C, 1 µl of EtBr solution (5 mg/ml) was added and gel was casted. Then it was placed in gel tank containing 1X TAE buffer. The restriction digested DNA solution was mixed with 10X gel loading buffer and whole solution was loaded in a well. In another well 1µl of 1X thermoscientific 1kb plus DNA ladder was loaded and run for nearly 1 hour at 50V. The gel was visualized under UV transillumintor and correct size DNA band of 1700bp insert and the 3797 bp of vector was cut with clean blades leaving other fragments in the gel for gel extraction.

3.19.1.6 DNA Extraction from Gel

Small segment of DNA after digestion was extracted from agarose gel. The DNA extraction protocols employed were as described below.

The weight of gel was measured and approximately 1.5 volume of TE buffer (pH 8.0) was added (For example, for 100 mg of agarose gel, 150 µl of TE buffer, pH 8.0 was added). The gel was melted by boiling the gel at 65° C for 5-10 minutes. Then, equal volume of chilled phenol (250 µl) was added to the solution, briefly vortexed and centrifuged at 13,000 rpm for 10 minutes at 4° C. The aqueous layer was carefµlly collected in fresh eppendorf tube avoiding carry off of lower organic phase and DNA was recovered by alcohol precipitation. The solution was mixed with 25 µl of 3M chilled sodium acetate (pH 5.2), 1 µl of glycogen (2 mg/ml) and 280 µl isopropanol solution. The mixture was incubated at room temperature for few minutes and centrifuged at 13,000 rpm for 15 minutes at 4° C. The pellet was washed with 1 ml of chilled 70% ethanol and again centrifuged at 13,000 rpm for 2 minutes at 4° C. Ethanol was evaporated by allowing the tube to air dry for 5-10 minutes. Then recovered DNA was re-suspended in 10 µl of MilliQ water and directly used for ligation reaction.

3.19.1.7 Quantification of Vector/Insert and Ligation

After purification of digested insert and vector, 2 µl of each sample is loaded in the gel along with the standard 1 Kbplus ladder. Concentration of loxp flanked Kan^r gene containing DNA fragment and YIplac211 plasmid recovered from agarose gel was determined by loading 2 µl of sample in gel and the intensity was compared with known amount of standard DNA ladder. The amount of 3.797 kb vector(7.445ng) DNA required to be added to 10ng of 1.7bp DNA fragment with molar ratio of 1:3 was calculated using formµla given below. Finally, the insert after complete digestion, purification and quantification was cloned in plasmid YIplac211 employing T4-DNA ligase (Thermoscientific).

3.19.1.8 Ligation reaction mixture:

Ligation reaction mixture was given below:

Insert (10ng)	4µl
Vector (20ng)	4 µl
T4 ligation buffer(10X)	2 µl
Ligase(1U/µl)	3 µl

Water	7 µl
Total	20 µl

The mixture was incubated at 16° C for overnight.

3.19.1.9 Transformation

After the ligation mixture was incubated at 16°C for overnight, the whole ligation mixture was used for transformation of *E. coli* DH5α competent cells by heat shock method *E. coli* DH5α competent cells was thawed on ice. Briefly, 20 µl of ligation mixture was added to 80 µl of *E. coli* DH5 α competent cells, mixed well and placed in ice bath for 30 minutes. Then it was subjected to heat shock by keeping in water bath at 42° C for 2 minutes and immediately transferred to ice bath and kept for 10 minutes. Then 1 ml of SOC medium as added to the transformed competent cells and incubated at 37° C for 90 minutes keeping the tube perpendicular and slightly shaking (100 rpm) to stabilize the plasmids in transformed cells. After incubation, 100 µl of transformed cells were directly plated into LB/ampicillin (100 µg/ml) agar plates whereas remaining cells were centrifuged at 10,000 rpm for 10 minutes at 4° C and cells were re-suspended in 100 µl of sterilized ice cold phosphate buffer (pH 7.0) and spread in LB/ampicillin (100 µg/ml) agar plates and plates were incubated at 37° C for overnight. The *E coli* cells grown in the LB ampicillin plate was considered as the transformed cell with the recombinant vector.

3.19.2 Confirmation of Cloning of Kanamycin resistance gene in YIplac211

3.19.2.1 Plasmid Extraction

Three ampicillin resistant colonies growing on plate were selected to inocµlate 3 ml of LB/ampicillin (100 µg/ml) medium and incubated at 37° C with shaking at 200 rpm. The plasmid was isolated as described previously by Alkaline lysis method, purified by chilled phenol-chloroform mixture and ethanol precipitated.

3.19.2.2 Restriction digestion

Thus recovered plasmid DNA was digested with restriction enzymes to verify cloning of

DNA insert containing kanamycin resistance gene to YIplac211 plasmid. The restriction digestion mixtures were prepared as follow:

Restriction digestion with *PstI*

Plasmid	5µl (20 ng)
PstI (5U/ µl)	1 µl
Tango (10X)	2 µl
MilliQ water	12 µl
Total	20 µl

The restriction digestion mixtures were incubated at 37°C for 3 hours and electrophoresed in 1% agarose gel as described previously. The positive transformant harboring desired insert cloned in YIplac211 vector was confirmed by electrophoretic movement of expected band size i.e. 3.797kb of YIplac211 plus 1.7kb of DNA insert containing loxp flanked kanamycin resistance gene, thus total band size was almost 5.547kb. Then the positive transformant was streaked in LB/ampicillin (50 µg/ml) agar plate and a single isolated colony was recultured in 10 ml of LB/ampicillin (100 µg/ml) medium. From this overnight culture, 2 ml of the culture broth was used to isolate plasmid DNA as described previously and restriction digested with *PstI* to re-confirm the cloning of insert in pUG6. Thus confirmed cloned plasmid DNA. The remaining culture (6 ml) was mixed with 4 ml of 40% glycerol and dispensed 2 ml aliquots in each 5 sterilized cryo vials and stored at -40° C after proper labelling.

3.20 Calibration curves

Calibration curve were used as standard curve for the determination of unknown parameter.

3.20.1 Xylose calibration curve

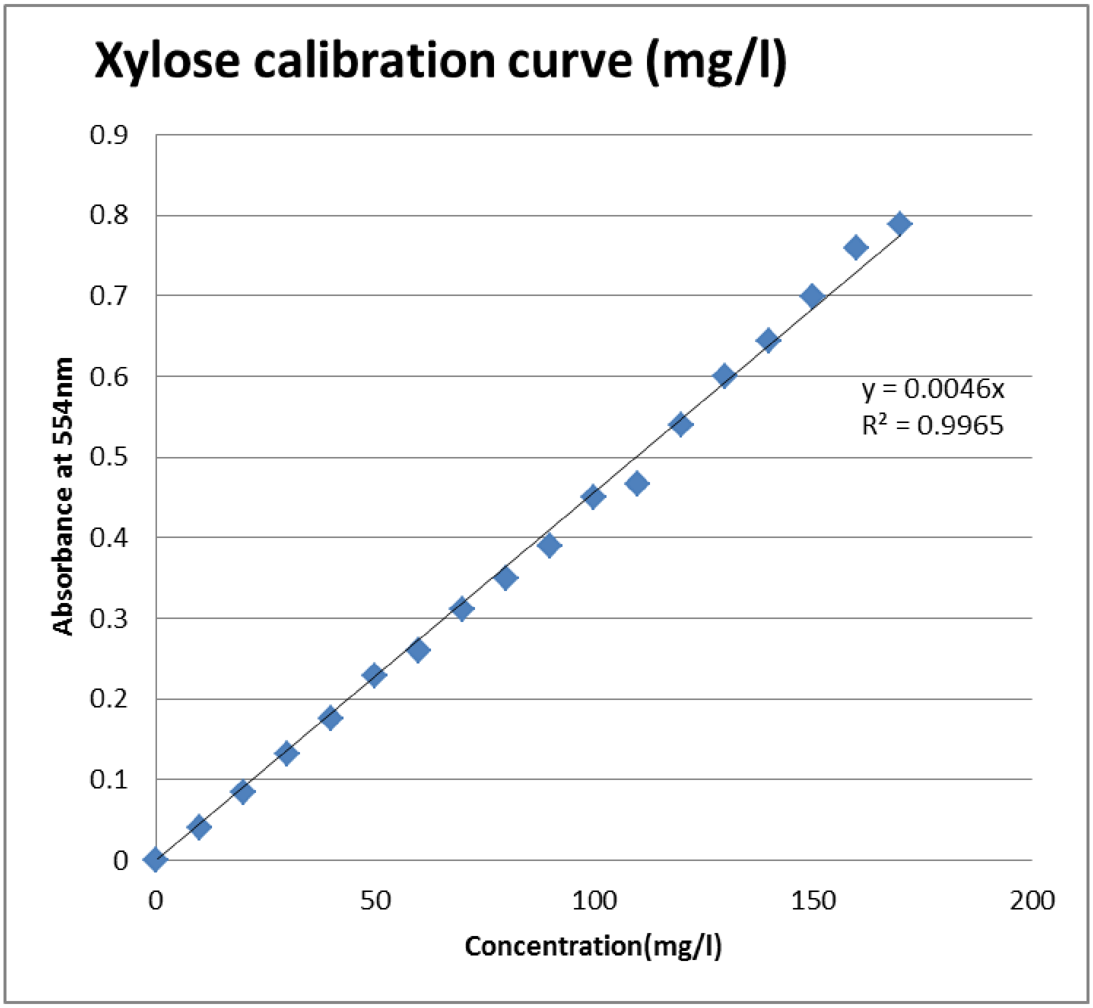

Figure 3.1: Calibration curve of xylose Absorbance at 554nm Vs. Concentration (g/l)

3.20.2 Glucose Calibration Curve

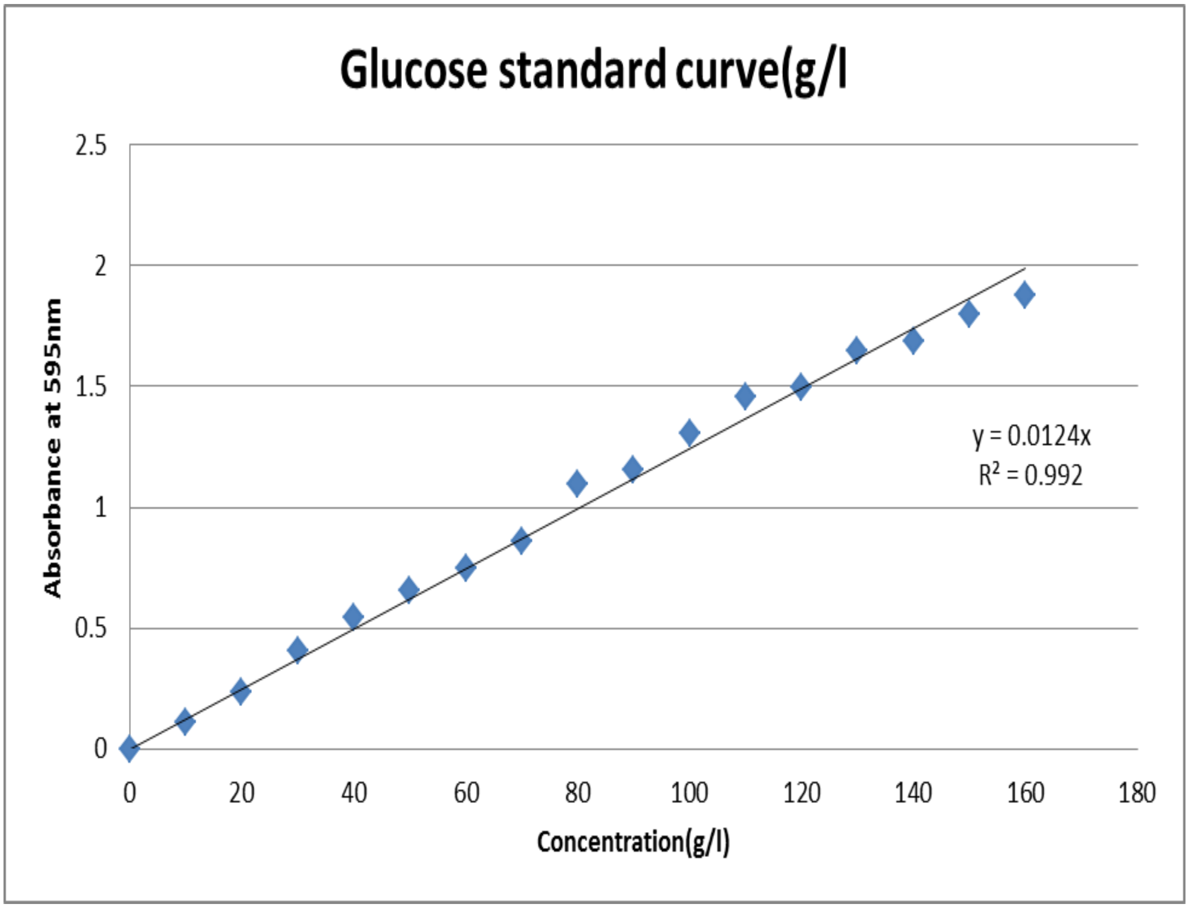

Figure 3.2: Calibration curve of glucose absorbance at 595nm Vs. Concentration(g/l).

3.20.3 Ethanol Calibration Curve

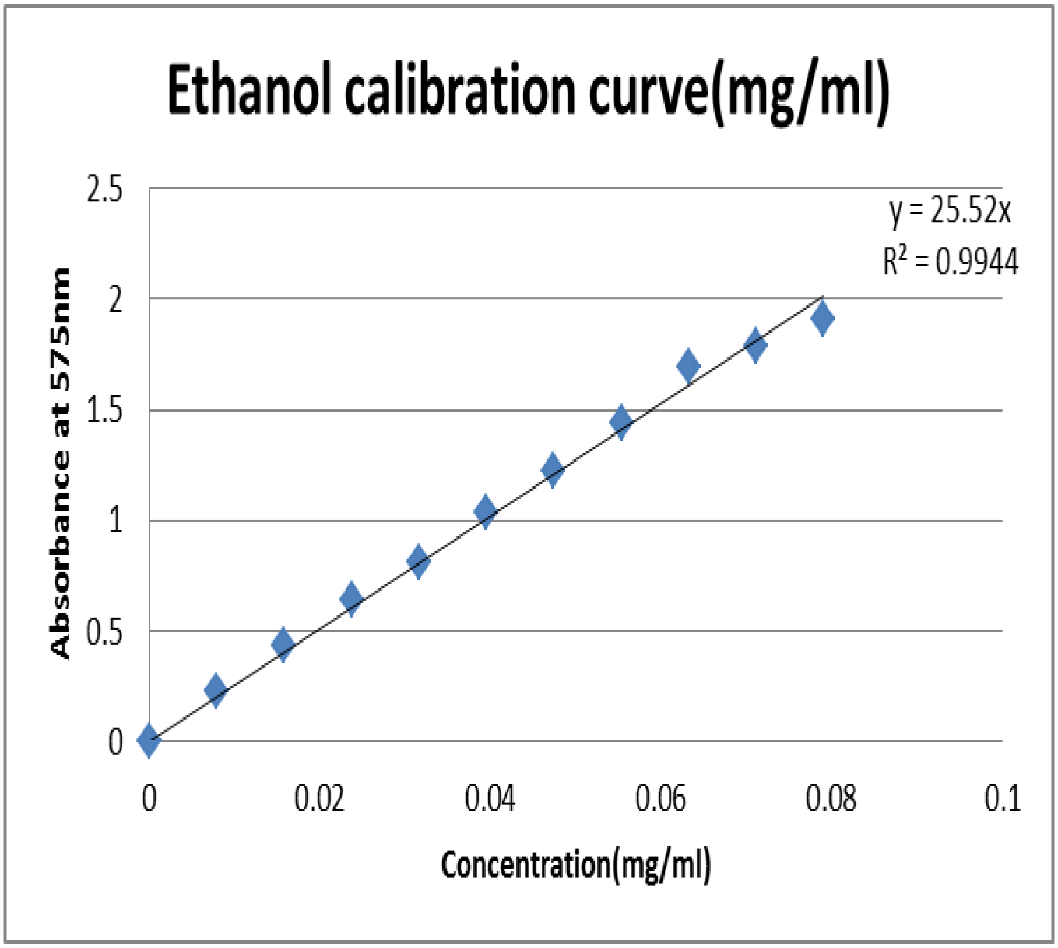

Figure 3.3: Ethanol calibration curve absorbance at 575nm vs concentration(mg/ml).

CHAPTER IV

RESULTS

4.1 TRANSFORMATION OF p*GXS1* IN YEAST

4.1.1 Cloning of p*GXS1* in *E. coli*

The plasmid *GXS1* was transformed into the electrocompetent cells of *E. coli* DH5 α by chemical method and selection was done by plating on the ampicillin containing media. The confirmation of the plasmid transformation was done by the restriction digestion and PCR.

4.1.2 Plasmid isolation and restriction digestion

Single colony was inoculated in the LB-ampicillin broth and incubated at 37C overnight. Plasmid was isolated from the culture by using the alkaline lysis method. Isolated plasmid was run in 1% agarose gel electrophoresis and visualized on UV-transilluminator. Then the plasmid was linearized with restriction enzyme *Pst*I. And it was run in 1% agarose gel electrophoresis and visualized by UV- transilluminator. The band observed was of the size of nearly 7.5kb compared to the Genei 1kb ladder. The size of plasmid with insert was 7.44kb. Then for the further conformation plasmid was done by digesting with the enzymes *Pst*I & *Sac*I and excised the insert. The band of insert was observed slightly greater than 2.0kb when run in 1% agarose gel electrophoresis which was visualized under the UV-transilluminator. The size of the insert was 2.2kb.

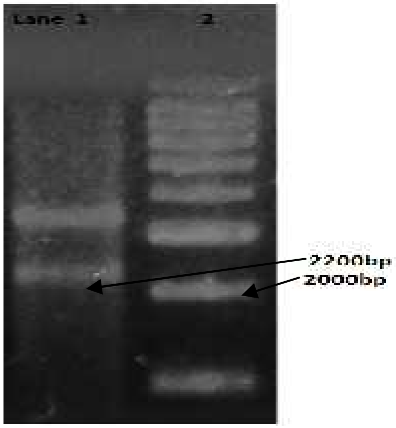

Figure 4.1 Restriction digestion of pGXS1 with *Pst*1 and *Sac*1, Lane1= Digestion of plasmid with *Pst*I & *Sac*I, Lane2= 1000bp ladder(Genei)

4.1.3 Primer amplification of transformants plasmid (Using two sets of primers TU*GXS1* Fw- TU*GXS1* Rv and TU*GXS1*int Fw-TU*GXS1*int Rv)

The plasmid of p*GXS1* transformant was further confirmed by the PCR using the two sets of primers(TU*GXS1* Fw-TU*GXS1* Rv and TU*GXS1*int Fw-TU*GXS1*int Rv). The plasmid was first amplified with the primer flanking the ORF primers TU*GXS1* Fw-TU*GXS1*. The PCR product was then run on 1.2% agarose gel electrophoresis and visualized under UV- transilluminator. The band was observed at the 1569bp comparing to the thermoscientific 1kb plus gene ruler. The PCR product of ORF was taken as template and amplified by using the internal primers(TU*GXS1*int Fw-TU*GXS1*int Rv).The PCR product was run in the 2% agarose gel electrophoresis and visualized under the UV transilluminator. The band observed was of 95bp size comparing to the 1kb plus generuler (Thermoscientific).

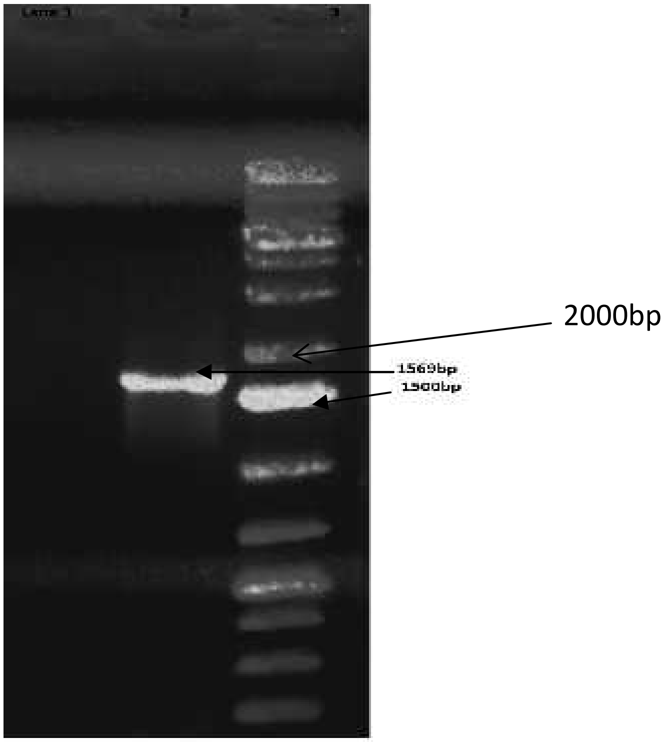

Figure 4.2 PCR amplification of the p*GXS1* plasmid by the primer of ORF (TU*GXS1*) Lane1=Negative control Lane2=PCR product of ORF of the *GXS1* Lane 3 1kb plus gene ruler(Thermoscientific)

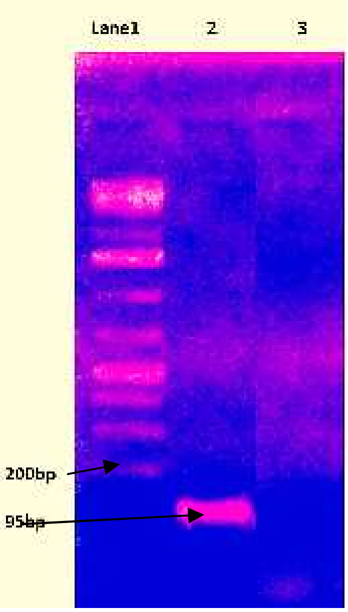

Figure 4.3 PCR amplification of the p*GXS1* by internal primer(TU*GXS1* int) Lane1=1kb plus gene ruler(Thermoscientific),Lane2=Internal PCR product of size 95bp, Lane3 Negative control

The result shows that the ORF of the glucose/xylose symporter was present in the plasmid.

4.1.4 Transformation in yeast

The isolated plasmid from p*GXS1* *E. coli* transformants by alkaline lysis method was mixed with electrocompetent cells of DTY165 haploid lab strain and transferred into the DTY165α (ura3$^-$) by electroporation technique. Selection was done by growing the transformants in uracil negative Yeast nitrogen base agar. The positive transformant was further grown on the xylose(5gm/l) containing YNB agar. This conform the transformation of p*GXS1* in the *S. cerevesiae.*

Figure4.4 Selection of transformant *S cerevesiae* , A) DTY-165 haploid lab strain yeast no growth was observed, B) DTY-165 yeast transformed with p*GXS1* growth was observed

4.1.5 Yeast plasmid isolation

Both transformed and untransformed yeast were grown on the YEPD broth for overnight at 28C with shaking at 220rpm.Then the cells were used for the isolation of plasmid. Lyticase and SDS was used for the disruption of the yeast cell wall and cell membrane. But the plasmid was not visible in size due to its low amount.

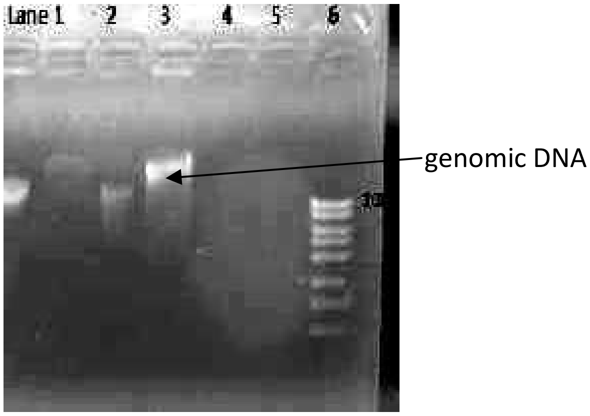

Figure 4.5 Isolation of the plasmid from the *S cerevesiae*
Lane1,Lane2, Lane3, lane4 and Lane5=Plasmid isolated from yeast, Lane6=1kb ladder(Genei)

4.1.6 Confirmation of transformation by PCR

Internal primer was used for the amplification of the 95bp band present in the transformed *S cerevesiae*. The isolated yeast plasmid was used as the template for the PCR. The band of size 95bp was observed compared to the 1kb plus generuler (Thermoscientific) when run on 2% agarose gel electrophoresis at 50v for 1hour.The gel was visualized under UV transilluminator.

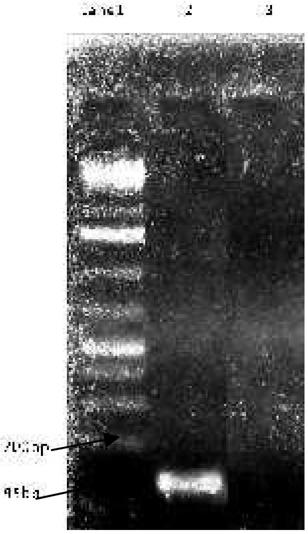

Figure 4.6 PCR amplification of the transformant *S cerevesiae* by using internal primer. Lane1= 1kb plus generuler, Lane2= PCR product of internal primer on yeast plasmid, Lane 4= negative control

The present of the specific band in the gel shows that the p*GXS1* plasmid has been transformed into the *S cerevesiae.*

4.1.7 Functional expression analysis

4.1.7.1 Growth kinetics on glucose (20g/l) and xylose(20g/l)

The transformed yeast (SX) and the non transformed yeast (DTYX) was grown in overnight at 28C with shaking at 220rpm.After incubation the cells were diluted to obtained the OD at 600nm around 0.1. Then the OD at 600nm was measured at every 3 hour interval as 0,3,6,9,….,36hours respectively. The results obtained were shown in figure Figure4.12/4.13.

The specific growth rate of the transformed and the untransformed *S cerevesiae* at the glucose(20g/l) was found to be 0.0820±0.00065/h and 0.092±0.00071/h respectively. In the xylose at the concentration of the 20g/l the specific growth rate of the transformed and the untransformed *S cerevesiae* was found to be 0.049±0.0033/hr and 0.0065±0.001860/hr respectively.

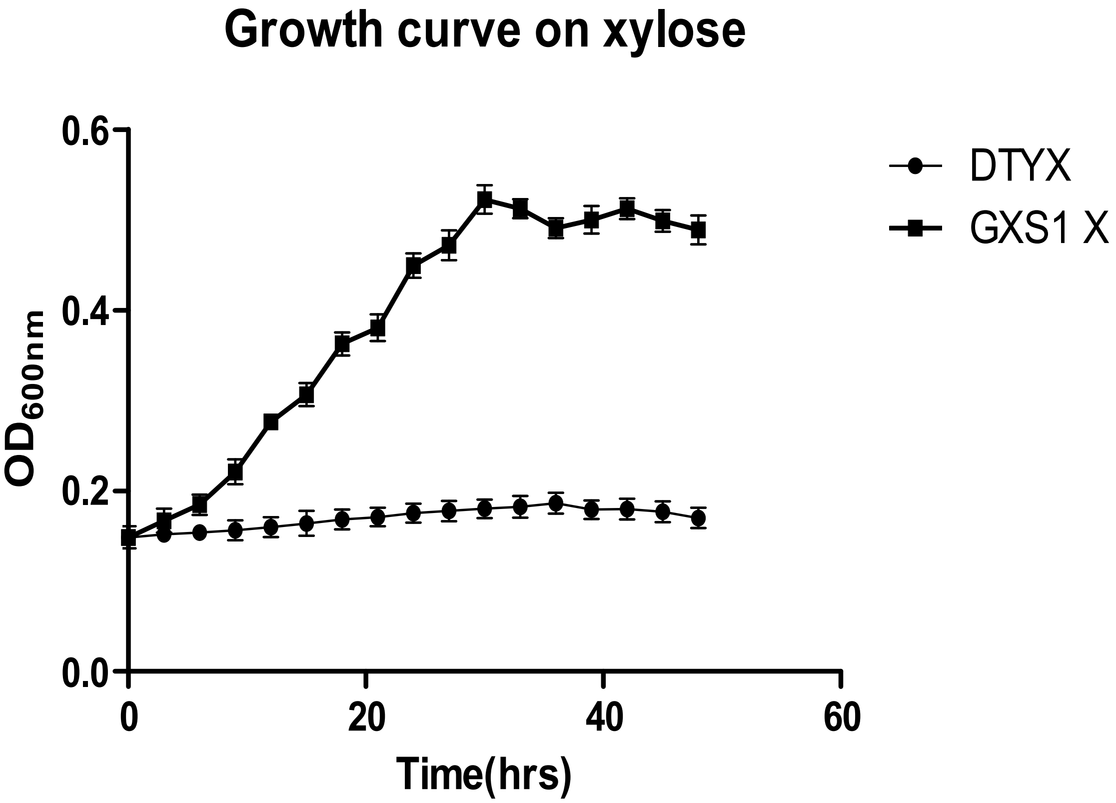

Figure 4.7 Growth curve of transformed(SX) and untransformed(DTYX) *S cerevesiae* cells at xylose concentration (20g/l).Graph was plotted the OD_{600} nm versus time in minute. ±All experiments were replicated for three times. P_{value} <0.001 and significant at 95% confidence interval.

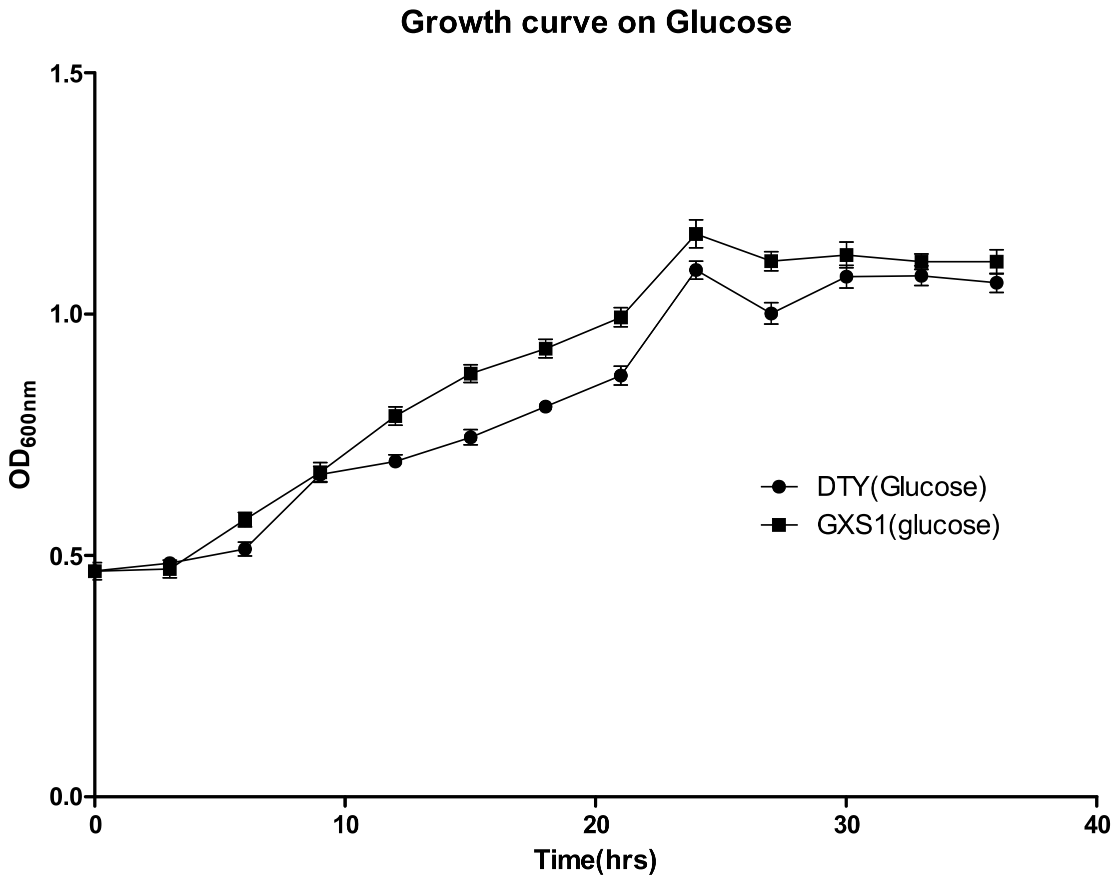

Figure 4.8 Growth curve of transformed(SX) and untransformed(DTYX) *S cerevesiae* cells at glucose concentration (20g/l). All experiments were replicated for three times. P_{value} <0.0001 for both replicates and significant at 95% confidence interval.

GXS1 transporter enhanced the growth of *S. cerevesiae* in xylose significantly but the growth in the glucose was higher slightly.

4.1.7.2 Extracellular xylose uptake

The transformed and untransformed yeast cells were grown O/N in YEPD broth at 28°C with shaking at 220rpm.Then the OD_{600} nm was made around 0.3 and cultured in the xylose containing 20g/l. The sample were taken at 0hr, 12hrs, 24hrs and 48hrs respectively and the extracellular concentration of xylose was measured by the phloroglucinol assay. The extracellular xylose concentration of the untransformed cell DTYX was found to be 20±0.00g/l,17.1525±0.3125g/l,15.254±0.405g/l and 14.23453±0.693g/l at 0 hrs, 12hrs, 24hrs and 48hrs respectively. The initial concentration of xylose was 20g/l. The uptake of the xylose was calculated by the initial concentration – Concentrtaion of xylose at 48hrs in the media. The result was divided by the 48hrs which yields the consumption rate. The untransformed *S cerevesiae* consumption rate of xylose was found to be 0.11±0.03291g/l/h during 48 hrs. The extracellular xylose concentration of *S. cerevesiae* cells harbouring *GXS1*, was found to be 20±0.00g/l, 15.2345±0.700g/l, 11.995±0.5775g/l and 9.033±0.49915 g/l at 0hrs, 12hrs, 24hrs and 48 hrs respectively. The transformed *S cerevesiae* consumption rate was found to be

S cerevesiae

erevesiae at

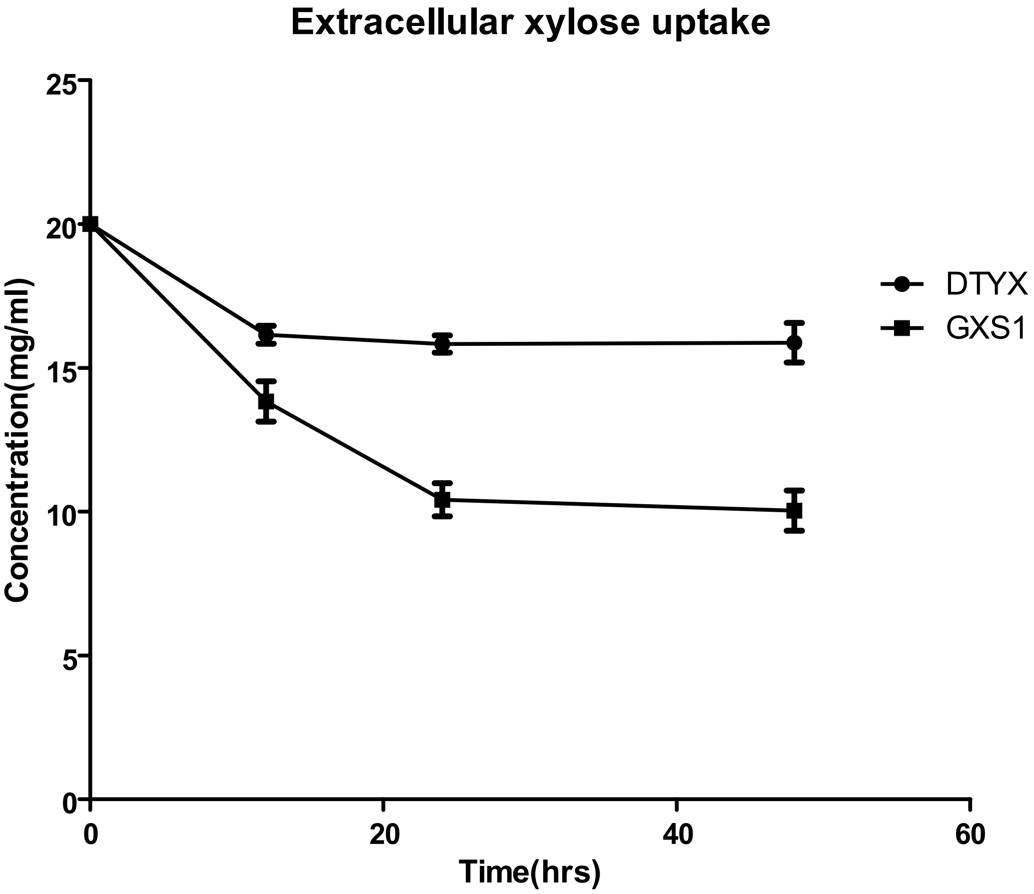

Figure 4.9 Extracellular xylose concentration of the *GXS1* transformed and untransformed *S. cerevesiae* grown in xylose concentration(20g/l). Graph was plotted concentration(g/l) vs time(hrs) when sample was taken. All experiments were done in three replicates. P_{value} for untransformed *S cerevesiae* was 0.0437 and for transformed *S cerevesiae* was found to be 0.0401 and found to be significant at 95% confidence interval.

4.1.7.3 Intracellular concentration of xylose

The O/N culture of the transformed and untransformed *S. cerevesiae* was washed twice with sterile water. Then the cells were incubated with the xylose at 28c with 220 rpm. Then the samples were taken at 30min, 60min, 120 min and 150 min respectively. Then the cells were washed twice with sterile water and incubated with the sterile water at 37^{0}C for 24hours for releasing the intracellular xylose. Then the xylose were measured by the phloroglucinol assay and OD600nm was measured for the cell dry weight. The concentration of xylose released from cells were measured.The untransformed *S cerevesiae* cell at 30min, 60min ,120min and 150min was found to be 16.27829±0.15466mg/l,16.36541±0.17041mg/l,16.5582±0.1702mg/l and 16.6209±0.1857mg/l per gram of cell dry weight. Intracellular concentration of xylose of the transformed *S cerevesiae*(*GXS1*) at 30min, 60min, 120min and 150 min were found to be 16.83907±0.2999mg/l,17.783981±0.1572mg/l,18.89555±0.2346mg/l and 19.565222± 0.22341 mg/l per gram of cell dry weight.

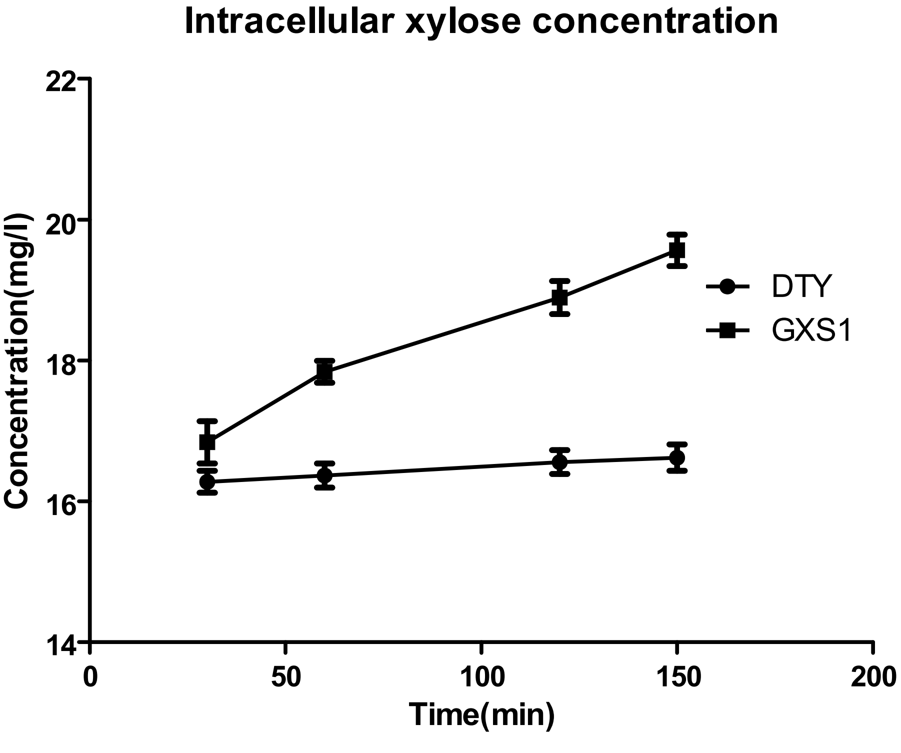

Figure4.10 Intracellular xylose concentration of transformed (*GXS1*) an wild *S. cerevesiae* (DTYX). The graph was plotted concentration vs time(mins). All experiments were done in three replicates. P_{value} was found to be 0.0022 for DTYX and 0.0076 for *GXS1* and result was found to be significant at 95% confidence interval.

The intracellular concentration was found to be significantly greater in the *GXS1* harbouring *S cerevesiae* comparing with the untransformed *S cerevesiae*.

4.1.7.4 pH elicitor experiment

When the xylose was added to the solution of pH 5.0. The symport activity of the symporter transport the xylose along with the H^+ inside the cells. So the pH of the extracellular solution should increased. The pH of the solution were cells was kept increases from 5.00 to 5.26±0.0001942 in 100 second interval in the *S cerevesiae* transformed with symporters. But in the untransformed *S cerevesiae* only slight increase in the pH was observed from 5.00 to 5.012±0.0003906. This showed that the increased transport of xylose was increased when *S. cerevesiae* was transported with *GXS1*, a symporters of xylose and proton.

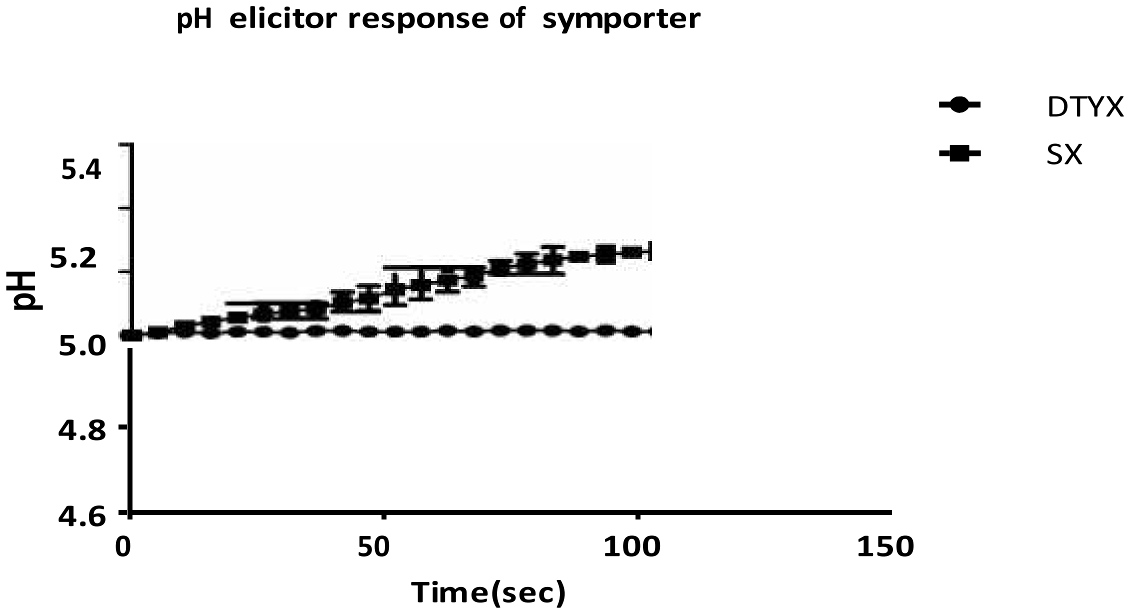

Figure 4.11 Determination of the symport activity of the *GXS1*.Graph was plotted pH vs time(sec). All experiments were done in three replicates. P_{value} was found to be 0.0004 for DX and P_{value} <0.0001 for SX and significant at 95% confidence interval and result was found to be significant.

4.1.7.5 X/G preference ratio

The O/N culture of transformed and untransformed *S. cerevesiae* were cultured in the xylose and glucose concentration of 20g/l each. Then the sample were taken out at 0hrs 12hrs , 24hrs and 48hrs respectively. Then concentration of glucose was determined by the DNS method and the concentration of xylose was determined by the phloroglucinol assay. The moles of the sugar remained in the broth was calculated by using molarity calculator. (http://www.endmemo.com/chem/molarity.php). Uptake of the monosaccharide was determined in moles of monosaccharide. Then the molar consumption rate was the slope of the data of the given monosaccharide uptake concentration in moles at 24hrs interval. Then the molar ratio of the xylose consumption rate to the glucose consumption rate was the X/G preference ratio. The concentration of the remaining xylose in the cultured broth with *GXS1* transformed *S. cerevesiae* was found to be 20g/l, 14.53 ±0.32g/l and 13.144±0.006g/l at 0hr, 12hrs, and 24hrs. The extracellular xylose concentration when glucose and xylose 20g/l each was supplied at 0hrs, 12hrs, 24hrs was found to be 0.13328moles, 0.09678279moles and 0.08755079 moles respectively. The molar uptake of the glucose was calculate and was found to be 0 moles, 0.03649721moles and 0.04569921moles at 0hr, 12hrs and 24hrs respectively. The slope of the xylose uptake was found to be 0.001904±0.0006568. So the molar consumption rate of xylose in co-consumption of xylose/glucose by *S cerevesiae* harbouring *GXS1* was 0.001904±0.002038. The molar consumption of the glucose was found to be 0 moles, 0.001499moles and 0.0024175 moles at 0hr, 12hrs and 24hrs respectively. The concentration of the remaining glucose in the cultured broth with *GXS1* transformed *S. cerevesiae* was found to be 20g/l, 2.4517 ±0.32g/l and 0.1696±0.006g/l at 0hr, 12hrs, and 24hrs. The extracellular glucose concentration when glucose and xylose 20g/l each was supplied at 0hrs, 12hrs, 24hrs was found to be 0.113012moles, 0.0136086moles and 0.0009413854 moles respectively. The molar uptake of the glucose was calculated and was found to be 0 moles, 0.0974034moles and 0.1100706146 moles at 0hr, 12hrs and 24hrs respectively. The slope of the glucose uptake was found to be 0.004586±0.002038. So the molar consumption rate of glucose by harbouring *GXS1* was 0.004586±0.002038. Then X/G preference ratio of transformed *S. cerevesiae* was found by calculating the molar consumption rate of xylose by the molar consumption rate by glucose. And the X/G preference ratio of *S cerevesiae* having *GXS1* was 0.415176. The percentage of sugar consumed was found to be 69.9%.

Similarly, The concentration of the remaining xylose in the cultured broth with untransformed *S. cerevesiae* was found to be 20g/l, 17.592 ±0.32g/l and 16.595±0.006g/l at 0hr, 12hrs, and 24hrs. The extracellular glucose concentration when glucose and xylose 20g/l each was supplied at 0hrs, 12hrs, 24hrs was found to be 0.13328 moles, 0.117178moles and 0.110538moles respectively. The molar uptake of the xylose was found to be 0 moles, 0.016102moles and 0.022742moles at 0hr, 12hrs and 24hrs respectively. The slope of the xylose uptake was found to be 0.0009476±0.0002276. So the molar consumption rate of xylose in co-consumption of xylose/glucose by untransformed *S cerevesiae* was 0.0009476±0.0002276. The concentration of the remaining glucose in the cultured broth with untransformed *S. cerevesiae* was found to be 20g/l, 2.03 ±0.32g/l and 0.1996±0.006g/l at 0hr, 12hrs, and 24hrs. The extracellular glucose concentration when glucose and xylose 20g/l each was supplied at 0hrs, 12hrs, 24hrs was found to be 0.113012moles, 0.0112676moles and 0.0011079041 moles respectively. The molar uptake of the glucose was calculated and was found to be 0 moles, 0.099744moles and 0.1099040959 moles at 0hr, 12hrs and 24hrs respectively. The slope of the glucose uptake was found to be 0.004579±0.004535. So the molar consumption rate of *S cerevesiae* harbouring *GXS1* in glucose was 0.004579±0.004535. Then X/G preference ratio of untransformed *S. cerevesiae* was found by calculating the molar consumption rate of xylose by the molar consumption rate by glucose. And the X/G preference ratio of untransformed *S cerevesiae* was 0.206. The percentage of sugar consumed was found to be 56.86%.

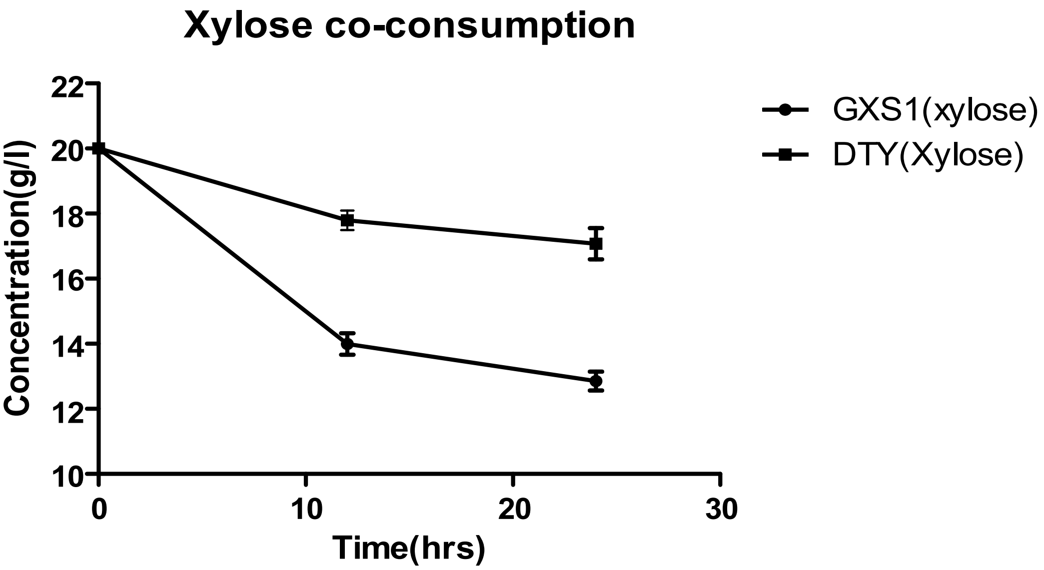

Figure 4.12 Determination of extracellular glucose concentration when glucose and xylose was used at 20g/l each. All experiments were replicated for three times. P_{value} was found to be 0.0032 and significant at 95% confidence interval.

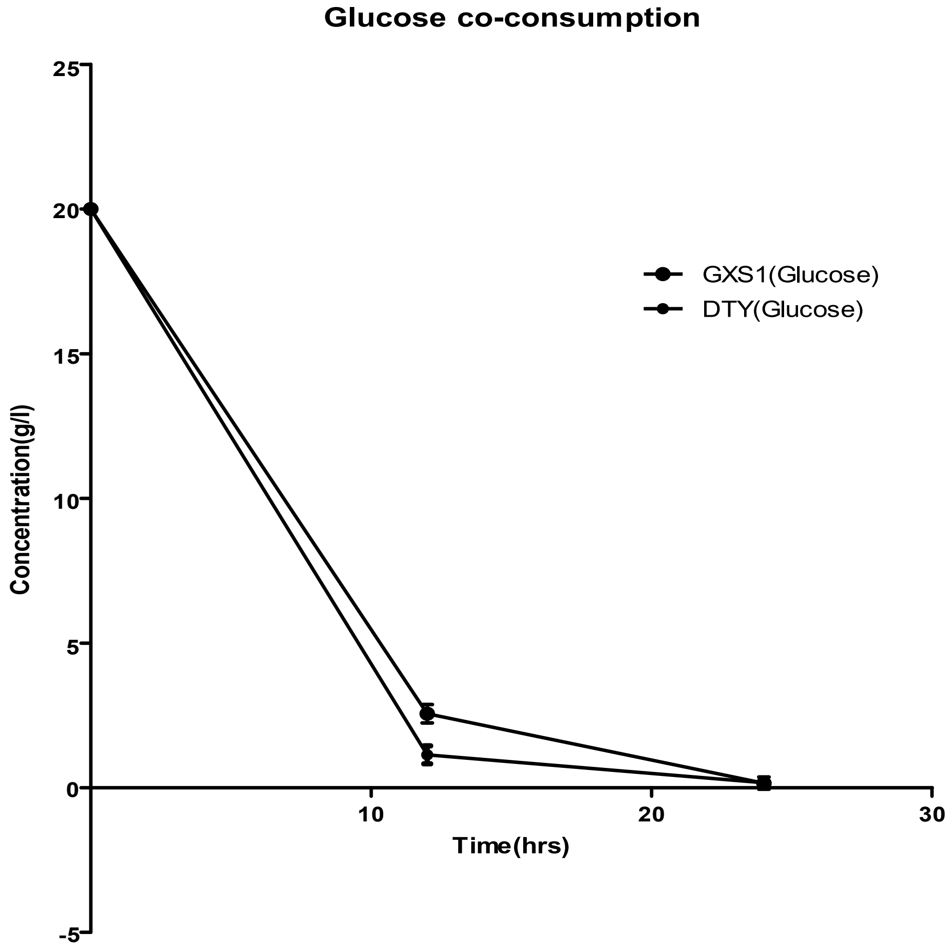

Figure 4.13 Determination of extracellular glucose concentration when glucose and xylose was used at 20g/l each. All experiments were replicated for three times. P_{value} was found to be 0.0045and significant at 95% confidence interval.

4.1.7.6 Ethanol production when growth in glucose20g/l or xylose 20g/l

Transformed and the untransformed *S cerevesiae* were grown at 20g/l of glucose and 20g/l of xylose separately for 7 days at 28C.The ethanol production by the untransformed *S. cerevesiae* from 20g/l xylose and 20g/l glucose was found to be 0.007621±0.0002603 mg/ml and 0.3259 ±0.0085mg/ml respectively. The ethanol production by the transformed *S. cerevesiae* from 20g/l xylose and 20g/l glucose was found to be 0.00794±0.0002603 and 0.3524±0.0010 respectively. The production of the bioethanol from the glucose by transformed *S. cerervesiae* was slightly greater than that of untransformed *S. cerevesiae*.But the production of the ethanol from the xylose by transformed *S cerevesiae* was nearly 100 fold greater than that of untransformed *S cerervesiae*. The production of the ethanol was much higher from glucose than that of the xylose.

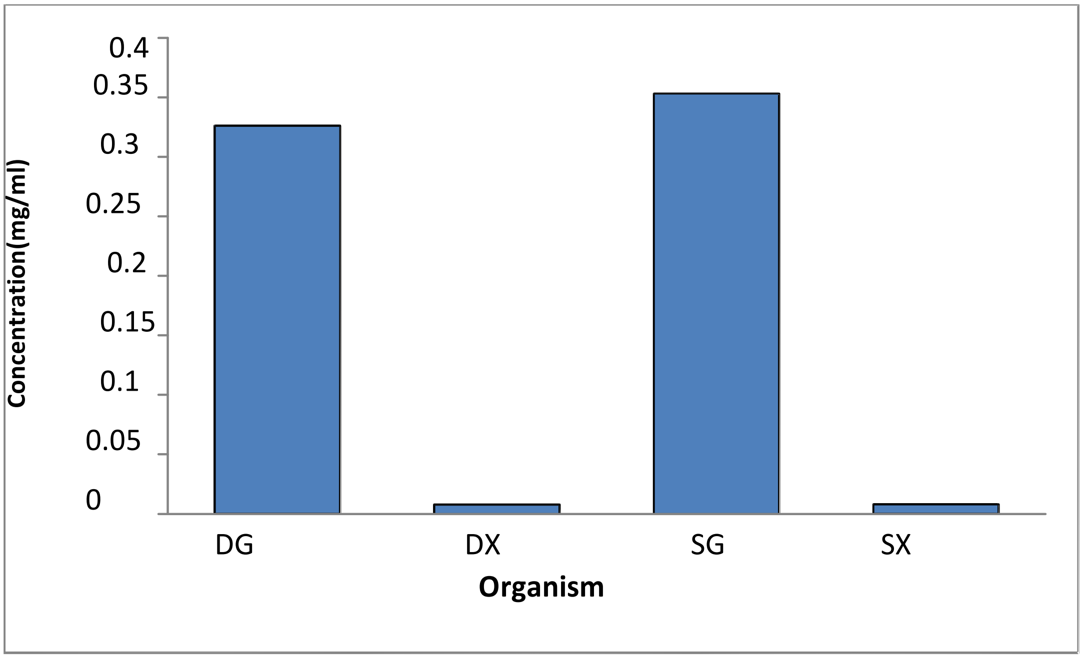

Figure 4.14 Estimation of ethanol concentration from the *S cerevesiae* grown on glucose and xylose. Graph was plotted of concentration of ethanol vs organism.

4.2 CLONING OF MARKER GENE(loxp-Kan^r –loxp)

PUG6 template vector was used as the template for the loxp flanked kan^r gene. The marker gene was amplified by using the primers.

FP’- 5’-: TCCAC AAGCTT CGTCGACAACCCTTAATATAACTTC (YTULOXPF)

RP- 5’: GAGTTG CTGCAG GAGTCAGTGAGCGAGGAAG (YTULOXPR)

The PCR condition was:

	Cycles	Temp	Time
Initial Denaturation	1 Cycle	95°C	5 min
Denaturation	5 Cycles	95°C	30 seconds
Annealing (oligoanalyzer)		**55°C**	30 seconds
Extension		72°C	**2 minutes**
Denaturation	25 Cycles	95°C	30 seconds
Annealing (oligoanalyzer)		**65°C**	30 seconds
Extension		72°C	**2 minutes**
Final Extension	1 Cycle	72°C	5 minutes
Hold	1 Cycle	4°C	
Amplicon size= 1700 bp			

The PCR product was run in the 1.2% agarose gel electrophoresis and visualized under UV transilluminator. The product was found to be 1.7kb compared to the 1kb plus generuler(Thermoscientific). This indicates the succesful amplification of the loxp flanked kan^r gene by the designed primer(YTUloxP)

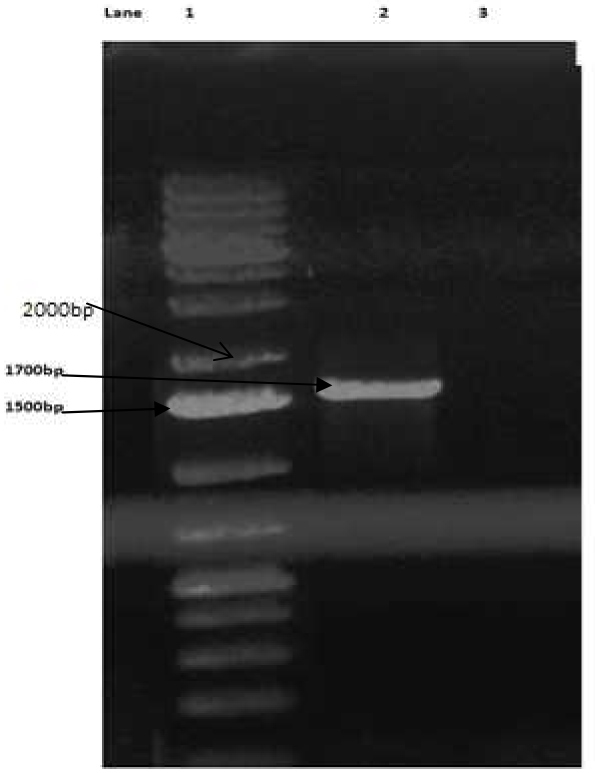

Figure 4.15 PCR amplification of the loxp-Kan^r gene (YTUloxP)
Lane1= Negative control, Lane2=PCR product of loxp flanking kanamycin resistance gene
Lane3=1Kbplus generuler (Thermoscientific)

4.2.1 Digestion of insert

The PCR product was gel purified and and partially digested with the *Pst*1 and *Hind*III. The digested product was run in the 1.2% agarose gel electrophoresis and visualized under the UV transilluminator. The 1700bp containing the loxp-Kan ^{r} with the ligation site for *Pst*1 and *Hind*III was used as insert compared to 1kbplus generuler (Thermoscientific).

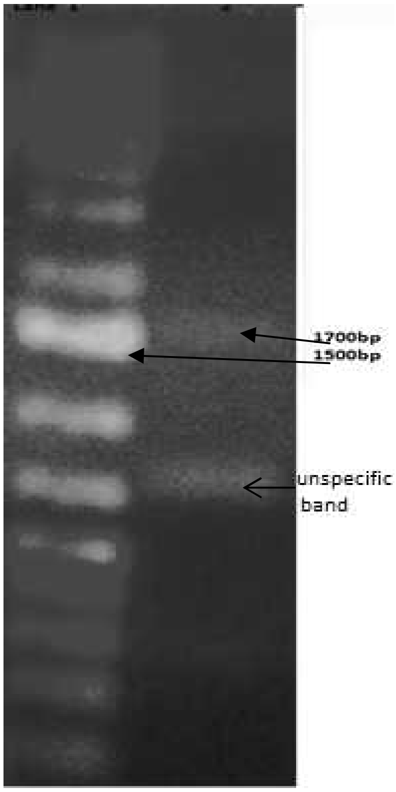

Figure 4.16 PCR quantification of insert.

Lane1= 1kb plus generuler(Thermoscientific), Lane2 =Partial digestion of loxp-Kan^r PCR product

4.2.2 Digestion of vector

The isolated plasmid of YIplac211 was double digested with *Pst*1 and *Hind*III and run in 1% agarose gel electrophoresis and visualized under UV transilluminator. The size of the vector was found to be 3797bp compared to the 1kbplus generuler (Thermoscientific).

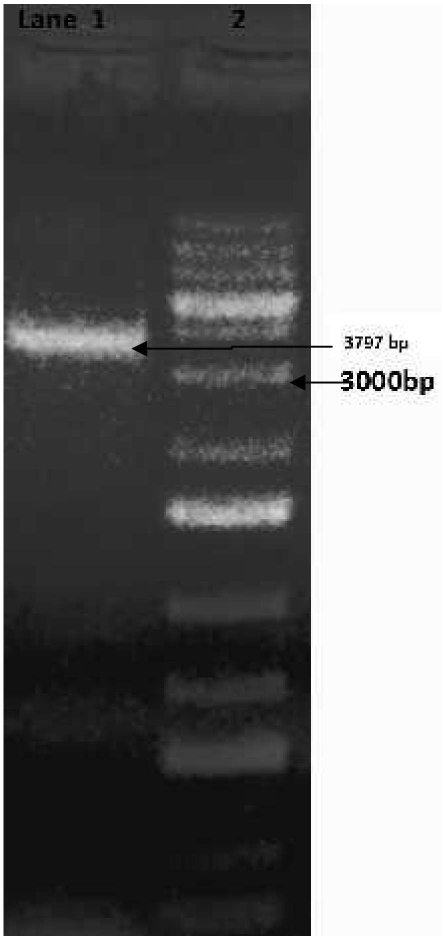

Figure 4.17 Vector digestion and quantification of vector. Lane1= YIplac211 double digested product with *Pst*I and *Hind*III, Lane2=1kbplus generuler (Thermoscientific)

4.2.3 Transformation of YIplac211-loxp-Kan^r

The YIplac211 and the insert was ligated and transformed with the ligation mixture by heat shock method(Sambrook et al,1989). The transformed colonies were seen in the LBA plate containing ampicillin conforming that the transformation was successful. The colonies were further used for the conformation by restriction digestion.

Figure4.18 Transformed E coli colonies with the ligation mix of YIplac211 and loxp flanked Kan^r gene

4.2.4 Plasmid isolation from transformants

The colonies obtained from the transformant was cultured in the LB(ampicillin) and the cells were used for the isolation of plasmid by alkaline lysis method.(Sambrook *et al*,1989)

Figure 4.19 Isolation of the loxp-kan^r transformants, Lane 1,2,3,4,5,6,7=Plasmid isolated from transformants, Lane8=1kbplus generuler(Thermoscientific)

4.2.5 Confirmation of transformants by linear digestion

The isolated plasmid was linearly digested with *Pst*1.The digested product was run in 1.0% agarose gel electrophoresis. The 5547 bp of the linearized plasmid was found, compared to the 1kbplus generuler(Thermoscientific).

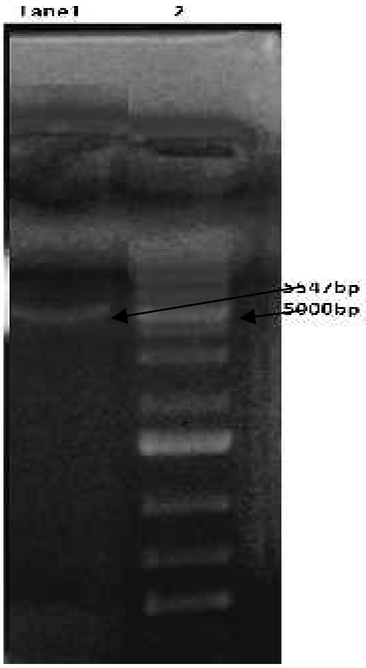

Figure 4.20 Conformation of loxp-Kan^r transformants by linear digestion Lane1=Plasmid digested with *Pst*1 and Lane2=1kb plus generuler(Thermoscientific)

The size of the linearized plasmid was equal to the sum of the insert and the vector, which indicates that the loxp-kan^r gene have been successfully cloned into the YIplac211. Further conformation can be done by the sequencing.

CHAPTER V

DISCUSSION

Bioethanol is the good alternative for the fossil fuel. The advantages includes the sustainability of the fuel production, reduction of the pollutants and have high value of the octane number. It permits to burn the biofuel in the high compression ratio, so it has the high power output efficiency and consequently favorable sustainable economy. Bioethanol has the simplest refining procedure than the fossil fuel (Calle R *et al*,1998). Lignocellulosic biomass are the cheapest , widely found and the largest source of the renewable feedstock. It can be obtained from number of sources such as agricultural and forestry residues(Bashir and Lee 1994). Utilization o f the biomass polymers such as cellulose, hemicellulose, lignin is beneficial in social aspects in terms of reducing solid waste, carbon- dioxide and green house gases. (Hatti *et al* and Saha 2003) . Unfortunately, *S. cerevisiae* cannot utilize pentose sugars contained in the hydrolysates of the biomass feedstock's hemicellulose component. On the other hand, there are many naturally existing fungal and bacterial species which can utilize pentose sugars efficiently. However, these microorganisms are not compatible with industrial ethanol fermentation due to their complicated physiology and low ethanol productivity (Chu and Lee 2007).

Molecular transport is a key process in cellular metabolism. This step is often limiting when using a nonnative carbon source, as exemplified by xylose catabolism in *S. cerevisiae*(Yong *et al* 2011). Native pentose-utilizing organisms exist but , a lack of well-developed genetic tools and low product tolerances (Hahn Hagedral et al 1991) limit their utility as hosts for industrial scale lignocellulosic conversion processes. So, significant effort has focused on the metabolic engineering of pentose catabolic pathways in the yeast *S. cerevisiae* to enable xylose and arabinose fermentation (Hahn Hagedral et al 2007). The lack of a dedicated xylose transport system in recombinant *S. cerevisiae* thus limits the capacity for dual xylose and glucose fermentation as well as high xylose catabolic pathway flux (Jefferies and T.W. 2006).

In this study, the plasmid containing glucose/xylose symporter(pGXS1) was transformed into the *S cerevesiae* for the expression of the GXS1 gene. Due to the low amount of the yeast plasmid DNA, it was not visible under UV transilluminator. The transformed *S cerevesiae* was confirmed by using PCR and southern blotting. The primer was designed for the amplification of 95bp internal region of GXS1 gene and this primer was also used for the preparation of the probe. The probe was used for the specific binding in GXS1 gene during southern blotting. The PCR was performed by using TUGXS1int primers amplifying the 95p internal fragment of the GXS1 gene. Then further conformation was done by the southern blotting. PCR and Southern blotting was the specific as well as sensitive test and widely used for the conformation of the transformants in the eukaryotic organism. In similar study of

Of screening transformants, internal region amplification of hygromycin phosphotransferase (*hph*) gene by PCR and the southern blot analysis was done for efficient selection of the transformants.(Xiao L and Ha S., 1997)

The specific growth rate of the transformed and the untransformed *S cerevesiae* at the glucose(20g/l) was found to be 0.0820±0.00065/h and 0.092±0.00071/h respectively. In the xylose at the concentration of the 20g/l the specific growth rate of the transformed and the untransformed *S cerevesiae* was found to be 0.049±0.0033/hr and 0.0065±0.001860/hr respectively. The specific growth rate of the transformed and untransformed *S cerevesiae* on glucose was slightly increased in transformed S cerevesiae but In xylose significant increment in the specific growth rate was observed. The specific growth rate in glucose is higher than comparison to that in xylose.In the similar study in the hexose transporter null mutants, the specific growth rate of the transformants of the GXS1 gene in HXT null mutant was found to be 0.049 ±0.004(Yong *et al*, 2011).

The growth of the *S cerevesiae* in the xylose is due to the presence of endogeneous NADPH- dependent aldose reductase GRE3(EC= 1.1.1.21) and D-xylulose reductase EC=1.1.1.9). Overexpression of this open reading frame in *S. cerevisiae* resulted in xylitol dehydrogenase activity. The enzyme is specific for NADH. The following Michaelis constants were estimated: D-xylulose, 1.1 mM; NADH, 240 microM (at pH 7.0); xylitol, 25 mM; NAD, 100 microM (at pH 9.0). Xylitol dehydrogenase activity with the same kinetic properties can also be induced by xylose in wild type *S. cerevisiae* cells.(Toivari *et al* 1999). The GRE3 reduces the xylose to xylitol and the D xylulose reductase further reduce xylitol to xylulose. The endogeneous enzyme xylulose kinase (2.7.1.17) converts the xylulose to the xylulose -5- p. which ultimately enters into the pentose phosphate pathway.(www.uniprot.org).

There was a rapid increment of the pH of the solution from 5.01 to 5.26433 in the transformant *S cerevesiae* when the xylose was supplied to the solution of pH 5. But In the non transformed *S cerevesiae* pH increases slightly from 5 to 5.012 only. Similar raising of the pH was observed when the xylose was supplied to the solution(Goncalves *et al*,2008). This is due to the xylose is symport along with the H^+ into the yeast cells and thus increasing the pH of the surrounding (Hagedral and

Karuhama 2007).It clearly indicates the expression of the symporter causing the pH increment. The extracellular xylose concentration of the untransformed cell DTYX was found to be 20±0.00g/l,17.1525±0.3125g/l,15.254±0.405g/l and 14.23453±0.693g/l at 0 hrs, 12hrs, 24hrs and 48hrs respectively. The initial concentration of xylose was 20g/l. The untransformed *S cerevesiae* consumption rate of xylose was found to be 0.11±0.03291g/l/h during 48 hrs. The extracellular xylose concentration of *S. cerevesiae* cells harbouring GXS1, was found to be 20±0.00g/l, 15.2345±0.700g/l, 11.995±0.5775g/l and 9.033±0.49915 g/l at 0hrs, 12hrs, 24hrs and 48 hrs respectively. The transformed *S cerevesiae* consumption rate was found to be 0.2200±0.04543g/l/h during 48hrs interval. The consumption rate of the xylose of *S cerevesiae* harboring GXS1 was increased by several fold comparing to untransformed *S cerevesiae* at 48hrs interval. This results showed that the xylose has been transported inside the cells and utilized by the *S cerevesiae* for the growth. The concentration of xylose released from cells were measured. The untransformed *S cerevesiae* cell at 30min, 60min ,120min and 150min was found to be 16.27829±0.15466mg/l, 16.36541±0.17041mg/l, 16.5582±0.1702mg/l and 16.6209±0.1857mg/l per gram of cell dry weight. Intracellular concentration of xylose of the transformed *S cerevesiae*(GXS1) at 30min, 60min, 120min and 150 min were found to be 16.83907±0.2999mg/l,17.783981±0.1572mg/l,18.89555±0.2346mg/l and 19.565222± 0.22341 mg/l per gram of cell dry weight. It was found that the intracellular concentration of the transformed *S. cerevesiae* was slightly greater than the untransformed one. The decreasing concentration of the extracellular xylose in the medium and the increasing intracellular concentration of the xylose indicates the transportation of the xylose inside the cells. In the study of the kinetics of transporters it was found that Km values for xylose transport were found to be 0.4±0.1 in *C. intermedia* but the heterologous expression strain of *S cerevesiae* shows the one order lower magnitude of xylose transportation.(Leonardo *et al* , 2006).

Then X/G preference ratio of transformed *S cerevesiae* was found to be 0.415176. The percentage of sugar consumed was found to be 69.9%. Then X/G preference ratio of untransformed *S cerevesiae* was found to be 0.206. The percentage of sugar consumed was found to be 56.86%. In the similar study , the X/G preference ratio of the GXS1 transformed in HXT null mutant was 0.51 per 14% of sugar consumed.(Yong *et al*,2011).The difference was due to the various other hexose transporter was present in our strain which has the affinity for the xylose too. The percentage of the sugar consumption was high in our transformant because of the high percentage of glucose was consumed relatively than HXT null mutant due of the presence of number of hexose transporter. *S. cerevisiae* has low and high affinity hexose transport systems, which accounts for 18 different transporters (Hxt1-17 and Gal2) that could transport a wide range of substrate including xylose and glucose (Bertillson *et al* 2007).

Preferential sugar uptake combined with the transcriptional and/or enzymatic censure of less preferred sugars turns out one of the major barriers in increasing the yield and productivity of fermentation process.(Vinuselvi *et al*,2012). The main reason for CCR observed in the heterologous xylose pathway in *S. cerevisiae* might be due to the lack of dedicated xylose transporter. *S. cerevisiae* has low and high affinity hexose transport systems, which accounts for 18 different transporters (Hxt1-17 and Gal2) that could transport a wide range of substrate including xylose and glucose. Hexose transporters are differentially expressed depending on the concentration of glucose. The specificity of these transporters to facilitate xylose uptake varies as a function of glucose concentration. Of the 18 transporters, Hxt4p, Hxt5p, Hxt7p and Gal2P exhibit a higher specificity towards xylose. *S. cerevisiae* expresses the xylose specific transporters, Hxt4p and Hxt7p, only at the low concentration of glucose. Hence, in the initial stages of fermentation glucose concentration will be relatively high and hence the expression of xylose specific hexose transporters would be inhibited. With time, glucose concentration would reduce leading to the expression of Hxt4p and Hst7p transporters and hence favor xylose uptake. Strong competition between glucose and xylose for a single transporter would also limit xylose uptake leading to CCR.(Bertilsson *et al* , 2008). The heterologous transporter conferred an improved growth on xylose and glucose-xylose co metabolism. However, glucose still remains to be more preferred carbon source indicating the need for more specific xylose transporter(Hector *et al*, 2008). In *Saccharomyces cerevisiae* the HXK2 gene, which encodes the glycolytic enzyme hexokinase II, is involved in the regulatory mechanism known as ‘glucose repression’. Its deletion leads to fully respiratory growth at high glucose concentrations where the wild type ferments profusely (L Rossell *et al* 2008).

The ethanol production by the untransformed *S. cerevesiae* from 20g/l xylose and 20g/l glucose was found to be 0.007621±0.0002603 mg/ml and 0.3259 ±0.0085mg/ml respectively. The ethanol production by the transformed *S. cerevesiae* from 20g/l xylose and 20g/l glucose was found to be 0.00794±0.0002603 and 0.3524±0.0010 respectively. In the glucose containing medium transformed *S. cerevesiae* produced slightly higher ethanol yield but there is the production of low amount of ethanol from xylose by both transformed and nontransformed yeast. The *GRE3* gene product which is capable of convert xylose to xylitol i.e, xylose reduction. *S. cerevisiae* also harbours a gene encoding a xylitol dehydrogenase, which converts xylitol to xylulose.(Richard *et al*.,1999). So that *S. cerevisiae* has a potential initial xylose pathway. However, native *S. cerevisiae* utilizes xylose very slowly probably as a result of too low activities of the enzymes. Xylose utilization at overexpression of endogeneous *GRE3* and *XYL2* (YLR070c) genes has been done for ethanol production(Toivari *et al*., 2002).The strain overproducing endogenous enzymes produced more xylitol than the strain overproducing the *P. stipitis* enzymes, which agrees with *GRE3* being strictly NADPH- dependent. Xylitol was formed from xylose in a reference *S. cerevisiae* strain CEN.PK113-7A, a deletion of the *GRE3* gene led to decreased xylitol formation, showing that Gre3 gene contributed to xylose reduction to xylitol. By overexpressing *XYL2 (XDH)* and *XKS1*, the xylose consumption increased, xylitol formation decreased below detection level and ethanol was formed from xylose. Which demonstrates that the gene products of *XYL2* and *XKS1* limit xylose utilization in wild-type *S. cerevisiae*, but also that a very low XR/XDH activity ratio (0.003 ±0.01) results in low xylitol formation(Bjerre et al 2004). The xylose reductase activity was found to be 0.006±0.001 U/mg of protein, whereas the xylose dehydrogenase activities was not detected. So the fermetation of the xylose was limited by the production of the xylitol in large amount. Whereas only limited amount of xylitol was converted by the endogeneous XDH to the xylulose and endogeneous xylulokinase has very low activity to funnel xylulose to the PPP, (Karuhama *et al*,2005). Enzyme xylulose reductase/ xylitol dehydrogenase converts the xylitol to the xylulose as follows:

$$\text{Xylitol} + \text{NAD}^{+} \longrightarrow \text{D-xylulose} + \text{NADH}.$$

which was a reversible reaction. The Km value of the xylulose reductase for D xylulose is 1.1mM whereas Km value for the xylitol is 25mM. So reaction favors the formation of high concentration of xylitol. Only low concentration of xylitol funnel through the PPP. (Richard *et al*,1999,www.brendaenzymes.org/literature/lit.ph4?e=1.1.1.9&r=246428) which may limit the production of ethanol. Production of ethanol from the pentoses is by far less efficient in terms of specific productivity as compared to the fermentation of glucose. There is clearly not a single limiting step in pentose fermentation by *S. cerevisiae* and therefore, strain engineering for enhanced flux from substrate to ethanol remains a challenge. Depending on the route explored for conversion of D-xylose and L-arabinose into D-xylulose, maintenance of a balanced ratio for oxidized and reduced forms of NADP$^+$ and NAD$^+$constitutes a fundamental issue of strain physiology during pentose fermentation. Utilization of (mainly) NADPH for reduction when NAD$^+$ is exclusively employed for oxidation results in a poor recycling of redox cofactors in the initial steps of pentose metabolism which in turn leads to a highly unfavourable distribution of fermentation products in which by products like xylitol are formed in excess.(Chu *et al*,2007,Hagedral *et al*,2007). XR catalyses the first step of a pathway that allows certain organism to metabolize xylose. After the reduction of xylose to xylitol by XR, xylitol is re- oxidized to yield xylulose by xylitol dehydrogenase, which is often specific for NAD$^+$. Xylulose can then be phosphorylated and enter general metabolic pathways. An efficient, high- flux pathway should recycle the co-substrate such that there is no net conversion of NADPH into NADH resulting from xylose metabolism. Perturbations in this ratio have been linked to cellular stress and xylitol excretion. (Lunzer *et al* 1998 and Osteegard *et al*, 2000). Depending on the route explored for conversion of D-xylose and L-arabinose into D-xylulose, maintenance of a balanced ratio for oxidized and reduced forms of NADP$^+$and NAD$^+$ constitutes a fundamental issue of strain physiology during pentose fermentation. Utilization of (mainly) NADPH for reduc tion when NAD$^+$ is exclusively employed for oxidation results in a poor recycling of redox cofactors in the initial steps of pentose metabolism which in turn leads to a highly unfavourable distribution of fermentation products in which by-products like xylitol are formed in excess.(Lee *et al* 2007,Hagedral *et al* 2007 and Jefferies W 2006). The different cofactor specificities of XR and XDH disturb the cellular redox cofactor balance during xylose metabolism, particularly under anaerobic conditions. An evolutionarily engineered strain with xylose isomerase from the anaerobic fungus *Piromyces* sp. E2 was shown to have improved anaerobic growth and fermentation on xylose compared with *S. cerevisiae* strains expressing the oxidoreductive pathway.(Kyuper *et al* 2002,Kuyper *et al* 2005). The low capacity of the pentose phosphate pathway (PPP) in *S. cerevesiae* have been identified as limitation of xylose metabolism in the yeast.(Jeffrries 2006,Karuhama *et al* 2005). Anaerobic growth on xylose was suggested to be limited by the rate of ATP production (Sondereger *et al* 2005). The reason behind the low production of the ethanol was may be due to the low capacity of xylulokinase, the enzyme in the xylose pathway (Toivari *et al*,2001).

CONCLUSIONS

The increasing global demand of the fossil fuel has always been an issues of its reserve depletion and also the global warming issues has been rising. The first generation biofuel has been derived from the sugarcane and grains which are the feedstocks for the human and animal. So the need of the second generation biofuel, i.e, produced from the lignocellulosic biomass which mitigate the food scarcity. But the high amount of the pentose sugar present and the lack of an efficient fermentation organism limits its fermentation.

Currently, research efforts of unraveling xylose fermentation in the process of bioethanol production from biomass are mainly focusing on two aspects: 1) genetic modification of glucose-fermenting microbial strains to ferment xylose; and 2) co-culture of glucose-fermenting and xylose-fermenting strains. The genetic modification of the strain was done mainly by the expressing the heterologous gene from the xylose fermenting organism. Which include the xylose reductase, the xylitol dehydrogenase and xylose isomerase for the production of the xylulose which ultimately funnel into the nonoxidative pentose phosphate pathway. The deletion of the endogeneous nonspecific aldose reductase(GRE3) has been done for the remedy of xylitol production and the cofactor imbalance. But the fermentation results was not satisfactory comparing the fermentation from the glucose due to the lacking of the xylose transporter in the wild type *S cerevesiae*.

This research presented some results for the solution of the limitation of the transport of the xylose in the lab strain of *S cerevesiae*. Successful expression of recombinant plasmid through genetic engineering makes it feasible for single strain to uptake of xylose compared to the control strain, there are some drawbacks that hinder application of recombinant strains on bioethanol production at industrial scale, such as pleiotropic effects caused by heterologous gene expression, non-stability in long-term culture, inhibition due to the undesirable co-products. The above drawbacks of recombinant strains may result from a lacking of understanding of cell metabolism on a system level. The drawbacks include the lack of the HXT null mutant for the accurate study of the functional expression of the GXS1 plamsid in *S. cerevesiae*. The expression of the yeast expression plasmid was only limited to the haploid lab strain. Because the transformation of the expression plasmid in the wild diploid strain get lost during successive generation. To overcome this limitation the loxp flanked kanamycin resistance gene was cloned in the integrative vector for the selection during the insertion of the targeted gene of interest in the wild diploid S cerevesiae. Loxp flanking gene can be easily removed from the recombinant *S cerevesiae* by the introduction of the Cre recombinase enzyme leaving the gene in the *S cerevesiae* chromosome.

The increase in the growth rate of transformant *S. cerevesiae* in the xylose containing medium and increase in intracellular transport rate of xylose may help to solve the limitation of transportation of xylose during the fermentation of xylose from lignocellulosic biomass.

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APPENDICES

Appendix 1. Basic information of oligonucleotides

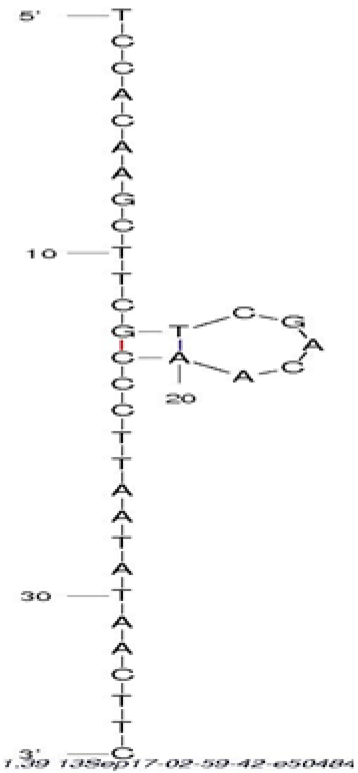
Appendix 1.1: Primers used for loxp-Kan^r amplification

FP'- 5'-: TCCAC ***AAGCTT*** CGTCGACAACCCTTAATATAACTTC (*Hind*III) (YTULOXPF)

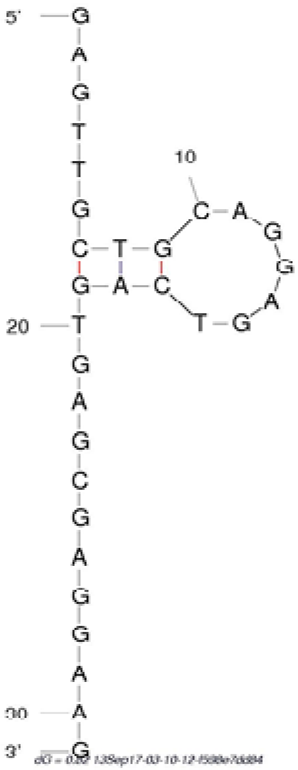
RP- 5': GAGTTG ***CTGCAG*** GAGTCAGTGAGCGAGGAAG (*Pst*I) (YTULOXPR)

S.No	Specifications		Forward Primer	Reverse Primer
Oligo Calculator Results				
1	Annealing sequenc	T_m	62.5	59.5
		G + C	40	58
		length	25	19
2	T_m	Basic	63.3	67
		Salt adjusted	73.3	76.2
		Nearest neighbor	64.05	68.34
3	G + C content		42	58
4	No. of base pairs		36	31
5	Secondary structure			
	T_m		14.2	28.8
	dG		1.39	0.82
	3` end		free	Free
DNA FOLD Results				
6	Secondary structure			
	Tm		24.9	39

	dG	0.71	- 0.20
	3` end	free	Free
<u>Oligo Analyzer Results</u>			
7	Self dimer: with restriction site	-10.23	-10.24
	without restriction site	-9.45	-5.09
8	Hetero dimer	-5.24	
9	Approximate Amplicon size	1730	



Forward primer



Reverse primer

Appendix 1.2: Primers used for *GXS1* gene amplification

Fw- *5′* -GTA C **GAGCTC** AT GGG TTT GGA GGA CAA TAG AAT GGT

Rv- *5′* -GG **TCTAGA** T TAA ACA GAA GCT TCT TCA GAC ATA ATA GC

S.No	Specifications		Forward Primer	Reverse Primer
Oligo Calculator Results				
1	Annealing sequence	T_m	64.6	65.1
		G + C	42	33
		length	26	30
2	T_m	Basic	65.6	65.5
		Salt adjusted	75.4	75.7
		Nearest neighbor	65.37	66.91
3	G + C content		42	41
4	No. of base pairs		36	41
5	Secondary structure			
	T_m		29.1	40.7
	dG		0.58	-0.38
	3\` end		free	Free
DNA FOLD Results				
6	Secondary structure			
	Tm		40.8	51.3
	dG		-0.34	-1.48
	3\` end		free	Free
Oligo Analyzer Results				

7	Self dimer: with restriction site	-9.49	-16.57
	without restriction site	-3.49	-16.57
8	Hetero dimer	-16.34	
9	Approximate Amplicon size	1561 bp	

Appendix 1.3: Primers used for GXS1 internal region amplification

Fw: *5′*-GTT CTT GCT CGT TAC CCT TC

Rv: *5′*- ACA AAG TGC ACC AAA GAA AG

S.No	**Specifications**		**Forward Primer**	**Reverse Primer**
Oligo Calculator Results				
1	Annealing sequence	G + C	50	40
		length	20	20
2	T_m	Basic	51.8	47.7
		Salt adjusted	58.4	54.3
		Nearest neighbor	50.85	49.14
5	Secondary structure			
	T_m		-31	40.7
	dG		2.70	-0.38
	3` end		free	Free
DNA FOLD Results				
6	Secondary structure			

	Tm	-20.6	16.4
	dG	2.19	0.91
	3` end	free	Free
Oligo Analyzer Results			
7	Self dimer	-3.61	-9.73
8	Hetero dimer	-7.06	
9	Approximate Amplicon size	95bp	

Appendix 2 Basic information of plasmids used in the study

Appendix 2.1 pUG6

The length of pUG6 plasmid is 4009 bp. It consists of kanamycin resistance gene flanked by the loxp sites. The size of the loxp-KanMX-loxp marker is 1700bp and resistance to G-418.Amp^r gene is used for the selection marker in *E. coli* for increasing the copy number.

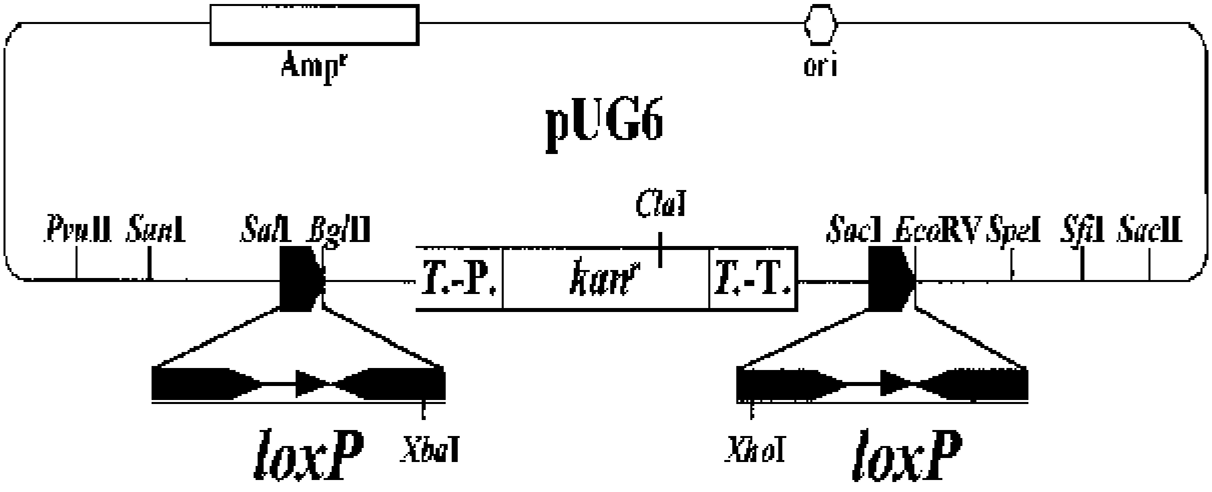


Figure **2.1** pUG6 plasmid containing loxp-KanMX-loxp marker (mips.helmholtz-muenchen.de)

Appendix 2.2 YIplac211

Yeast integrating plasmid is used for the chromosomal integration of the gene in *S cerevesiae*. The length of YIplac211 is 3797 bp. It consists of Amp^r marker for increasing the copy number of plasmid in the *E. coli.*

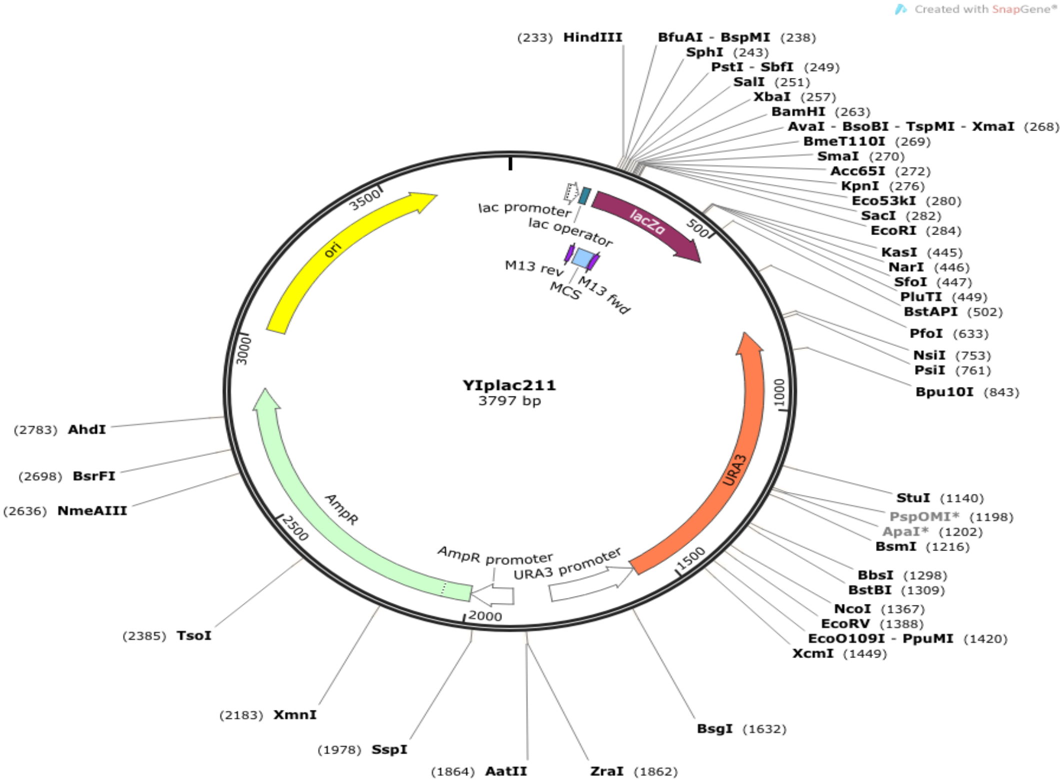


Figure 2.2 Yeast integrating plasmid with URA3 marker (www.snapgene .com)

Appendix 2.3

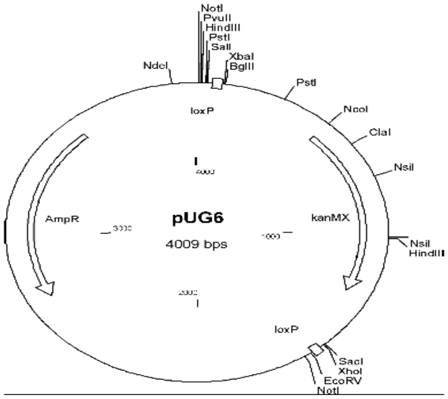


Figure 2.3 Restriction map of pUG6 template vector, Which is used as a template for loxp-Kan^r – loxp amplification.

Appendix 2.4 YEplac195

YEplac195 was used as a vector in pGXS1 plasmid. The HXT7 promoter-GXS1- PGK terminator was coned in the *pst*I and *Sac*I site.

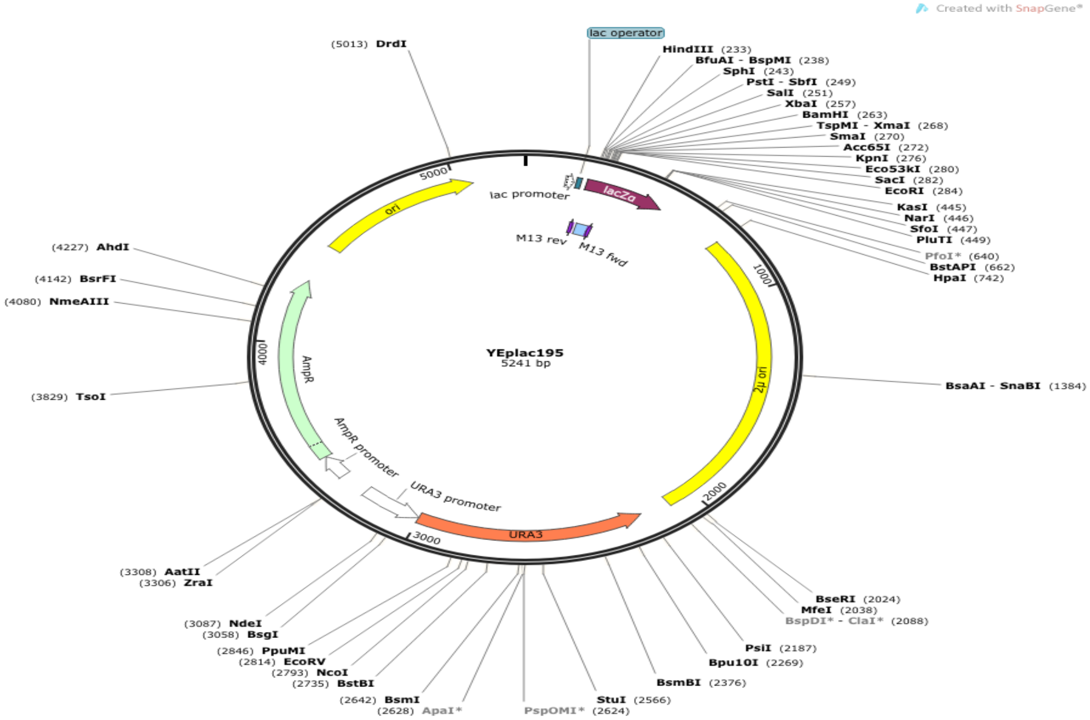


Figure 2.4 Yeast episomal plasmid with URA3 marker

Appendix 2.5

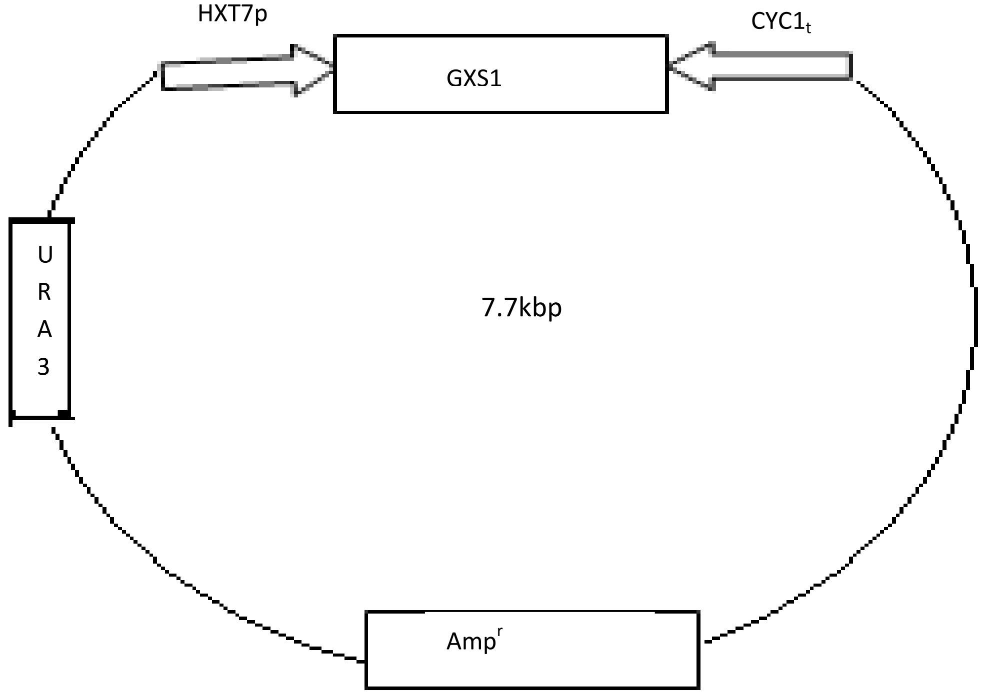


Figure 2.5 GXS1 expression plasmid, HXT7p- Hexose transporter promoter(300bp) GXS1-Glucose/xylose symporter gene(1569 bp), $CYC1_t$ – Cyclin C1 terminator(300bp)

The expression plasmid containing GXS1 gene under the control of HXT7 promoter and CYC1 terminator was used in this study.

Appendix 3: Bioinformatics Tools used for Primer Designing and other purposes

NCBI for genome map of *GXS1* gene from *Candida intermedia,*pUG6 template vector sequence and YIplac211 vector sequence.

http://www.ncbi.nlm.nih.gov/nucleotides

http://www.ncbi.nlm.nih.gov/guide/proteins/

a. Integrated DNA technologies; Oligo analyzer 3.1 for Tm, hetero/homo dimer
http://eu.idtdna.com/analyzer/applications/oligoanalyzer/default.aspx

b. The mfold Web server- DNA folding form for folding structure of primer DNA
http://mfold.rna.albany.edu/?q=mfold/dna-folding-form

c. NCBI- Standard Nucleotide BLAST
http://www.ncbi.nlm.nih.gov/blast/Blast.cgi?PAGE=Nucleotides

d. New Englands Biolabs webtool: NEB cutter for restriction map analysis of amplicon

e. www.uniprot.org

f. www.brenda enzymes.org

Appendix4: Preparation of Stock solutions, Buffers and Reagents commonly used in Molecular Biology

Tris-Cl (1 M, pH 7.5 and 8.0)

Tris-Cl buffer was prepared by adding 12.11 g of Tris base in 80 ml of MilliQ water and pH was adjusted to 7.5 or 8.0 by adding concentrated HCl. Then final volume was maintained to 100 ml. The solution was sterilized by autoclaving and stored at 4^0C.

EDTA (0.5 M, pH 8.0)

18.61 g of disodium EDTA.$2H_20$ was added to 80 ml of MilliQ water, stirred vigorously on magnetic stirrer and pH was adjusted to 8.0 with NaOH. The final volume of the solution was made upto 100 ml and sterilized by autoclaving and stored at 4^0C.

Tris EDTA (TE) buffer (pH 8.0)

10 mM Tris-Cl (pH 8.0)
1 mM EDTA (pH 8.0)

Potassium Phosphate buffer (0.1 M, pH 7.0)

1 M K_2HPO_4	61.5 ml
1 M KH_2PO_4	38.5 ml
MilliQ water	upto 1000 ml

Sodium Acetate (3 M, pH 5.2)

40.83 g of sodium acetate.$3H_20$ was dissolved in 80 ml of MilliQ water and pH was adjusted to 5.2 with glacial acetic acid and final volume was made upto 100 ml. The solution was sterilized by autoclaving and stored at 4^0C.

<u>Preparation of solutions for Plasmid DNA extraction by Alkanline-lysis method</u>

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 4^0C.

Alkaline Lysis Solution II (Freshly prepared)

0.2 N NaOH
1% (w/v) SDS

Alkaline Lysis Solution III

5 M Potassium acetate	60.0 ml
Glacial acetic acid	11.5 ml
H20	28.5 ml

Yeast plasmid isolation

20% SDS – 2gm of SDS was weighed and dissolved in 10ml of water

Preparation of solutions for Agarose gel electrophoresis

10 X TAE Buffer

Tris base	4.84 g
Glacial acetic acid	1.142 ml
0.5 M EDTA (pH 8.0)	2 ml
MilliQ water	upto 100 ml

The working solution (0.5 X) of TAE buffer was prepared by diluting 17.5 ml of 10 X TAE stock solutions with MilliQ water to make the final volume upto 350 ml.

10X Gel loading buffer

Bromophenol blue	10.5 mg
0.5 M EDTA (pH 8.0)	2 ml
98% Glycerol	2.5 ml
MilliQ water	upto 5 ml

Ethidium Bromide (10 mg/ml)

100 mg of ethidium bromide was weighed and dissolved in 10 ml of MilliQ water. The solution was protected from sun light by wrapping with aluminum foil and stored at room temperature.

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Antibiotics

	Stock Solution (-20^0C)	Working solution	
		LB broth	LB/Agar plate
Ampicillin	50 mg/ml in H_20 (filter sterilized)	50 µg/ml	50 µg/ml

Appendix 6: List of media used in study

Luria Bertani (LB) media

2.5 g of LB medium was weighed and dissolved in 100 ml MilliQ water and sterilized by autoclaving. Once the medium is cooled (25^{0}C), appropriate antibiotics were supplemented if required and stored at 4^{0}C.

LB/Agar plate

LB medium	2.5 g
Agar	1.5 g

The volume was adjusted to 100 ml with MilliQ water and sterilized by autoclaving. Once the medium is cooled (60^{0}C), then appropriate antibiotics were added to the desired concentrations (as mentioned above) and the plates were stored at 4^{0}C. pH was adjusted to 7.0, final volume was made upto 1000 ml and autoclaved. Then 10 ml of separately autoclaved 20% glucose and 1 M $MgCl_2$ was added respectively.

YEPD medium

Yeast extract	10 g
Peptone	20 g
Glucose/Xylose	20 g
dH_2O	1000 ml

YNB medium

(10X)

YNB	6.7gm
Ammonium sulphate	5g

The solution was sterilized by syringe filter of 0.2µm membrane.

Yeast plasmid isolation

20% SDS – 2gm of SDS was weighed and dissolved in 10ml of water.

Solution required for southern blot detection

Blocking/Washing Buffer. 1 volume of the concentrated 10X Blocking/Washing Buffer was diluted with 9 volumes of water. The diluted buffer was stored at 4°C.

Blocking Solution. The Blocking Solution was prepared as a 1% (w/v) solution of Blocking Reagent in 1X Blocking/Washing Buffer. The necessary amount of the Blocking Reagent was weighed , and the required volume of the Blocking/Washing Buffer was added , and the suspension was stirred on a magnetic stirrer until the Blocking Reagent completely dissolved at 60°C with shaking. The blocking solution was stored in aliquots at -20°C.

Dilution of the Streptavidin-AP Conjugate. The concentrated Streptavidin-AP

Conjugate was dilute 5000-fold in Blocking Solution. Prepare sufficient solution to allow the membrane to float freely in a tray. The diluted solution should be prepared freshly.

Detection Buffer. One volume of the concentrated 10X Detection Buffer was diluted with 9 volumes of water. The diluted buffer was stored at 4 C .

Substrate Solution. 1 volume of 50X BCIP/NBT Solution was diluted with 49 volumes of 1X Detection Buffer. The Substrate Solution should be prepared freshly.