ENHANCEMENT OF XYLOSE TRANSPORT IN *SACCHAROMYCES CEREVISIAE* BY TRANSFORMATION OF GXF1, A XYLOSE TRANSPORTER GENE, FROM *CANDIDA INTERMEDIA*

M. Sc. Thesis

2014

Submitted to

CENTRAL DEPARTMENT OF BIOTECHNOLOGY

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OUTLINE

List of Abbreviations

µg	microgram
µl	microliter
µM	micromolar
ADW	Autoclaved deionized water
ATP	Adenosine Tri phosphate
BLAST	Basic local alignment search tool
bp	Basepair
CCR	Carbon catabolite repression
DNA	Deoxy ribonucleic acid
dNTPs	Deooxynucleotide triphosphate
EDTA	Ethylene Diammine Tetraacetic acid
EtBr	Ethidium Bromide
g	Relative centrifugal force
g	Gram
hr	Hour
IDT	Integrated DNA Technologies
Kb	kilo base pairs
L	litres
LB	Luria Bertani
m	Milli
M	Molar
min	Minute
mM	Millimolar
N	Normality
NADPH	Nicotinamide Adenosine Diphosphate
NCBI	National center for Biotechnology Information
NEB	New England's Biolabs

ng	Nanogram
nm	Nanometer
OD_{600}	Optical density at 600nm
ORF	Open reading frame
PPP	Pentose Phosphate Pathway
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Revolutions per minute
sec	Second
TAE	Tris- Acetate EDTA
TCA	Tri- Carboxylic Acid
TE	Tris EDTA
T_m	Melting Temperature
Tris	Tris- (hydroxymethyl) – aminomethane
U	Units
UV	Ultraviolet
w/v	Weight per volume
YEPD	Yeast Extract Peptone Dextrose

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Abstract

Rising of energy costs and the increased awareness of global warming have inspired production of renewable, biomass-derived chemicals and fuels. Plant biomass is potentially an inexhaustible source of bioenergy. To produce the fuel for the motor vehichles, industrial bioethanol production from cheap renewable lignocellulosic substrates has been regarded as an important attempt. However, efficient production of bioethanol is attributed to the ability of microbial cell to utilize the abundant glucose and pentose sugar present in lignocellulosic biomass. Because of the lack of xylose transporter in wild *S. cerevisiae*, they are unable to utilize xylose sugar which is the second most abundant sugar in lignocellulosic biomass. Several successful researches have been performed in *Sachharomyces cerevisiae* to induce efficient xylose transport, and xylose fermentation. One of the limiting steps in xylose fermentation is the xylose transportation step. Different xylose transporter encoding gene are naturally occurring in several species, the integration of those xylose transporting genes in *Sachharomyces cerevisiae* would led to the transportation of xylose inside the cells. GXF1 is the glucose/xylose faciliator occurring in fungus *Candida intermedia* which transports the xylose by facilitated diffusion. The yeast cloned with GXF1 xylose transporter, in the present study, has been shown for improved xylose growth kinetics (specific growth rate, $\mu = 0.02\ hr^{-1}$ and xylose transporting phenotypes compared to the control cells. However, very less amount of ethanol production 0.09mg/ml is found which might be due to lack of xylose metabolizing pathways in wild yeasts.

The aim of this resarch is the heterologous expression of GXF1 in *Sachharomyces cerevisiae* for efficient xylose transport inside the cell for the production of bioethanol. Good xylose transport kinetics has been shown by the expression of GXF1, however, ethanol production is quite impossible by just expression of transporter gene, as yeast lacks xylose metabolizing pathways too. Hence further improvement of the strain with the xylose metabolizing pathways is believed to uplift the production of bioethanol from cheap lignocellulosic substrates.

Keywords: Transformation, *Sachharomyces cerevisiae*, *Candida intermedia,* GXF1, xylose transporter, Bioethanol.

1.INTRODUCTION

1.1) Background

Due to the depletion of the petroleum refineries and the rising atmospheric pollution, and increasing CO accumulation in atmosphere with the use of fossil fuels, ethanol has been considered as the most important petroleum alternative. Ethanol can be produced from substrate like lignocellulosic biomass as well as agricultural, industrial and urban waste which are renewable, economic and less expensive for production (Prasad *et al.,* 2007). Shapouri (Shapouri *et al.,* 1995 and Shapouri *et al.,* 2002) concluded that the energy content of ethanol is higher than the energy required for producing ethanol. The production of bioethanol will be commercially favourable when the cost of pretreatment can be reduced along with the improvement of microorganisms used for fermentation of sugar (Gray *et al.,* 2006).

Bioethanol production relies upon plant sources containing carbohydrate as a main component which can be easily fermented to ethanol by use of microorganisms (Naik *et al.,* 2010). Plant sources like oil seed potatoes, corn and other valuable food crops containing high amount of starch with glucose as a monomer when used are easily fermented and gives high production of ethanol (de Vries *et al.,* 2010). But these food crops led to the food crisis and social disturbance, food starvation in the world (Gray *et al.,* 2006; Bacovsky *et al.,* 2010). Hence production of ethanol should be prompted with such biomass which is less valuable, less expensive, renewable and easily available, such as lignocellulosic biomass, microalgal oils which led the fuel alternative bioethanol (Schenk *et al.,* 2008; Mata *et al*., 2010). Lignocellulosic biomass could produce ethanol up to 442 GL year^{-1} which is about 16 times higher than the current world ethanol production. Potential bioethanol production can replace 353 GL of gasoline (32% of global gasoline consumption) when bioethanol used in E85 fuel for a midsize passenger vehicle. Asia being the largest producer of bioethanol from lignocelluloses can produce 291 GL year^{-1} of bioethanol (kim *et al.,* 2004).

Use of lignocellulosic biomass although taken as a good alternative in area of bioethanol production, but its complex structural composition makes it difficult to ferment the available sugars (Moiser *et al.,* 2005). Lignocellulosic biomass is composed of cellulose, hemicelluloses, and lignin where lignin seals the hemicelluloses, making hemicelluloses sugar locked, and hence the removal of lignin being essential (Galbe and Zacchi 2007) and is performed with several pretreatment process (Mc Millan 1994) which eases the release of cellulose and hemicelluloses sugar present in lignocellulosic biomass.

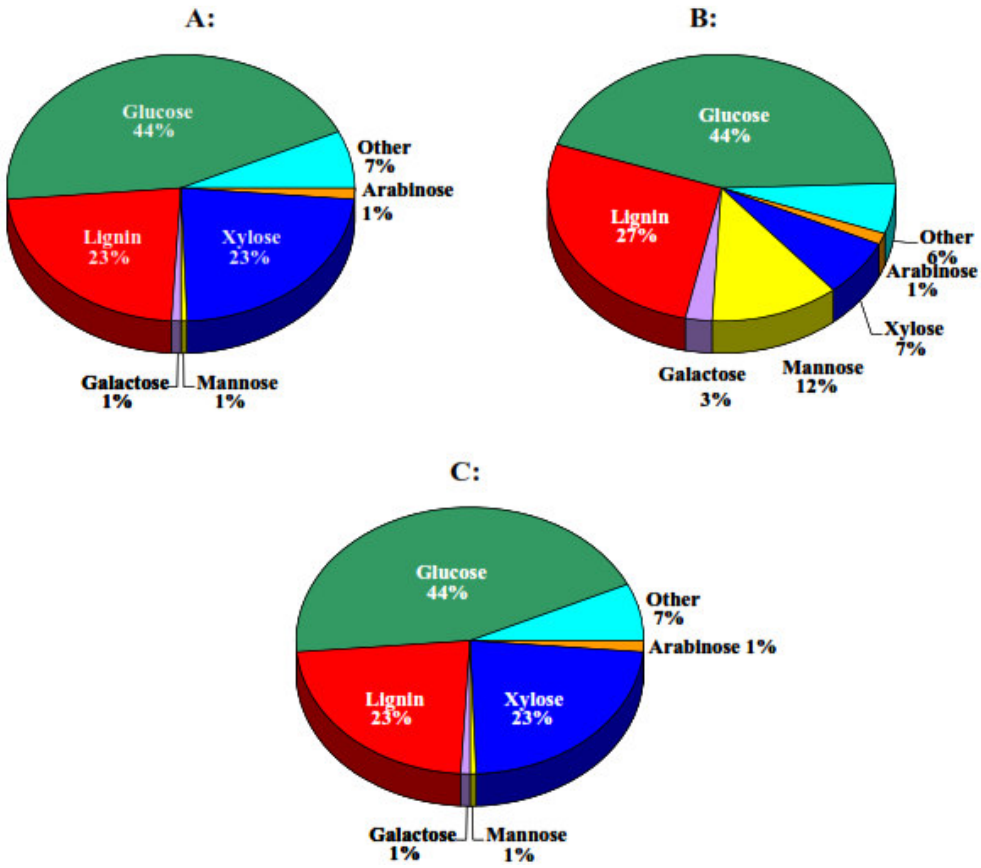


Figure1 Schematic representation of the dry weight composition of (A) Hardwood, (B) Softwood and (C) bagasse (Puls and Shuseil, 1993).

Fermentation of sugar using yeast is preferred because of their good fermentating capacity as, larger sizes, thicker cell walls, better growth at low pH, less stringent nutritional requirements, and greater resistance to contamination (Jeffries, 2006). Bioethanol production from the lignocellulosic biomass refers to the fementation of sugar molecules to from ethanol with the use of microorganisms. Hexose such as glucose are easily taken up by the *Saccharomyces cerevisiae* through the 18 proteins (HXT1- 17, Gal2), and three maltose transporters (Wieczorke *et al.,* 1999). The problem of using *S. cerevisiae* has been studied for its inability to ferment pentose sugars (Subtil and Boles 2011). Some of the hexose transporting protein Hxt4, Hxt5, Hxt7 and Gal2 has been discovered for its xylose transporting properties (Hamacher, *et al.,* 2002) but the over expression of hexose transporters neither increased growth on xylose nor enhanced xylose fermentation thus limiting the role of hexose transporter for transportation of xylose (Hamacher, *et al.,* 2002). High level of glucose in the substrate also hinders the xylose transport explained by CCR, hence lignocellulosic biomass containing high amount of glucose will inhibit the xylose transportation by *S. cerevisiae*

which results only glucose utilization (Gancedo, 1998). Because the transportion of D-xylose is the limiting step in the D- xylose utilization by yeast (Eliasson *et al.,* 2000a) and, the pentose being the abundant fraction of the hemicellulosic content of lignocellulosic biomass, the engineering approaches of the *S. cerevisiae* for the utilization of both hexose and pentose sugar will be the most effective approach for eminent production of bioethanol.

Saccharomyces cerevisiae is unable to metabolize the five carbon xylose sugar contained as a higher fraction in lignocelluloses biomass (Barnett 1976; Saha 2003) which might be assumed due to the absence of xylose transporters and xylose metabolizing pathways inside. The heterologous expression of xylose transporter genes and xylose metabolizing genes is a technique for making *Saccharomyces cerevisiae* able to use up xylose sugar along with hexose sugar for the production of bioethanol. *Candida intermedia* is a good source of xylose transporting genes, GXF1 and GXS1 (Leandro *et al.,* 2006). GXF1 transports xylose along with the glucose sugar by facilitated diffusion mechanism. The expression of GXF1 in *Saccharomyces cerevisiae* is a good approach for making *Saccharomyces cerevisiae* as a good D- xylose transporting yeast(Leandro *et al.,* 2006) . After being transported inside, the baker's yeast cannot utilize D-xylose sugar as a carbon source. Although being unable to utilize the D-xylose sugars, bakers yeast can metabolize xylulose, the ketoisomer of xylose (Wang and Schneider 1980). Introduction of a xylose isomerization step from a xylose utilizing microorganism is done by heterologous expression of xylose isomerase encoding gene which converts D-xylose to D-xylulose (kuyper *et al.,* 2005) which then goes to PPP for metabolism. The expression of XR (xylose reductase) and XDH (xylitol dehydrogenase) is found to be another approach for oxidoreductase conversion of D-xylose to D-xylulose which is then entered to PPP metabolism (Kotter and Ciriacy 1993).

Thus, the major approach relies upon the cloning of xylose transporter encoding gene in *S. cerevisiae* for the utilization of pentose sugars. Several pentose fermenting microorganisms have been isolated which are naturally pentose fermenters, *Candida shehatae*, *Pachysolen tannophilus* and *Pichia stipitis* are the D- xylose utilizing yeasts which have capacity to produce ethanol microaerobically using D-xylose as a substrate (Jeffries and Jin, 2000). *P. stipitis* and *C. shehatae* are described as the best D-xylose fermenting yeasts (Jeppsson *et al.,* 1999). Several transporters have been isolated from several species such as *A. thaliana* (Hector *et al.,* 2008), *C. intermedia* (Leandro *et al.,* 2006), *S. cerevisiae* (Saloheimo *et al.,* 2007 and, Hamacher *et al.,* 2002), *Trichoderma reesei*(Saloheimo *et al.,* 2007) for the D- xylose transportation. The expression of the xylose transporter encoding gene in the favourable host microorganism would possibly result the development of D-xylose transporting yeast strain with high performance.

Thus, metabolic engineering of the *S. cerevisiae* through the heterologous expression of the gene encoding xylose transporter can be an advent for the production of bioethanol from lignocellulosic biomass using *S. cerevisiae* as fermenting microorganism.

1.2) Current Studies

Expression of several heterologous D- xylose transporters have been performed with *S. cerevisiae*. Two xylose transporter from fungus *Candida intermedia* , one high affinity D-xylose/D-glucose-5 symporter (GXS1) and the other low affinity D-xylose/D-glucose facilitator(GXF1), was characterized for their improved growth phenotypes (Leandro et al., 2006) and xylose transporting properties with Km 0.2mM and 50mM respectively (Gardonyi *et al.,* 2003). Resarches reveal GXF1 overexpression shown to improve fermentation performance 2 times in recombinant D- xylose utilizing *S. cerevisiae* compared to the control strain (Runquist, *et al.,* 2009). *Candida intermedia* GXS1 when expressed along with *Scheffersomyces stipitis* XUT3, directed evolution showed 70% increased growth rate and enhanced simultaneous sugar utilization with glucose and xylose mixtures in *Saccharomyces cerevisiae* (Young *et al.,* 2012). Comparative analysis of three xylose transporters Gxf1, Sut1 and At5g5920 when expressed in *S .cerevisiae*, the best xylose transport phenotype was shown by GXF1 , while SUT1 as least xylose transporter, Km of GXF1, At5g5920 and SUT1 being 166mM ,148mM , 96mM respectively (Runquist, *et al.,* 2010). SUT1 has been studied for its enhanced xylose uptake and ethanol production phenotype when over expressed in xylose assimilating *S. cerevisiae* (Katahira *et al.,* 2008). In recombinant D-xylose utilizing *S. cerevisiae*, two *Arabidopsis thaliana* xylose transporters were studied which showed xylose uptake rate and xylose consumption rate up to 46% and 40% respectively and xylose consumption rates increased by 2.5 fold compared to the control strain. (Hector, R.E *et al.,* 2008). Successful heterologous expression of xylose transporter gene xylT was also observed in *L. lactis* (Nyyssola *et al.,* 2005) and *L. plantarum* (chaillou *et al.,* 1998) for enhanced xylose transport kinetics. Apart from the typical xylose transporters, ATP independent xylose uptake has been discovered by overexpression of galactitol transporter (GatC) in an engineered *E coli* (Nduko *et al.,* 2013). In *E. coli* the role of the mutated and adaptively evolved GatC for production of D- lactate through the increased xylose transportation is yet another approach on discovery of xylose transporter (Utrilla *et al.,* 2012). A new D-xylose transporter encoding gene Trxlt1 from *Trichoderma reesei* have been discovered to be evolved from adaptive evolution able to grow on xylose (Saloheimo *et al.,* 2007).

1.3) Hypothesis

The hypothesis of current study is the heterologous expression of xylose transporter GXF1 of *Candida intermedia* in *S. cerevisiae* for the enhancement of xylose transport. Transformation of GXF1 in wild *Saccharomyces cerevisiae* is presumed for the enhanced pentose transporting phenotype that metabolize D-xylose along with the hexoses .In this way metabolization of majority of sugar present in lignocellulosic biomass will be possible and can enhance the production of bioethanol from lignocellulosic biomass. The research therefore aims at producing recombinant *S. cerevisiae* capable of enhanced pentose transportation characteristics.

1.4) Objective

1.4.1) General objective

1) Cloning of GXF1 gene from fungus *Candida intermedia* in yeast *Saccharomyces cerevisiae*
2) Functional expression analysis of Xylose transporter GXF1 in *Saccharomyces cerevisiae*

1.4.2) Specific objectives

1) Transformation of GXF1 of *Candida intermedia* in *Ecoli* DH5α
2) Transformation of GXF1 in yeast *Saccharomyces cerevisiae*
3) Test of transformants by Southern blot of GXF1 cloned plasmid DNA using biotin labeled probe
4) Test of recombinant *Saccharomyces cerevisiae* for xylose transport and utilization alone and with glucose.
5) Test for ethanol production by recombinant *Saccharomyces cerevisiae.*

1.5) Significance

The main aim of this research is to perform cloning of D-xylose transporter in yeast and evaluating the transporting efficiency of pentose sugar by the recombinant yeast. Lignocellulosic biomass is composed of cellulose, hemicelluloses and lignin (Glazer & Nikaido, 2007) where cellulose is composed of glucose monomers, hemicelluloses are

composed of hexose and pentose sugar in a greater fraction, and lignin is the complex structural moiety which locks the hemicelluloses fraction. The complexity of lignin can be relieved by several pretreatment processes, such as acid pretreatment, alkaline pretreatment, ionic liquid pretreatment and other physical methods too (Mc Millan 1994). For the sugar utilization by microorganisms, transportation of sugars is highlighted for being the rate limiting step (Hector, *et al.,* 2008). Two types of sugar molecules are the main constituent of lignocellulosic biomass, hexose sugar and pentose sugars. Transportation of hexose sugar is performed by the different types of hexose transporters present in the wild *S .cerevisiae* (Wieczorke *et al.,* 1999). But, unfortunately wild *S. cerevisiae* lacks the pentose transporter. Pentose sugars are transported in very low amount through hexose transporters and the pentose transport is repressed in presence of glucose (Belinchon and Gancedo 2003). Hence, transportation of pentose being a major concern, the main objective of current research is to engineer the yeast that can transport pentose sugar. Different sugar transport proteins which acts as xylose transporter from *A. thaliana* (Hector *et al.,* 2008)*, C. intermedia* (Leandro *et al.,* 2006)*, S. cerevisiae* (Saloheimo *et al.,* 2007 and, Hamacher et al., 2002), *Trichoderma reesei* (Saloheimo *et al.,* 2007) has been studied for their D–xylose transportation properties. Hence, the heterologous expression of the D- xylose transporting gene in yeast has been taken as greatest attempt for the pentose transportation in wild baker's yeast. *Candida intermedia* encoding two D- xylose transporters GXF1 and GXS1, has been a regarded as a best D- xylose transporting fungus and thus used in this research. GXF1, which is a D-glucose/xylose facilitator having capacity to transport D-xylose by facilitated diffusion mechanism. In the present research work cloning of the heterologous xylose transporter GXF1 will be performed in yeast *S. cerevisiae* and the recombinant yeast will be studied for their efficiency in D- xylose transport and utilization of pentose for the production of ethanol.

This research work aims to promote the utilization of second generation biomass, especially lignocellulosic biomass, and the co utilization of pentose sugar along with hexoses for bioethanol production. The current study will also unveil the functional characterization of GXF1 gene and reveal its efficiency as xylose transporter. The functional expression of *C. intermedia* gene on *S .cerevisiae* strain will also help to widen the compatibility of the gene on baker's yeast too. Comparative analysis of D-xylose transport kinetics and the ethanol production by the wild yeast and recombinant pentose transporting yeast will help to screen out the scope of the researches in the field of metabolic engineering through the heterologous expression of gene in *S. cerevisiae* for bioethanol production.

Furthermore, this resarch has a tremendous scope not only for utilization of unused, cheap, unvaluable lignocellulosic substrates but also the production of bioethanol from the agro industrial wastes and several pentose rich substrates which would make the production of bioethanol economic and sustainable. It also aims to compensate the ever rising demand of the energy all over the world.

2. LITERATURE REVIEW

2.1) Ethanol from lignocelluloses

2.1.1) Bioethanol: a renewable alternative to fossil fuel.

Recent increasing problem of atmospheric pollution is due to the accentuated growth of world's consumption of energy originated from the fossil fuels and thus released gases are the main cause of green house effect (Wang *et al*., 2012). Also the rising cost of petroleum and global warming are provoking the suggestion for alternative and renewable low cost fuel (Ragauskas *et al*., 2006). Oil prices have been rising around the world in 1970s and as the world supply is finite, hence many researches has been attempted to produce ethanol in an economic way and aimed for the use of renewable resources (Lynd *et al.,* 1991). In order to solve the problem of rising cost of petroleum and the depletion of these non renewable resources, the obtaining of fuels from renewable sources, such as lignocellulosic biomass, has been a keen interest in the last years, and currently it has been believed ethanol to be the main form of bioenergy and is taken as a best alternative to the use of fossil fuel (Wang *et al.,* 2011). Bioethanol has several advantages such as it burns clearly and it minimizes air pollution by lowering the amounts of ozone precursors and it is also less toxic than methanol (gasoline) (Wyman *et al*., 1990). Reports also shows that 700 million light duty vehicles, automobiles, light trucks, SUVs and minivans , on roadways are around the world and are thought to increase to 1.3 billion by 2030, and to over 2 billion vehicles by 2050, most of increase are to be categorized in developing countries (Hansen, 2004), hence the production of a sustainable, renewable and high efficient fuel energy is the major concern for the present scenario.

2.1.2) Bioethanol: a sustainable fuel

Bioethanol had been a best target to solve the rising problem of petroleum because the wastes can be manipulated and the use of biomass will maintain a balance for emission of $C0_2$ by motor vehichles. Earth surface receives 2.5×10^{21} Btu/year amount of solar energy which is far more than that the human utilization i.e, 2.0×10^{17} Btu/year, and thus the plant stores the solar energy as carbon via photosynthesis which is 10 times of the world usage (lynd *et al*., 2003). Because, the depletion of petroleum refiniries will increase energy demand there is a need of lignocelluloses derived ethanol for future fuel named as carbohydrate based economy (Gnansounou 2009). Lignocellulosic residue comprises lignin rich fermentation residue which is the coproduct of bioethanol and can generate both 458 terra-watt-hours (TWh) of electricity (about 3.6% of world electricity production) and 2.6 EJ of steam (Kim and Dale ,2004). The use of plant biomass for bioethanol is sustainable because it give a proper cycle mechanism as the plant will be used for biofuel and Co_2 emitted by vehichles will be used by the plant for photosynthesis.

2.1.3) Bioethanol from biomass

Utilization of lignocellulosic biomass such as agricultural waste, wood, or energy crops, to fuel ethanol provides renewable means to meet future fuel demand which do not depend upon edible commodities, and simultaneously reduce the green house gases emission (Perlack et al., 2005). Biofuel are economical because it can be produced anywhere in the world and it can be produced from the ranges of biomass from household wastes to industrial wastes. Brazil produces ethanol from sugarcane, Sweden, The UK , and Austria uses wood , Canada uses corn and wheat , US uses corn kernels and corn stovers (Herrera, 2006). Bioethanol can be produced from the carbohydrate based plant product which are first generation and second generation plants. Oil palm (South East Asia), sugarcane (Brazil) and sweet sorghum (china) appears to be most sustainable for bioethanol production determined from several sustainable determining factors (de Vries *et al.,* 2010) in contrary to which researches have shown probable unsustainability of the use of first generation crops because of the possible impact and its competition with food (Naik *et al.,* 2010).

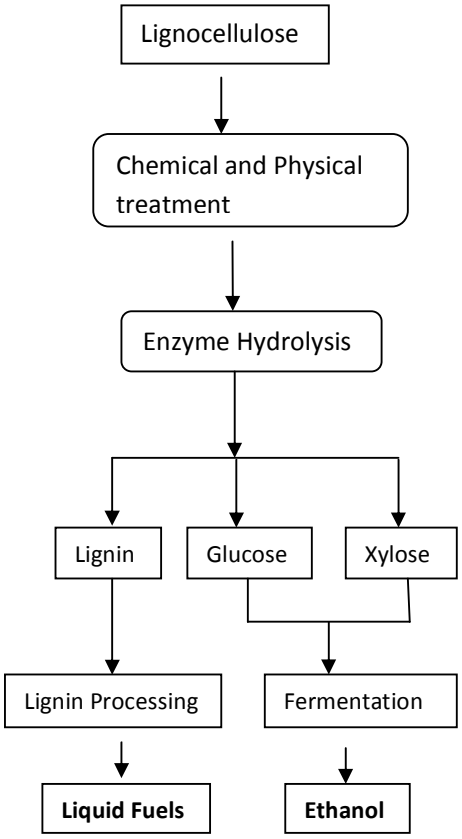


Figure2: Lignnocellulosic biomass for production of Liquid fuels and Ethanol (Aristidou and Penttila 2000)**.**

2.1.4) First generation and second generation biofuel

Biofuels are categorized into first generation and second generation , the former biofuels are produced form edible crops such as oil seeds and several valuable crops but it is limited to compensate the energy demand of all over the world (Schenk *et al.,* 2008; Mata *et al.,* 2010). First generation crops play an important role in animal and human food chains, and use for biofuel may rise the prices of the foodstock resulting in social disturbance all over the world (Gonzalez-Garcia *et al.,* 2009; Mussatto *et al.,* 2010; Xavier *et al.,* 2010). These resources are limited for biofuel because of the need of reassignation of arable lands from food to fuel which directly leads to food crisis (Gray *et al.,* 2006; Bacovsky *et al.,* 2010). Production of ethanol dependent on cane sugar (sucrose) and/or starchy grains and tubers (corn , potatoes) are first generation process of production which is not effective for long term use as it needs high amount of cultivable land , and its use for biofuel may increase the food prices and may lead to food crisis (Mitchell, 2008).

Hence, it is essential to look up another alternative resources for production of bioethanol which lead to attention towards second generation biofuels which are produced from the other unused feedstocks, which is not highly valuable (Schenk *et al.,* 2008; Mata *et al*., 2010). Additional research in this area is needed in concern to search for non food crops like agricultural / industrial wastes as raw material for bioethanol production. European Union strongly encourages research on focusing the biotechnological solutions for energy and chemical demands from renewable sources such as, lignoocellulosic biomass, forestry wastes, agricultural biomass residues and food industrial wastes which is "second generation" biofuels. In biofuel production, the term plant biomass refers to the lignocellulosic material as it is the major constituent of the cheap, abundant renewable and economic non food materials available from plants (Gomez *et al.,* 2008; Zabaniotou *et al.,* 2008).

2.1.5) Chemical composition of plant biomass

Lignocelluloses material is composed of cellulose, hemicelluloses and lignin (Jeffries and Jin, 2000) whose composition greatly varies between species (Jeffries and Jin, 2000; Aristidou and Penttila, 2000). Crop based materials containing high amounts of glucose can produce good amounts of biofuel but it may lead to food crisis, hence the use of crop which are regarded as wastes because of their structural complexity can be a good constituent for production of ethanol as second generation biofuel (Peters 2006). Lignocellulosic biomass consists of 45% of cellulose, 30% of hemicellulose and 25% of lignin (Glazer & Nikaido, 2007). Cellulose is composed highly of glucose and it contributes about half of plant biomass (Peters 2006). Hemicelluloses is composed of

various polymer among which xylan polymer which consists of D-xylose backbone joined by β-1,4 bonds, and has a variety of side chains including acetyl groups.

Table 1: Lignocellulose biomass composition (% dry mass) (Hamelinck *et al*., 2005)

Feedstock	Hardwood			Softwood	Grass
	Black locust	Hybrid poplar	Eucalyptus	Pine	Switchgrass
Glucan 6C	41.61	44.7	49.5	44.55	31.98
Galactan 6C	0.93	0.97	0.76	2.56	0.95
Xylan 5C	13.86	14.56	10.73	6.30	21.09
Arabinan 5C	0.94	0.82	0.31	1.6	2.84

2.1.6) Pretreatment of the lignocellulosic biomass for release of fermentable sugars

Production of bioethanol from the second generation is a quite difficult task because of the need of hydrolysis of hemicelluloses and cellulose to monomer sugars. The complex physio- chemical structure does not allow the easy fermentation of the lignocellulosic biomass hence different means of pretreatment led to the change in physical structure and alter the complex chemical composition and hence makes the lignocellulosic biomass to be fermented by microorganism with a high efficiency (Moiser *et al.,* 2005). The most stringent seals around cellulose is the lignin – hemicelluloses –pectin complex which forms a barrier for hydrolysis of cellulose – hemicelluloses matrix and its removal is performed by several process [Taherzadeh and Karimi (2007) ,Xu *et.al* (2009); Li and Kim (2011); (Galbe and Zacchi 2007)] to release the fementable sugar of cellulose – hemicelluloses complex sealed by lignin moiety. Different pretreatment process developed includes mechanical comminution, alkali swelling, acid hydrolysis, steam and other fiber explosion techniques, and exposure to supercritical fluids which are destined to render carbohydrate components of lignocellulosic materials to enzyme hydrolysis and microbial conversion (Mc Millan 1994), and hence making the more fermentable sugar available and thus converted to ethanol.

2.1.7) Complete utilization of lignocellulosic biomass by microorganisms, a major challenge

Due to the heterogenecity of lignocellulosic components, lignocellulosic biomass fermentation is not as easy as glucose or beer wort fermentation, and hence a specialized fermentative microorganism is required (Zaldivar *et al.,* 2001). The microorganism must be resistant to the toxins of lignocellulosic hydrolysate, should ferment both pentoses and hexoses without catabolite repression, and should produce high amount of ethanol making this industrially feasible. But unfortunately there is absence of naturally occurring microorganism with all those specifications.

2.1.8) Economics of bioethanol

According to Goldemberg (Goldemberg 2008), motor vehicles account for more than 70% of global carbon monoxide (CO) emissions and 19% of global carbon dioxide (CO_2) emissions. CO_2 emissions from a gallon of gasoline are about 8 kg. Development of modern technologies and rapid economic growth has drastically altered the living style of many people since few decades. And, large quantities of non renewable natural resources, especially fossil fuels are consumed in a very high rate to meet the rising demand of energy which results in the release of large amounts of green house gases in atmosphere every day, also the limited supply of crude oil is the cause of energy supply crisis (Hahn-Hagerdal, *et al.,* 2006).

2.1.9) Bioethanol: a practical alternative

In terms of market value (Caesar, 2008), and in terms of volume (Hong and Nielsen, 2012), ethanol is the largest product in industrial biotechnology. “Clean Technologies” has been recently a rising concern for the clean environment and the production of chemicals which is a good alternative as it is economic and requires less energy for the production of commodities of importance to chemical, energy and food industries and reduces pollutants in industrial effluents(Sanchez, 2009).

2.1.10) Problem of using lignocelluloses for bioethanol production

Lignocellulosic biomas is composed of: cellulose ,hemicellulose , and lignin (Glazer and Nikaido 2007). After cellulose and hemicelluloses, lignin is the most abundant polymer present composed of three phenylpropane units (p- coumaryl, coniferyl and sinapyl alcohol) that are held together by different linkages which are water insoluble and degradation being very tough (Fengel and Wegener, 1984). Hemicelluloses links with cellulose microfibrils, and forms a network to provide structural backbone to plant cell wall, this structure impedes bioethanol production by weakening the access to the

available carbohydrates. Hence, it is very essential to destroy the the complexity of polymer structure of the lignocelluloses. For this several pretreatment process is developed , they helps on the reduction of biomass particles, preserves the pentose (hemicellulosic) fractions and avoids the formation of by products which would otherwise inhibit growth of fermentable microorganism, minimizes energy demands and limits cost (National research council 1999).

2.2) Metabolism of D- Xylose by yeast

2.2.1) Natural D-Xylose fermenting yeasts

Several naturally D-xylose utilizing bacteria and yeasts have been isolated (Jeffries, 1983). Inspite of the ability to ferment xylose, microorganisms have many limitations as sensitivity to compounds in the lignocellulosic hydrolysate, lack of manipulating genetic systems, and conferring special requirements for ethanol production (Aristidou and Penttila, 2000). There are different types of yeasts isolated which can grow on D-xylose , but only some are able to catalyze conversion to ethanol anaerobically (Jeffries, 1985; Kuyper *et al.,* 2004). Three most studied D-Xylose utilizing yeasts *Candida shehatae*, *Pachysolen tannophilus* and *Pichia stipitis* which are able to produce ethanol microaerobically (Jeffries and Jin, 2000). Best D-Xylose fermenting yeasts are *P. stipitis* and *C. shehatae* yet described (Jeppsson *et al.,* 1999). *P. stipitis* is able to ferment all the sugars found in wood including D-Xylose by D-Xylose utilizing pathway which consists of several enzymes that are repressed by glucose and it requires D-Xylose for induction (Ho *et al.,* 1998). There are several naturally occuring D- xylose transporting proteins and have been isolated for their good D- xylose transportation phenotype as from A*. thaliana* (Hector *et al.,* 2008)*, C. intermedia* (Leandro *et al.,* 2006)*, S. cerevisiae*(Saloheimo *et al.,* 2007 and, Hamacher *et al.,* 2002), *Trichoderma reesei* (Saloheimo *et al.,* 2007). Active high affinity transport activity was shown by efficient pentose fermentor fungus *Candida succiphila* grown on xylose. Yeast Kluyveromyces *marxianus* showed low affinity facilitated diffusion when grown on xylose microaerobically and both low affinity and high affinity transport activity when grown on xylose under aerobic condition (Stambuk *et al.,* 2003).

2.2.2) *S. cerevisiae* as a good fermenting microorganism

Saccharomyces cerevisiae is the preferred microorganism because of its high ethanol productivity, ethanol tolerance, inhibitors tolerance present in the lignocelluloses hydrolysate(Linden and Hahn- Hagredal, 1989). Yeasts are superior to bacteria in several ways such as larger sizes, thicker cell walls, better growth at low pH, less stringent nutritional requirements, and greater resistance to contamination (Jeffries, 2006). *S. cerevisiae* can ferment D-glucose 6 to 35 times faster than native D- Xylose utilizing

yeasts can ferment D- Xylose (Jeffries, 1985) which is an evidence for supporting *S. cerevisiae* as a good fermenting microorganism. Presently, *Saccharomyces cerevisiae* is the most preferred organism for commercial production of bioethanol.

2.2.3) *S. cerevisiae* for fermentation of sugar and production of ethanol

Naturally D- Xylose fermenting yeasts like *P. stipitis* requires low level of oxygen under anaerobic conditions (Jeppsson *et al.,* 1999), while *S. cerevisiae* can grow in conditions of aerobic to anaerobic conditions and have potential to produce vast amount of ethanol, hence regarded as a best microorganism to produce ethanol from the fermentable substrate in shortest time (Jeppsson *et al.,* 1996). Also, *S. cerevisiae* can survive under high ethanol concentration and its products are toxic for other microorganisms, and the technique to harvest ethanol are also efficient and of low cost and hence *S. cerevisiae* is regarded as the efficient and economic to produce bioethanol (Jeffries and Jin, 2000). *S. cerevisiae* shows growth also in presence of many toxic compounds present in lignocellulosic hydrolysates, while other microorganisms shows sensitivity to those compounds making *S. cerevisiae* more usable (Zaldivar *et al.,* 2002). The major limitation of *S. cerevisiae* is that it cannot grow naturally on D-Xylose and thus fermentation of pentoses cannot occur. Inspite of this, *S. cerevisiae* is the more preferable organism of choice to work because of its good food grade status from centuries of use in bread and alcohol fermentation industries. Besides this, *S. cerevisiae* is a model organism because its whole genome has been sequenced and is available (Anonymous, 1996). Yeasts metabolism is better understood than others since research has been done on its biochemistry, genetic modification and other selection techniques are thus its study are easy to perform.

2.2.4) Xylose metabolism in microorganisms

Metabolism of xylose to xylulose occurs in microorganism through two separate routes. One step pathway is catalyzed by xylose isomerase occurs in bacteria, whereas the two step reaction xylose reductase (XR) and xylitol dehydrogenase (XDH) occurs in yeast. Xylulose is subsequently phosphorylated with xylulokinase then phosphorylates xylulose and forms xylulose 5- P which is then further catabolized through the Pentose phosphate pathway and Embden-Meyerhof-Parnas(EMP) pathway(Aristidou and Penttila 2000). PPP in yeasts is aimed at production of reducing power in the form of NADPH and precursors such as D-ribose – 5 phosphate and D-erythrose 4- phosphate for nucleotide and amino acid biosynthesis (Horecker and Mehler 1955). PPP being efficient producer of NADPH and precursors for fuel biosynthesis pathway, it also serves as an entry point for various carbon sources metabolism. Several yeasts species such as *Candida, Pichia, Pachysolen, Kluyveromyces and Debaryomyces* are able to utilize xylose sugar present

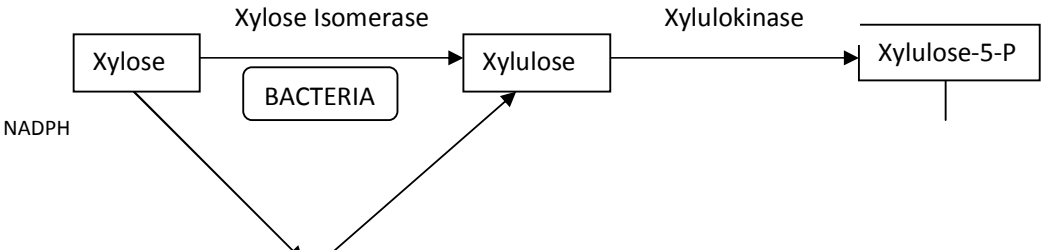
in hemicelluloses fraction of plant material. These sugars enter the metabolism through PPP. In D-xylose metabolism, part of the carbon channelled to glyceraldehyde 3-phosphate and D-fructose 6-phosphate is directed to D-glucose 6-phosphate which enter the PPP to produce NADPH (Toivari, 2007). Due to the lack of xylose transporters in *Saccharomyces cerevisae,* it cannot utilize the abundant xylose sugar present in lignocellulosic biomass (Hector *et al.,* 2008). Hence incorporation of xylose transporter can lead to the efficient conversion of D xylose to D xylose -5 P which is then assimilated to PPP.

Table2: Pentose utilizing properties of different microorganisms

Species	Advantages	Drawbacks	References
Saccharomyces cerevisiae	Naturally adapted to ethanol fermentation High alcohol yield(90%) High tolerance to ethanol Amenability to genetic modification	Cannot ferment xylose and arabinose sugars. Cannot survive at high temperature	McMillan, 1994 Talebnia *et al.,* 2010; Jorgensen 2009; Gamage *et al.,* 2010 Hahn-Hägerdal 2007 Rogers *et al.,* 2007
Candida shehatae	Ferment xylose	Low tolerance to ethanol Low yield of ethanol Requires microaerophilic conditions Does not ferment xylose at low pH	McMillan, 1994 Zaldivar *et al.,* 2001 Banerjee *et al .,* 2010 Ligthelm *et al.,* 1988
Zymomonas mobilis	High ethanol yield(97%) High ethanol tolerance Amenability to	Cannot ferment xylose sugars Low tolerance to inhibitor	McMillan, 1994 Herrero 1983 Balat 2008

	genetic modification Does not require additional oxygen	Neutral pH range	
Pichia stipitis	Best xylose fermenter Ethanol yield high (82%) Ferment most of cellulosic material including glucose, galactose , cellobiose Possess cellulose enzymes for SSF	Ethanol intolerant above 40% Cannot ferment xylose at low pH Sensitive to chemical inhibitors Requires microaerophilic conditions Reassimilates formed ethanol	McMillan, 1994 Zaldivar *et al.,* 2001 Nigam 2001 Jeffries *et al.,* 2007
Pachysolen tannophilus	Ferment xylose	Low yield of ethanol Requires microaerophilic conditions Does not ferment xylose at low pH	Zayed,, & Meyer (1996) Zaldivar *et al.,* 2001
Escherichia coli	Ability to use both pentose and hexose Amenability to genetic modification	Repression catabolism interfere to cofermentation Low ethanol tolerance Narrow pH and temperature growth range Production of organic acids Low tolerance to	Gamage *et al.,* 2010 Weber and Boles 2010 Zayed,, & Meyer (1996)

		inhibitor Genetic stability not proven yet	
Kluveromyces marxianus	Can grow at high temperature 52˚C Ferment variety of sugars Reduce cooling cost Reduce contamination Suitable for SSF process Amenability to genetic modification	Excess of sugar affects its ethanol yield Low ethanol tolerance Poor xylose fermentation and high xylitol formation	Banat *et al.,* 1992 Kumar *et al.,* 2009 Weber *et al.,* 2010
Thermophilic bacteria *Thermoanaerobacterium sachharolyticum* *Thermoanaerobacter ethanolicus* *Clostridium thermocellum*	Resistance to extremely high temp 70˚C Ferment variety of sugars Displays cellulolytic activity Amenability to genetic modification	Low ethanol tolerance	Zeikus *et al.,* 1981 Georgieva *et al.,* 2008 Kumar *et al*., 2009 Shaw *et al.,* 2008 Banerjee *et al.,* 2010



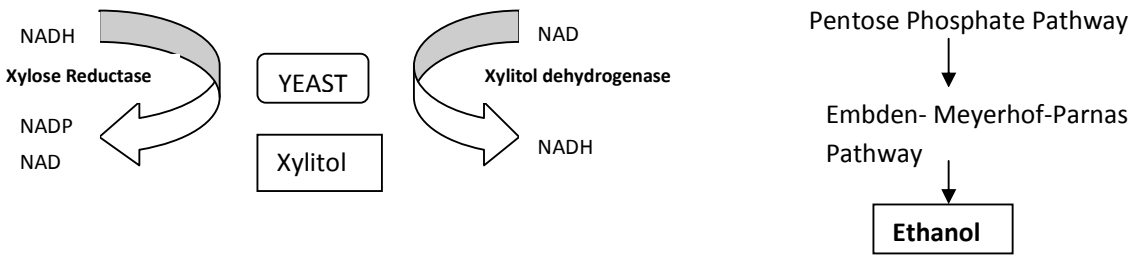


Figure3: Comparision of xylose – utilizing pathways in bacteria and yeast. (Aristidou and Penttila 2000)

2.2.5) Xylose metabolism in *saccharomyces cerevisiae* and role of transporter and enzymes related to metabolism

Based on the widespread large scale application for bioethanol production from hexoses, *Saccharomyces cerevisiae* appears to be the most promising metabolic engineering platform for bioethanol production from plant hydrolysates. The disadavantage of the *Saccharomyces cerevisiae* is the absence of xylose transporting properties and lacks xylose utilizing pathway inside, many metabolic engineering approaches has been a keen interest for making *Saccharomyces* able to metabolize D-xylose.

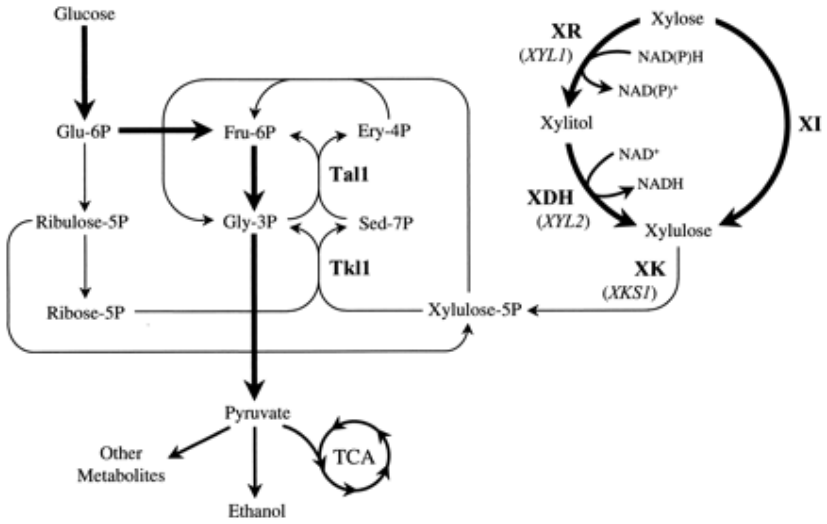


Figure4: Overview of metabolic pathways for glucose and xylose metabolism. The Embden-Meyerhof-Parnas pathway and the xylose utilization pathway are illustrated by thick lines. The PP pathway is indicated by thin lines. Abbreviations: TCA, tricarboxylic acid cycle; Tkl1, transketolase; Tal1, transaldolase; Glu-6P, glucose-6-phosphate; Fru-6P, fructose-6-phosphate; Gly-3P, glyceraldehyde-3-phosphate; Ery-4P, erythrose-4-phosphate; Sed-7P; sedoheptulose-7-phosphate (Ostergaard *et al.,* 2000).

One of the limiting step in xylose utilization by *Saccharomyces cerevisiae* is the lack of xylose transport inside the cell. (Hector *et al.,* 2008). Wild *Saccharomyces cerevisiae* transports D-xylose by hexose transporter but with very low affinity (Hamacher *et al.,* 2002) and only after the depletion of more preferred sugar glucose (kim *et al.,* 2012). Thus the introduction of heterologous xylose transporter in *Saccharomyces cerevisiae* is being important for making it able to take up xylose.

The assimilation of xylose requires two oxidoreductase steps which are not available in *Saccharomyces cerevisiae*. One of the best xylose ferementing yeast *Pichia stipitis* when used as a source for xylose metabolizing genes, the XYL1 and XYL2 encoding XR and XDH respectively when expressed in *S. cerevisiae* (Ho *et al.,* 1998, Hagerdal *et al.,* 2001, Jeffries and Jin 2000, Jin *et al.,* 2000) , the resulting transformants were able to use xylose oxidatively and produced xylitol without addition of a co- metabolizable carbon source . Although, the presence of xylose metabolizing genes from oxidoreductive pathway, XR and XDH, in *S. cerevisiae* , the inability to metabolize xylose sugar by this microrganism is attributed due to the different cofactor requirement of XR and XDH for NADPH and NAD^+ respectively, and thus creating cofactor imbalance problem (Kotter and Ciriacy 1993 ; Kuhn *et al.,* 1995; Richard *et al.,* 1999). The cofactor imbalance can be alleviated by giving low aeration but it is more costly and not feasible industrially (Jin *et al.,* 2004, Toivari *et al.,* 2001).

By passing the cofactor imbalance problem of the XR and XDH can be alleviated by introducting the xylose isomerase pathway which directly converts D-xylose to D-xylulose and is phosphorylated to enter PPP. Successful expression of xylose isomerase gene in *Saccharomyces cerevisiae* has been done from different microorganism like *Clostridium phytofermentants* (Brat *et al.,* 2009), *Piromyces sps*. (Kuyper et al., 2003), *Opiromyces sps.*(Madhavan *et al.,* 2009) .

The phosphorylation of D-xylose to D-xylulose is done by another enzyme xylulokinase (XK). Wild *Saccharomyces* although contains XKS1 gene encoding XK (Deng and Ho 1990, Rodriguez *et al.,* 1998) but the XK activity is very low to support the ethanolic xylose ferementation in strains engineered with xylose utilizing pathway (Deng and Ho 1990, Eliasson *et al.,* 2000; Toivari *et al.,* 2001) . Thus, the fine tuned overexpresssion of XKS1 gene in *Saccharomyces cerevisiae* will make the xylose utilizing recombinant *Saccharomyces cerevisiae* to produce ethanol from xylose (Jin *et al.,* 2003 , Johansson *et al.,* 2001)

The central pathway for the metabolization of pentose sugar is the Pentose Phosphate Pathway (PPP). PPP is the only way to introduce xylulose to central metabolism. It also provides the anabolic intermediates such as ribulose 5 –P, erythrose 4-P, and NADPH for biosynthesis and cell growth. Flux through the non oxidative PPP in *S. cerevisiae* is much

lower than other yeast (Gancedo *et al.,* 1973) which might be due to prolonged selection of *S. cerevisiae* for $C0_2$ and ethanol production from hexose sugar. Four non oxidative PPP genes were shown to improve xylulose consumption by *S .cerevisiae* (Jeppsson *et al.,* 2002, Johansson and Hagerdal 2002). The overexpression of four genes functioned high compared to when single gene was overexpressed (Johansson and Hagerdal 2002).

In *S .cerevisiae* the reduction of the xylose to xylitol is performed by endogenous xylose (aldose reductase) encoded by GRE3 gene (Kuhn et al., 1995) .Xylitol inhibited XI (Yamanaka 1969, Brat *et al.,* 2009) and thus the deletion of GRE3 gene minimized xylitol production and shown to improve xylose metabolism in *S. cerevisiae* using XI pathway (Traff *et al.,* 2001). However, GRE3 gene is stress induced protein (Kuhn *et al.,* 1995) and thus deletion resulted in decrement in growth rate by 30 % (Lonn *et al.,* 2003).

2.2.6) Approaches for efficient D-xylose metabolism in *S. cerevisiae*

For the efficient bioethanol production several metabolic engineering approaches are done in this field (Chu & Lee 2007; Karhumaa, *et al.,* 2007; Matsushika *et al.,* 2009). As *Saccharomyces cerevisiae* lacks xylose transporter, cloning of the xylose transporter leads to transportation of xylose inside the cell. But this is not sufficient for the production of bioethanol, there occurs several problems. After the xylose enters inside there is a need of the corresponding enzymes for xylose metabolism. Wild type *S .cerevisiae* cannot ferment D- xylose but can ferment D xylulose, hence conversion of D xylose to D xylulose is required (van Maris *et al.,* 2007). Heterologous enzymes for this step are developed through expression of heterologous xylose reductase and xylitol dehydrogenase (Walfridsson, *et al.,* 1997), but this is alone not efficient as this leads to the unnecessary production of xylitol due to the cofactor imbalance. To solve this problem , several strategies have been implemented as, shifting cofactor utilization in XR from NADPH to NADH , and also the mutuations can be introduced to reduce affinity of XR for NADPH (Jeppsson *et al.,* 2006, Bengtsson, *et al.,* 2009; Zeng, *et al.,* 2009) and XDH for NAD^+ (Metzger *et al.,* 1995). Apart from this , the most effective way developed is the expression of Xylose Isomerase gene which converts D- xylose to D-xylulose (Van Vleet *et al.,* 2009) directly without any problem of cofactor utilization too as well as minimal production of xylitol., various XI genes when overexpressed with genes of non-oxidative PPP, the recombinant strain can grow anaerobically on D-Xylose with same ethanol yield as glucose (van Maris *et al.,* 2007, Kuyper, *et al.,* 2005). Several researches has been done in this work and various pentose utilizing pathways have been integrated in baker's yeast allowing the fermentation of pentoses like D-xylose and D-arabinose which showed enhanced pentose fermentation for ethanol production (Fonseca, *et al.,* 2007; Brat, *et al.,* 2009; Wisselink, *et al.,* 2007; Wiedemann, *et al.,* 2008).

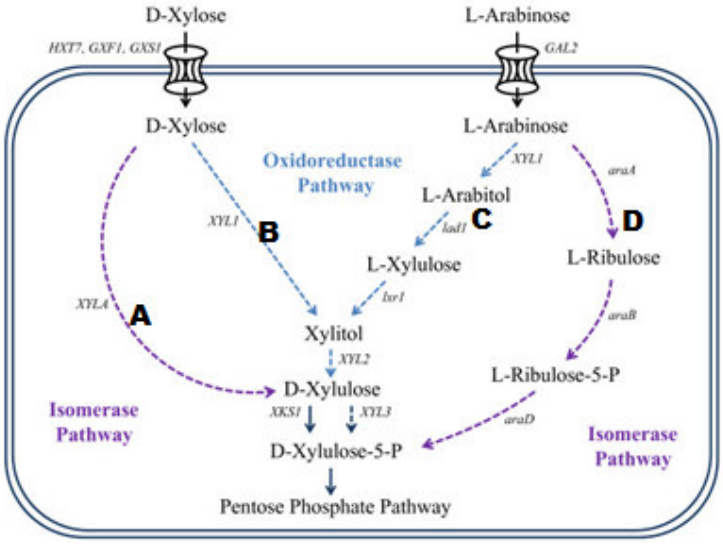


Figure5 Exogenous transport and metabolic pathways introduced in *S. cerevisiae* (A): bacterial xylose isomerase pathway (B) fungal oxido reductase pathway; (C) Fungal arabinose oxidoreductase pathway ; (D) Bacterial arabinose isomerase pathway. {Symbols: araA: arabinose isomerase; araB: ribulokinase; araD: ribulose-5-P-4-epimerase; lxr1: L-xylulose reductase; lad1: arabitol 4-dehydrogenase; XYLA- Xylose isomerase; XYL1: xylose reductase; XYL2: xylitol dehydrogenase XYL3: Xylulokinase; XKS1: Xylulokinase}. (Young *et al.,* 2010)

2.3) Metabolic engineering of *Saccharomyces cerevisiae* for efficient D- xylose transportation

2.3.1) D-xylose transportation in wild *Saccharomyces cerevisiae*

Theoritical study suggests the hexose and pentose sugar both to yield maximum amount of ethanol, 0.51 g ethanol.g $^{-1}$ sugar (Ostergaard *et al.,* 2000) , but practically it is not seen relevant because of the unnecessary by products production production from pentose as xylitol, at levels close to those of ethanol. One of the major limitations of pentose utilization by yeast is unable to transport D-xylose into the yeast cell (Eliasson *et al.,* 2000a). Decrease of glucose increase the xylose uptake rate on recombinant xylose utilizing *S. cerevisiae* (Eliasson *et al.,* 2000a). Activation energy for D – xylose also suggests the movement of sugar to cell limited at temperatures of 15˚C to 30˚C. Hexose sugar transport is performed in *S. cerevisiae* by hexose and galactose transporters (Wieczorke *et al.,* 1999) which if deleted showed no D-xylose transport that directs the use of hexose and galactose transporter acting as xylose transporter (Hamacher *et al.,* 2002). But the overexpression of hexose transporter did not increase the xylose

transportation (Hamacher *et al.,* 2002), and hence there is need of alternative approach for development of pentose utilizing yeast.

2.3.2) Limitation of *S. cerevisae* for efficient xylose transportation.

Biofuel production relies upon the ability of microorganism, *Saccharomyces cerevisae* to convert the majority of sugars present in the lignocellulosic biomass as hexose sugars and pentose sugars. Xylose transport acts as a limiting step for xylose utilization (Hector, *et al.,* 2008). *Saccharomyces cerevisiae* transports xylose by use of hexose transporter with very low affinity (Km 100-190mM) (Busturia and Lagunas, 1986; Hamacher *et al.,* 2002; Heredia *et al.,* 1968., Kotyk 1967) .Wild type *Saccharomyces cerevisae* lacks the potentiality to ferment pentose sugars (Thorsten Subtil and Eckhard Boles 2011), also it can ferment glucose to ethanol but unable to utilize pentose until glucose is completely consumed (kim *et al.,* 2012). Unless the hemicellulosic fraction of lignocellulosic material is consumed along with cellulose, biofuel production by the biomass is not economical (Wyman *et al.,* 2003). This is because *Saccharomyces cerevisae* lacks specific pentose transporter and transportation of pentose occuring through hexose transporter and galactose transporter system (Sedlak and Ho 2004). Yeast engineering for the xylose transport and xylose fermentation using cheap unusable substrates has been a research target to meet the rising energy demand (Jeffries, 2006).

2.3.3) Hexose transporters and their low affinity for xylose transportation

Glucose transport has been shown through the 18 hexose transporter poteins (Hxt1–17, Gal2) and 3 maltose transporting proteins (Wieczorke *et al*., 1999). Study of the xylose transporting hexose transporters Hxt1, Hxt2, Hxt4, and Hxt7 showed Km values ranged between 130 and 900 mM (Saloheimo *et al.,* 2007). Further studies showed the hexose transporter playing the role of xylose transporting properties with low affinity such as, Hxt4, Hxt5, Hxt7 and Gal2 which acted as xylose transporting proteins (Hamacher *et al.,* 2002). But the overexpression of these proteins did not result in faster growth on xylose under aerobic conditions nor did it enhance in xylose fermentation rate under anaerobic conditions. (Hamacher *et al.,* 2002).

2.3.4) Glucose mediated repression of xylose transporter

Sugars like glucose inhibits the transcription of genes required for utilizing the alternative carbon sources like pentoses (Belinchon and Gancedo 2003) which is carbon

catabolite repression (CCR), where preferable carbon sources lowers the preferability for alternative carbon by producing different cascade pathway or modification of required proteins (Gancedo, 1998). Genetic study of CCR shows the involvement of genes for repressing synthesis of sensitive enzymes required during growth on repressing carbon sources and another way by regulating the derepression process (Zimmermann, *et al.,* 1977). Several engineering strategies for the development of CCR negative yeast have been developed, which allowed the usage of unpreferrable carbon sources including pentoses, however it involves variety of cascade chain pathway, regaulatory pathways and several signaling molecules (Roca *et al.,* 2004). Repression of xylose transport by presence of glucose has also been shown to be relieved by cofermentation on the mixture of cellobiose and xylose, which lets increment in xylose transport (S-J *et al.,* 2011). A mutant of Glf transporter (2-RD5) from *Zymomonas mobilis* was shown to relieve the inhibition of xylose transport by glucose when expressed in *E coli* (Ren *et al.,* 2009).

2.3.5) Expression of Xylose Transporters in *S. cerevisiae*

Nature has gifted many pentose assimilating fungal strains for pentose uptake including high affinity and low affinity as well (Fonseca *et al.,* 2007; Leandro, *et al.,* 2006; Stambuk *et al.,* 2003). The most successful improvement of xylose transport is shown through the expression of *Candida intermedia* PYCC 4715 transporter proteins (Leandro *et al.,* 2006). Improvement of D-xylose uptake is conducted by introduction of heterologous D-xylose transporters in *S. cerevisiae* strains. One high affinity D-xylose/D-glucose 5 symporter (GXS1) and one low affinity D-xylose/D-glucose facilitator (GXF1) has been discovered in fungus *Candida intermedia* and its characterization was performed in *S. cerevisiae* at molecular level (Leandro, *et al.,* 2006). Two transporters of *Candida intermedia, GXF1 and GXS1 showed the improved growth phenotypes in recombinant S. cerevisiae* when glucose and xylose was used as sole carbon sources (Leandro *et al.,* 2006). The expression of mutant transporter of *C. intermedia* GXS1 and *S. stipitis* SUT1 in *S. cerevisiae* showed improved growth rate on xylose by 70% (Young *et al.,* 2012). Overexpression of GXF1 transporter improves the fermentation performance in recombinant D- xylose utilizing *S. cerevisiae* (Runquist, *et al.,* 2009) and two D-xylose transporter gene from *Arabidopsis thaliana* when expressed in *S. cerevisiae* increased 2.5 fold D- xylose consumption rate and 70 % increment in ethanol production in recombinant D-xylose metabolizing *S. cerevisiae* (Hector, R.E *et al.,* 2008). Comparision of three D- xylose transporters GXF1, At5g5920, SUT1 from *Candida intermedia* , *Arabidopsis thaliana* and *Pichia stipitis* ,where GXF1 showed best xylose transporting phenotypes and then At5g5920 and negligible by SUT1(Runquist, *et al.,* 2010). In a genetically engineered yeast strain with integrated xylose isomerase, several heterologous xylose transporters introduced showed improved cell growth and

fermentation among which RGT2, XUT7, XUT6 showed xylose consumption rate ranging from 0.399 to 0.535g/L/h comparable to the native D-xylose utilizing yeast *Scheffersomyces stipitis*. Also introduction of XUT7, RGT2 and SUT4 showed 50% increment in ethanol productivity compared to the parental strain (Moon *et al.,* 2013).

Along with different hexose transporters, a galactose transporter,Gal 2, has been revealed for its capacity for xylose uptake (Sedlak and Ho 2004). Through the approach of mutation and adaptive evolution, a mutated GatC has been shown for its efficient xylose transportation ability (Utrilla *et al.,* 2012), and shows the ATP independent manner of xylose uptake in an engineered *E.coli* (Nduko *et al.,* 2013). SUT1, a hexose transporter of *Pichia stipitis* showed the increased xylose uptake and ethanol productivity during glucose xylose cofermentation when expressed in xylose assimilating *S. cerevisiae* (Katahira *et al.,* 2008). Another heterologous expression of xylose transporter xylT when observed in *L. lactis* (Nyyssola *et al.,* 2005) and *L. plantatum* (Chaillou *et al.,* 1998) showed the increased xylose transport phenotype. In a xylose utilizing recombinant yeast a transporter homologue permease encoding gene, Trxlt1 from *Trichoderma reesei* have been discovered through adaptive mutuation showed the ability to grow on xylose thus is specific for being the xylose transporter (Saloheimo *et al.,* 2007).

Table3 Transport protein used for xylose uptake

Organism name	Gene	Xylose transporting properties	Ethanol production
A. thaliana	At5g59250 At5g17010 (Hector *et al.*, 2008)	+ +	+ +
C. intermedia	GXF1 GXS1 (Leandro *et al.*, 2006; Tanino et al., 2012)	+ +	+ +
Trichoderma reesei	Trxlt1 (Saloheimo *et al.,* 2007)	+	
S. cerevisae	HXT1(Saloheimo *et al.,*	+	

	2007) HXT2(Saloheimo *et al.,* 2007) HXT4(Saloheimo *et al.,* 2007; Hamacher *et al.,* 2002) HXT5(Hamacher *et al.,* 2002) HXT7(Saloheimo *et al.,* 2007; Hamacher et al., 2002) GAL2(Hamacher *et al.,* 2002)	 + + + + +	 - - - -
Pichia stipitis	SUT1 (Katahira *et al.,* 2008, Runquist *et al.,* 2010) SUT2 (Weierstall *et al.,* 1999) SUT3 (Weierstall *et al.,* 1999)	- + +	
Scheffersomyces stipitis	RGT2 (Moon *et al.,* 2013)	+	+
Lactobacillus brevis	XYLT (Chaillou *et al.,* 1998)	+	

2.3.6) *Candida intermedia* as a good candidate source for pentose specific transporters

Among the various organism isolated, naturally occurring D- xylose transporter containing fungus *Candida intermedia* is characterized as one of the best D- xylose transporter and preffered for heterologous expression of xylose transporter in *Saccharomyces cerevisiae. Candida intermedia* contains two types of transporters one is high affinity xylose proton symporter, GXS1 which transport xylose by proton symport mechanism and another low affinity glucose xylose faciliator GXF1 which transports through facilitated diffusion mechanism (Leandro *et al.,* 2006). Successful expression of

GXF1 transporter has been performed in *Saccharomyces cerevisiae* which showed a good xylose transport in the recombinant compared to the wild type strains (Leandro, *et al.,* 2006; Runquist, *et al*., 2009). The high efficiency of *C. intermedia* PYCC 4715 in role of xylose assimilation was confirmed by same growth kinetics and biomass yields in glucose and xylose medium (Gardonyi *et al.,* 2003). Studies have revealed the role of RGT2 from *Scheffersomyces stipitis* as efficient xylose uptake and fermentation for producing ethanol, the high similarity of RGT2 with GXS1 from *Candida intermedia* is a good supporting evidence of GXS1 as a good xylose transporter (Moon *et al.,* 2013).

2.3.7) Xylose transporter encoding gene GXF1

Several native D-xylose utiliziing yeasts have been isolated and screened for high xylose transport capacity, one of the fastest growing yeast *Candida intermedia* PYCC 4715 showed the highest xylose transport. Two types of transporter are present in *Candida intermedia* PYCC 4715, one operating through xylose/proton symport mechanism, and another through facilated diffusion mechanism. High affinity xylose/proton symport system system (Km= 0.2 mM, Vmax=7.5 mmol $h^{-1}g^{-1}$) was more repressed by glucose, while the less specific low affinity transport system operated through faciliated diffusion mechanism (K= 50 mM, Vmax= 11 mmol $h^{-1}g^{-1}$) and it expressed constitutively (Gardonyi *et al.,* 2003). Screening of the high xylose uptake in various fungus showed highest xylose uptake in *Candida intermedia, the cDNA library screening in S. cerevisiae HXT* null strain TMB 3201 identified the GXF1 transporter gene for highest xylose uptake (Leandro, *et al.,* 2006). Hence ,this gene can be used for heterologous expression in *S. cerevisiae* (Gardonyi *et al.,* 2003). GXF1 also showed a good sequence identity with the Sut1–Sut3 transporters found in *P. stipitis and it showed high affinity for xylose with same order of magnitude* (approx. 50 mM compared with 50–150 mM)(Weierstall, Hollenberg *et al.,* 1999).

2.3.8) Significant expression of GXF1 in *S. cerevisiae*

Expression of Glucose/ Xylose facilitator, GXF1 along with other two transporters SUT1 and At5g59250 has been performed in *Saccharomyces cerevisiae* where GXF1 showed best xylose transport kinetics (Runquist, *et al.,* 2010). Expression of glucose/xylose facilitator GXF1 from *Candida intermedia* in the recombinant D-xylose-fermenting *Saccharomyces cerevisiae* strain TMB 3057 showed two times lower K_m for xylose and increase in specific growth rate at xylose 4g/l compared to a control strain not expressing GXF1 (Runquist *et al.,* 2009). GXF1 expression showed enhanced xylose uptake during the SSCF of pretreated wheat straw (Fonseca, *et al.,* 2011). GXF1 gene when overexpressed together with xylulokinase expressing gene xylB in xylose isomerase overexpressing strains showed 37% increase in consumption of xylose and

24% increase in the aerobic growth when growth on xylose; whereas only overexpression of xylB in XI strain showed only 21% increment in aerobic growth rate and 27 % increment in the xylose consumption when compared to control strains (Parachin, *et al.,* 2011). Expression of GXF1 was seen fully functional in *S .cerevisiae* and when GXF1 overexpressed with GXS1 it showed decreased level of GXS1 mRNA transcripts in glucose grown cell, which is because of enhanced glucose flux mediated by GXF1 proteins (Leandro *et al.,* 2008). Four transporters HXT1 and HXT7 from *S. cerevisiae* and GXF1 and GXS1 from *C. intermedia* when overexpressed in xylose isomerase pathway harbouring *S. cerevisiae* showed the enhanced consumption of sugar and ethanol productivity, among all GXF1 showed efficient fermentation of both glucose and xylose (Tanino *et al.,* 2012).

3.Materials and methods

3.1) Sources of Materials and Chemicals

Clone of pGXF1 was gifted with thanks from Prof. Dr Paula Goncalves, the University of Lisbon, Portugal (Leonardo *et al.,* 2006). *E. coli* DH5α and *S .cerevisiae* DTY165 (Leucine auxotroph) gifted by Prof. Dr E Boles; University of Frankfurt, Germany was used as host for transformation. The source of chemicals, employed in this study are: DNA polymerase, dNTPs, DNA ladder, (Thermoscientific); Agarose (Fischer Scientific agarose Low eeo) was used for electrophoretic separation of DNA samples and DNA was visualized by using UV transilluminator (Major Science). Southern blotting was performed by Biotin chromogenic detection kit (Fermentas). Primer was ordered from Macrogene Korea. All PCR works were performed in TECHNE ^{5}Prime PCR machine gifted by TWAS. Electroporation was performed by Electroporator BioRad Micropulser^{TM}.

3.2) Primer designing

A set of primer (YTUF1F and YTUF1R) was designed using different online web tools oligocalc (Oligonucleotides properties calculator), mfold (The mfold web server) and IDT (Integrated DNA Technologies), mentioned in appendix 2.

Set of primer amplifying 1.6Kb insert

Fw: 5' GACTA ***GAGCTCCATATG*** TCACAAGATTCGCATTC 3' (NdeI/SacI) (YTUF1F)

Rv: 5' TACTGT ***GGATCC*** CAT***TCTAGA*** TTAAACCTGTTCGTCGGTG 3' (XbaI/BamHI) (YTUF1R)

Set of primer amplifying the 163bp of insert

Fw : 5'-CTTTGCTTCCACCTTCGTTG-3' (CiGXFL1)

Rv : 5'-AGTGTTGGAGGTCTCGTTGG-3' (CiGXFR1)

3.3) GXF1

GXF1, naturally present in *Candida intermedia,* is a glucose /xylose transporter which transports glucose and xylose sugar by faciliated diffusion mechanism. A GXF1 cloned expression vector was gifted with thanks from Prof. Dr. Paula Goncalves, which has ampicillin resistant marker and Leu2 marker gene (Leucine synthesizing gene) designed for the selection of cloned transformants of *E. coli* and yeast cell respectively. The GXF1 gene is 1664bp long nucleotide sequence and GXF1 protein is of 547 amino acid length(NCBI). It belongs to major facilitator superfamily, sugar transporter family (uniprot).

Figure: GXF1 cassete with HXT7 promoter and PGK terminator in pGXF1

3.4) PCR amplification of GXF1, (Glucose/ Xylose facilitator)

Two sets of primers have been used for the confirmation of plasmid. The basic information about the two sets of primer (YTUF1F, YTUF1R) and (CiGXFL1, CiGXFR1) has been mentioned in appendix 1.1 and 1.2 respectively. One set amplified the 163bp of insert (CiGXFL1, CiGXFR1), for which the annealing sequence has been determined ($T_{m)}$ regarding the whole primer sequence. Whereas, for the primer which amplifies the 1.6Kb fragment (YTUF1F, YTUF1R), for the first 5 PCR cycles the annealing temperature was targeted to only priming region (3'- end nucleotide complimentary to template DNA) was used whereas for the rest of 25 PCR cycles, annealing temperature (T_m -10˚C) was determined regarding the whole primer sequence. The general protocol for preparing PCR reaction mixture and PCR conditions have been mentioned in appendix 5 and 6. This strategy increases overall PCR efficiency and yield of PCR product. PCR was performed in TECHNE ^{5}Prime PCR. Then, 5μl of PCR product was subjected to 1% agarose gel electrophoresis along with 1Kb plus thermoscientific DNA ladder in a separate well.

3.5) Agarose gel electrophoresis

The agarose concentrations used in electrophoretic separation were choosen based on the size of the DNA to be resolved. Agarose (Fisher Scientific Agarose Low eeo) was melted in 1×TAE buffer (40mM Tris, 20mM acetic acid, and 1mM EDTA, pH 8.0) by heating and was cooled to about 50˚C before adding 0.5μg/ml of ethidium bromide. The molten agarose was poured in a tray and allowed to set. After the gel had set, DNA samples were loaded and electrophoresed in 1×TAE in appropriate electric field strength for optimum separation. The DNA was visualized by UV transilluminator (Major Science).

3.6) Transformation of *Ecoli* DH5α

Transformation was done by heat shock method using the competent cell prepared by $CaCl_2$ method as mentioned in Sambrook (Sambrook and Russel 2001).

3.6.1) Preparation of competent cell

Competent cells were prepared using $CaCl_2$ method. A single colony of *E coli* DH5 α was picked up from a plate grown for 16-20 hrs at 37 °C and transferred into 10 ml of LB broth in sterile 15-ml polypropylene tube. The culture was incubated overnight at 37 °C with vigorous shaking (>250 cycles/min in a rotary shaker). 500 μl of seed culture was inoculated into 50 ml of LB broth in sterile 250-ml flask and incubated for about 2 h at 37 °C with vigorous shaking (>250 cycles/min in a rotary shaker) until the OD_{600} reached 0.3-0.4 (< 10^8 cells/ml). The cells were aseptically transferred to sterile, ice-cold 50-ml polypropylene tube and cooled to 0 °C by storing the tube on ice for 10 min. It was centrifuged at 4100 rpm for 10 min at 4 °C. The media was decanted from the cell pellets and the tube was inverted for 1 min to allow the last traces of media to drain away. Then the cell pellets was resuspended in 30 ml of filter-sterilized ice-cold $Mgcl_2$-$CaCl_2$ solution (80mM $MgCl_2$, 20mM $CaCl_2$) .It was then centrifuged at 4100 rpm for 10 min at 4 °C. The media was decanted from the cell pellets and the tube was inverted for 1 min to allow the last traces of media to drain away. The cell pellets were resuspended by gently vortexing in 2 ml of filter-sterilized ice-cold 0.1 M $CaCl_2$ and 15 % glycerol. 200 μl of suspension of competent cells was transferred to a chilled sterile microcentrifuge cells and immediately stored at –70 °C.

3.6.2) Transformation

Transformation was done using the competent cell prepared form Calcium Chloride method. 1µl of the plasmid DNA and 100 µl of ice cold competent cell was pipetted into a new fresh eppendorf tube. Two other vials were taken with DH5α cells only .It was incubated in ice for 30 minutes. Then heat shock was given by keeping in 42˚C water bath for 2 min and chilled in ice immediately for 5-10 minutes. The cells were then grown in 1ml of LB by incubating at 37˚C with shaking at 80 rpm for 1hr. The grown cell was then centrifuged at 10000rpm for 10mins and the cell pellet was mixed with 100µl LB. Plating of DH5α only cells were done in 2 different plates one with 100μg/ml ampicillin (negative control); and another without ampicillin(positive control); Plating of GXF1 transformation cells were done in LB with ampicllin (100μg/ml).

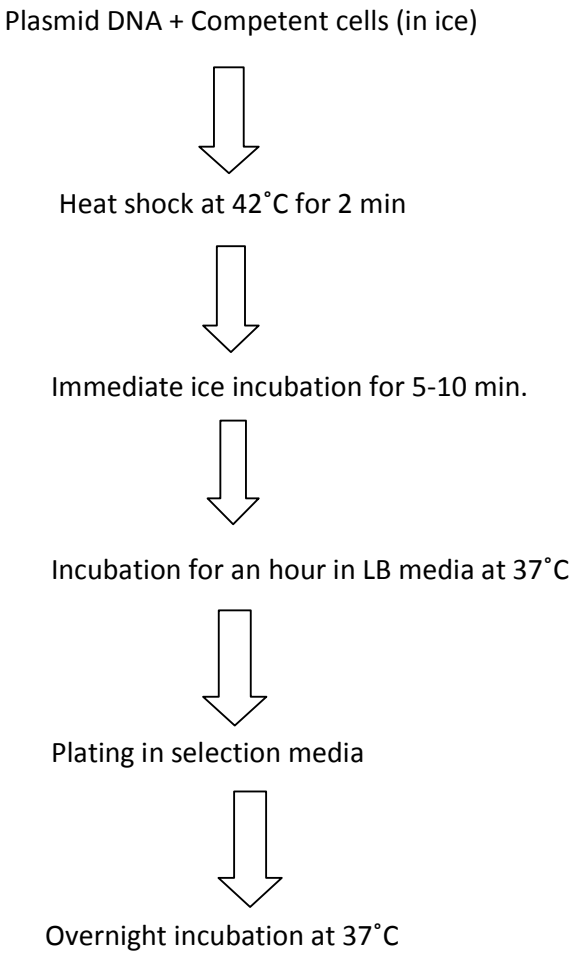


Figure6 Flow chart of transformation

3.7) Confirmation of *E coli* transformants

Transformants are confirmed by the plasmid extraction and PCR method.

3.7.1)Plasmid isolation

Plasmid from Ampicillin resistant cells was extracted using alkaline lysis method. Six well grown colonies were picked up from the transformation agar plate and a colony from

positive control plate (untransformed E coli DH5α cells) was taken and inoculated in the LB with 100μg/ml ampicillin along with making a replication plate simultaneously and it was incubated at 37˚C for O/N. The broth culture was used for plasmid isolation. Culture was centrifuged and cell pellet obtained was dissolved with 200µl alkaline solution I and mixed by pipetting. It was then mixed with 200µl of alkaline solution II and mixed by gently inversion 5 times followed by 200µl of alkaline solution III. The solution was centrifuged at 13000rpm for 10 minutes. Supernatant containing plasmid DNA was transferred to fresh Eppendorf tube and 2μl of 10mg/ml RNase A was added and incubated for 20 minutes at room temperature for the removal of RNA. Then, equal volume of phenol: chloroform 3:1 was mixed and centrifuged for 10 minutes. The upper aqueous phase containing DNA was transferred to fresh tube and equal volume of chloroform was mixed and centrifuged at 13000rpm for 2 minutes. Again upper phase was transferred to fresh Eppendorf tube the addition of sodium acetate (3M, pH 8) as $1/10^{th}$ volume of solution followed by double volume of isopropanol. DNA was allowed to precipitate at -20˚C for 1-2 hour. The precipitated DNA was then centrifuged and the DNA pellet obtained was washed twice with 70 % ethanol. Then the pellet was allowed to air dry for 5- 10 mins, and the plasmid DNA was dissolved in water /TEbuffer and stored at -20˚C.

3.7.2)Confirmation of *E coli* transformants by PCR

Transformants were confirmed by PCR using two set of primer (CiGXFL1 and CiGXFR1) and the other one (YTUF1F and YTU1R) used to amplify 163 bp and 1.6Kb of the GXF1 gene respectively.

3.7.2.1) PCR amplification of 163bp of insert

Plasmid DNA of *E coli* and a negative control (untransformed *E coli* DH5α cells) was used as template for PCR amplification of 163bp of GXF1 gene using a set of primers F_w - CiGXFL1 and R_v - CiGXFR1 . PCR amplification was carried with specialized PCR conditions as mentioned in appendix. PCR reaction mixture and specific PCR condition used for amplification is given in appendix 5.1 and 6.1. Then, 5μl of PCR product was subjected to 1% agarose gel electrophoresis along with 1Kb plus thermoscientific ladder in a separate well.

3.7.2.2) PCR amplification of 1.6Kb ORF

The isolated plasmid DNA and a negative control (untransformed *E coli* DH5α cells) was taken as template for PCR amplification of 1.6 Kb fragment GXF1 gene. A set of primer F_w - YTUF1F ; R_v - YTUF1R was used for PCR amplification of 1.6Kb fragment of GXF1. PCR amplification was carried in two step reaction conditions as mentioned above. PCR reaction mixture and specific PCR conditions used for amplification is given in appendix

5.1 and 6.2. Then, 5μl of PCR product was subjected to 1% agarose gel electrophoresis along with 1Kb plus thermoscientific DNA ladder in a separate well.

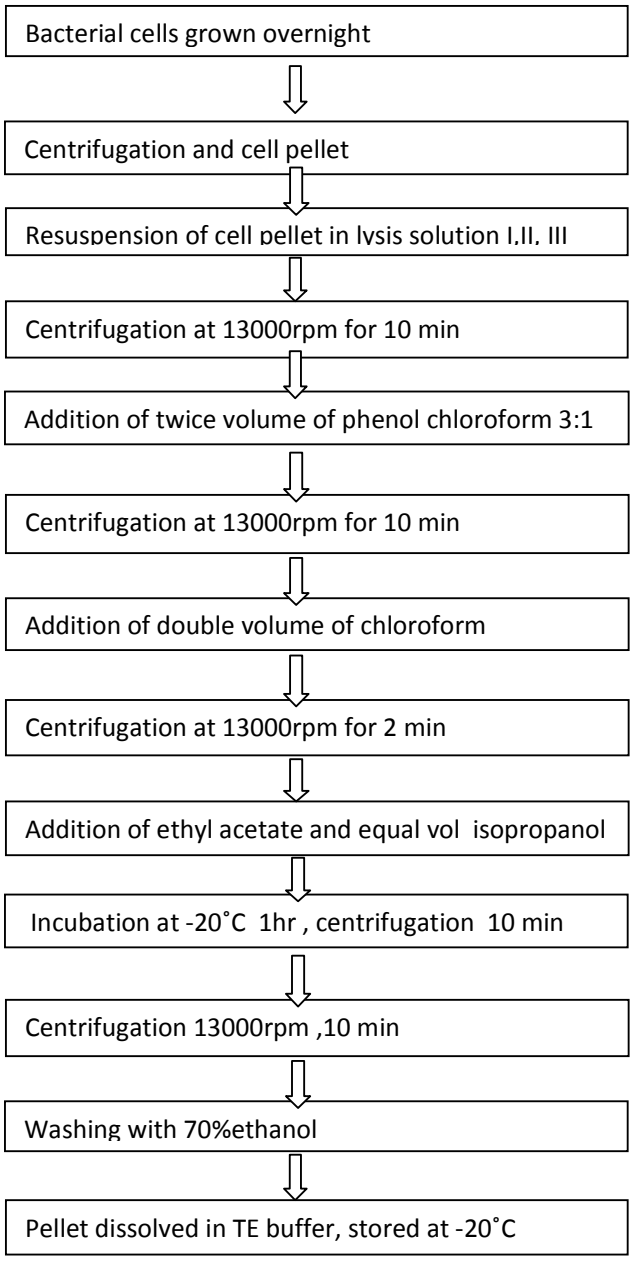


Figure7 Flow chart of bacterial plasmid extraction using alkaline lysis method

3.8) Transformation in *Saccharomyces cerevisiae*

Transformation in yeast was done by electroporator (Electroporator BioRad Micropulser^{TM}) using the elctrocompetent cells.

3.8.1) Preparation of electrocompetent cells

Yeast colonies of *Saccharomyces cerevisiae* DTY165 was inoculated in 10ml of autoclaved sterile YPD media and incubated at 30˚C, 200rpm for 2 hours. 5ml of the culture was then transferred to 200ml autoclaved sterile YEPD media and incubated at 30˚C, 250rpm for O/N and the OD was checked to reach 1×10^8 cells/ml. Then the cells were chilled in ice water bath for 15 minutes to stop growth. The culture were then aseptically transferred to 50ml ice cold polypropylene tubes and centrifuged at 3000g for 5 min at 4˚C. The media was decanted and the cell pellet was resuspended in 50ml sterile ice cold water by gently vortexing and the final volume was made up to 250ml. It was centrifuged at 3000g for 5 min at 4˚C. The washing step with ice cold water was repeated once. Then, the cell pellet was resuspended in 20ml of sterile ice cold 1M sorbitol. It was centrifuged at 3000g for 5 min at 4˚C. Finally, the cell pellet was dissolved in 0.5ml sterile ice cold 1M sorbitol which is the required electrocompetent cells for performing electroporation.

3.8.2) Electroporation

5μl of DNA (5-100ng) GXF1 plasmid DNA was pipetted into sterile ice cold 1.5ml polypropylene tubes and kept in ice for chilling. Electroporation was also performed with the GXF1 untransformed yeast colony as a control. Electrocompetent cells of volume 40μl were pipetted into the polypropylene tubes with DNA .It was mixed gently and incubated in ice for 5 min. The mixture was transferred in 0.2cm electroporator cuvettes and a pulse with voltage of 1.5 KV was applied. Immediately, cuvette was removed from the chamber and 1ml of ice cold 1M sorbitol was mixed. The diluted cells were transferred to sterile polypropylene tubes. Then the electroporated cells and untransformed cells (control) were plated on YNB 4% agar plates supplemented with 20g/l xylose and 30 mg/ml Leucine for selection. Plates were incubated for 48-72 hours at 30˚C.

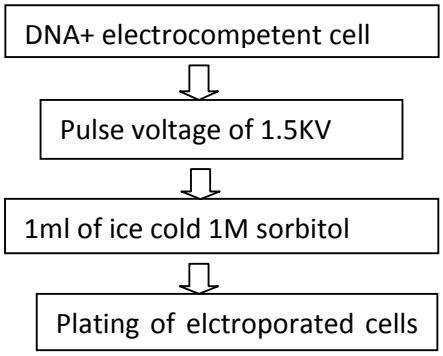


Figure8 Flow chart for transformation of yeast cells by Electroporation

3.9) Confirmation of *S. cerevisiae* transformants

3.9.1) Plasmid isolation from *Saccharomyces cerevisiae*

Four yeast colonies was picked up from the YNB transformation selective agar plates and one positive control colonies were also taken inoculated in 5 ml YEPD medium and incubated at 30˚C for 18-24 hours shaking at 250rpm. Picking up the colonies is followed by making a replication plate in a YNB agar plate. The culture was then centrifuged at 13000rpm for 5 min to obtain the cell pellet. 10μl of lyticase (5U/μl) was mixed with cell pellet along with the residual 50μl media by pipetting for the degradation of yeast cell wall. It was incubated at 37˚C for 30-60 minutes with shaking at 200-250 rpm. Then, 10 μl of 20% SDS was mixed with vigourous vortexing for 1 min. One freeze thaw cycle was done. It was vortexed to ensure the lysis of cell. Then, 200μl of TE buffer (pH -7) was put followed by the 200μl of PCI (25:24:1) and vortexed maximum for 5 minutes. It was centrifuged at 14000rpm for 10 minutes. Upper layer was transferred to fresh polypropylene tubes. DNA precipitation was done with 8μl of 10M ammonium acetate and 500μl of absolute ethanol with incubation at -70˚C for 1 hour. It was then centrifuged at 14000 for 10 min. The pellet was air dried and resuspended in 20μl of nuclease free water and stored at 4˚C.

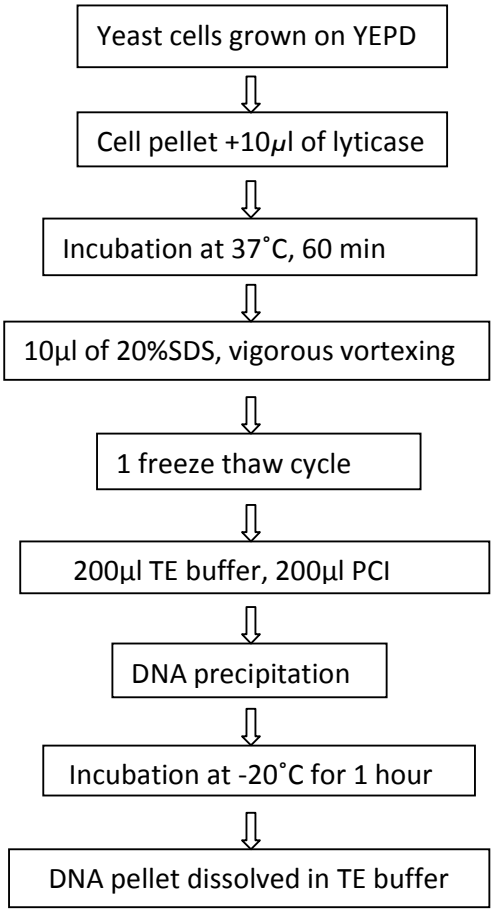


Figure9 Flow chart for yeast plasmid extraction

3.9.2) Confirmation of *S. cerevisiae* transformants by PCR and Southern Blotting

Confirmation of *S. cerevisiae* transformants was performed using a set of primer (CiGXFL1 and CiGXFR1) for the amplification of 163 bp of the GXF1 gene.

3.9.2.1) Confirmation of cloning in *S.cerevisiae* by PCR

Plasmid DNA of transformed *S. cerevisiae* DTY165 and a negative control DNA (untransformed *S. cerevisiae* DTY165) was used as template for PCR amplification of

163bp fragment of GXF1 gene using a set of primers CiGXFL1 and CiGXFR1. PCR amplification was carried with specialized PCR condition. PCR reaction mixture and specific PCR condition used for amplification is given in appendix 5.1 and 6.1. Then, 5μl of PCR product was subjected to 1% agarose gel electrophoresis along with 1Kb plus thermoscientific ladder in a separate well.

3.9.2.2) Southern blotting of GXF1

Southern blotting was performed by the Biotin chromogenic detection kit (Fermentas).

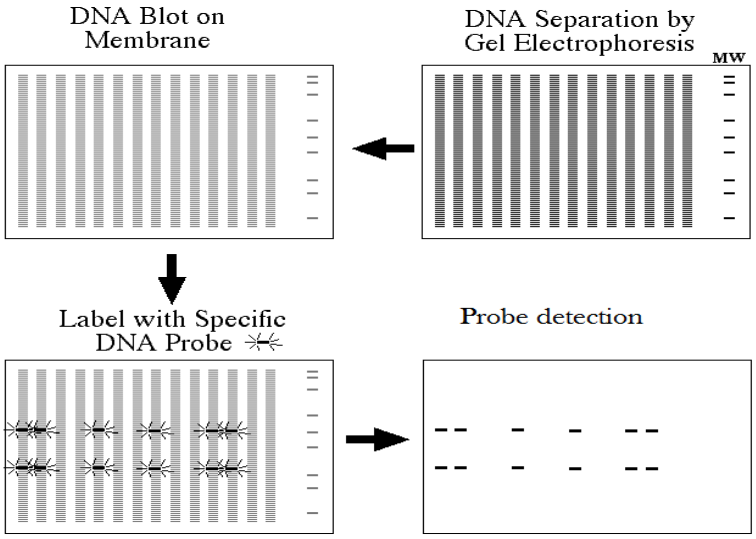


Figure10 General outline of Southern Blotting

3.9.2.2.1) Preparation of probe

Probe was prepared using PCR method using a set of primer amplifying 163bp of the insert. Transformed yeast plasmid DNA and another untransformed yeast colonies (negative control) were taken as a template for PCR. PCR reaction mixture and thermocycling conditions have been mentioned in appendix 5.2 and 6.1. Two PCR for transformed plasmid DNA was done simultaneously one with labelled nucleotides and another with unlabelled nucleotides. PCR product was run in 2% agarose gel and visualized by UV spectrophotometer.

3.9.2.2.2) Blotting of DNA

Transformed plasmid DNA and a control untransformed cells was run in 1% agarose gel and run for 1-2 hours at 50V. Then DNA was depurinated by soaking the gel in 0.2N HCl until the bromophenol band begins to change its colour slightly to yellowish. The gel was

rinsed once with deionized water. The DNA was then denatured by soaking it in denaturation solution (0.4N NaOH /0.6M NaCl) for 30 minutes. Gel was rinsed with deionized water and added with neutralization solution gently shaked for 30 minutes. The gel was then immediately placed in blotting apparatus and left for 2 days in 10× SSC. The membrane was removed from the blotter and rinsed in 5×SSC for 5 min with agitation DNA side upwards. The membrane was blot dried on a piece of whatmann paper and sandwiched in between two sheets of dry whatmann paper and dried in oven at 80˚C for 2 hours for crosslinking of DNA to membrane.

3.9.2.2.3) Prehybridization

Prehybridization was performed with the prehybridization solution. Firstly, 0.5mg/ml aqueous solution of DNA was denatured by heating at 100˚C water bath for 5 minutes and then chilling on ice. The denatured DNA was added to the prehybridization solution making the final concentration of DNA 50μg/ml. The membrane was then placed in plastic bag with the prehybridization solution containing denatured DNA, prehybridization was performed by shaking it at 42˚C for 2-4hours.

3.9.2.2.4) Hybridization

The biotin labeled probe was denatured by keeping in 100˚C for 5 minutes and then chilled on ice. The denatured probe was added to the prehybridized solution to make the final concentration of probe 25-100ng/μl. The prehybridization solution was discarded and hybridization solution was added to the membrane (60μl/cm^2). It was then incubated O/N at 42˚C with shaking. The membrane was washed twice with 2×SSC, 0.1% SDS, each wash was performed for 10 minutes at room temperature with shaking. Again, washing was performed twice with 0.1×SSC, 0.1% SDS for 20 min at 65˚C with shaking. Membrane was then taken out and excess liquid was drained by placing membrane in filter paper.

3.9.2.2.5) Probe detection

The membrane was washed with 30ml of blocking/ washing buffer for 5 minutes at room temperature with moderate shaking. Blocking of the membrane was done with 30ml of blocking solution with moderate shaking at r.t for 30 min. The solution was then discarded and the membrane was incubated in freshly prepared 20ml diluted streptavidin- AP conjugate for 30 min at r.t with moderate shaking. Then, membrane was washed twice by incubating with 60ml of blocking/washing buffer for 15 mins. The solution was discarded and incubated with 20ml of detection buffer for 10 min. The solution was discarded and the membrane was incubated with 10ml freshly prepared substrate BCIP/NBT solution in dark for 30 minutes or O/N for the observation of blue purple precipitate. To stop the reaction the substrate solution was discarded and rinsed

with water for few seconds. Then water was discarded and membrane was air dried to visualize the membrane.

3.10) Functional Expression Assay

Functional expression of GXF1 on S cerevisiae is performed using the glucose and xylose sugar transportation assay. Growth rate of *S. cerevisiae* is determined by growth on glucose as well as xylose. Xylose transport assay is performed by analyzing the depletion of xylose sugar in media as well as increase in intracellular xylose concencentraion inside cells. Glucose and Xylose co fermentation is also analysed by depletion of the respective sugar in media.

3.10.1) Growth kinetics on glucose

Yeast colonies of control and transformed *S. cerevisiae* DTY165 of same OD and were inoculated in YEP with 20 g/l glucose and grown at 28˚ C with shaking at 250rpm. 1ml of sample was harvested at different 3 hour time interval up to 36 hours. Specific growth rate was calculated by measuring the OD at 600nm by Spectrophotometer (Genesys 10vis Thermoscientific). Experiment was repeated 5 times and the average mean was taken for the analysis of result.

Specific growth rate (μ) = ($\ln N_t - \ln N_0$) / Δt

Where, N_t = Number of cells at time t

N_0= Number of cells at initial time

Δt= difference in time interval ($T_t - T_0$)

3.10.1.1) Standard curve of glucose

Standard glucose concentration of 1mg/ml to 18mg/ml was made using DNS method. 1ml of known concentration of glucose sample was mixed with 3 ml of DNS reagent and boiled for 5 minutes and then adding 1ml of 40% potassium sodium tartarate solution for the colour stabilization. The OD was then measured using Spectrophotometer (Genesys 10vis Thermoscientific) at 575nm .

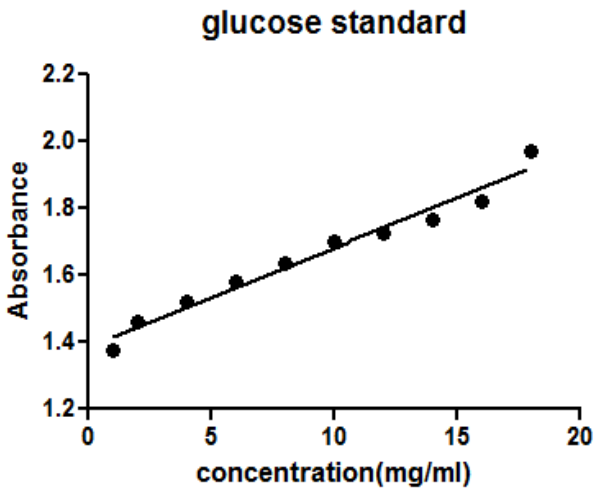


Figure11 standard graph of glucose from1mg/ml to 18mg/ml.

3.10.2) Growth kinetics on xylose

Yeast colonies of control and transformed *S. cerevisiae* DTY165 of same OD were inoculated in YEP with 20 g/l xylose and grown at 28˚ C with shaking at 250rpm. 1ml of sample was harvested at different 3 hour time interval up to 36 hours. Then, the specific growth on xylose was estimated by measuring the OD at 600nm by Spectrophotometer (Genesys 10vis Thermoscientific). The process was repeated five times and the average mean was taken for the data analysi

Specific growth rate (μ) = ($\ln N_t - \ln N_0$) / Δt

Where, N_t = Number of cells at time t

N_0= Number of cells at initial time

Δt= difference in time interval (T_t-T_0)

3.10.2.1) Standard curve of xylose

Standard xylose curve of the concentration from 0.1mg/ml to 0.3mg/ml and 1mg/ml to 6mg/ml was prepared using phloroglucinol assay. 200μl of standard xylose samples were mixed with 5ml of freshly prepared phloroglucinol reagent and boiled for 5-10 minutes. Then the absorbance was taken at 554nm using Spectrophotometer (Genesys 10vis Thermoscientific).

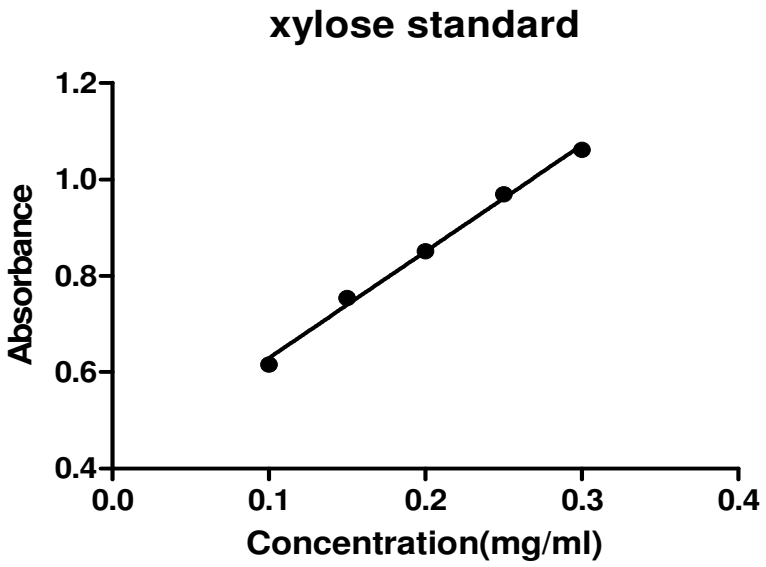


Figure12 Standard graph of xylose from 0.1mg/ml to 0.3mg/ml.

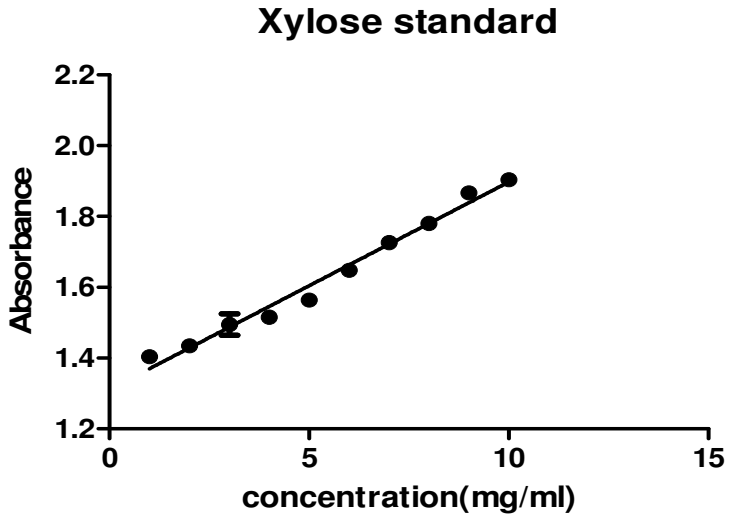


Figure13 Xylose standard graph from 1mg/ml to 6mg/ml.

3.10.3) Carbohydrate utilization test

3.10.3.1) Extracellular Xylose assay

Transformed and untransformed colonies were separately picked up and inoculated in YEPD medium and allowed to grow at 28˚ C O/N . The cell culture was harvested and made for same OD. Then, the culture was was centrifuged and cell pellet was washed with autoclaved deionized water, to remove the excess glucose of the medium. The cell pellet was then grown in YEP medium with 20g/l xylose at 28˚C with 250 rpm and 1ml cultures were harvested at 6 hour time interval up to 30 hours. The culture was tested for xylose uptake by measuring xylose remaining in the broth by phloroglucinol assay. 200µl of the sample was taken and treated with 5 ml freshly prepared phloroglucinol reagent and boiled for 5-10 minutes .Standard xylose was also made of 0.1mg/ml to 6 mg/ml. The absorbance was taken at 554nm in a Spectrophotometer (Genesys 10vis Thermoscientific). The experiment was performed five times and the average mean was further calculated for data analysis. Xylose quantification was performed by the standard data of xylose.

3.10.3.2) Intracellular xylose assay

Intracellular xylose accumulation was determined essentially as described (Hector *et al.,* 2008). Transformed yeast colonies and untransformed control colonies were picked up and inoculated in YEPD medium with 20g/l glucose grown at 28˚C for O/N. Control and transformed cell were made for same OD. The culture was centrifuged and cell pellet was washed with autoclaved deionized water for 3-4 times for complete removal of traces of glucose from the medium , and grown in YEPX medium with 20g/l xylose incubated at 30 ˚C, and 1ml of culture was harvested at 6 hour time interval up to 30 hours. The cell pellet was washed with deionized autoclaved water 2-3 times, and then incubated in 2ml of ddwater at 37˚ C at 250 rpm for 2 days for the lysis of cell and liberation of intracellular xylose from the cell. Then the xylose concentration was measured using phloroglucinol assay; 200µl of sample was mixed with 5 ml of phloroglucinol reagent and boiled for 5-10 mins and the absorbance was measured at 554nm using spectrophotometer (Genesys 10vis Thermoscientific). The experiment was repeated for five times and the average mean was taken for the data analysis. Quantification of xylose was done using standard data of xylose.

3.10.4) Ethanol production test

Ethanol production assay was done using dichromate test (Seo *et al.,* 2009). Transformed and untransformed yeast colonies were separately inoculated in YEP containing 20g/l xylose , and grown for same cell OD . The cell culture was then grown anaerobically at 28˚ C for 7 days. Then ethanol production was tested by dichromate

test. 1ml of culture was mixed with 1 ml of TBP (Tributyl Phosphate) with vigorous vortexing. The 700µl of upper phase was transferred to new tube and 700µl of dichromate reagent was added and then mixed by vortexing vigorously. Then, lower phase was taken for ethanol test by measuring absorbance at 595nm in Spectrophotometer (Genesys 10vis Thermoscientific). Experiment was done for five times and the average mean was calculated for data analysis. Ethanol concentration measurement was done using standard ethanol calibration chart.

3.10.4.1) Ethanol standard

Standard chart of ethanol was prepared from 0.1mg/ml to 1mg/ml. Ethanol of known concentration was used and ethanol estimation was done by dichromate test. 1ml of the ethanol sample was was mixed with 1 ml of TBP (Tributyl Phosphate) with vigorous vortexing. The 700µl of upper phase was transferred to new tube and 700µl of dichromate reagent was added and then mixed by vortexing vigorously. Then, lower phase was taken for ethanol test by measuring absorbance at 595nm in Spectrophotometer (Genesys 10vis Thermoscientific). Experiment was done for five times and the average mean was calculated for data analysis

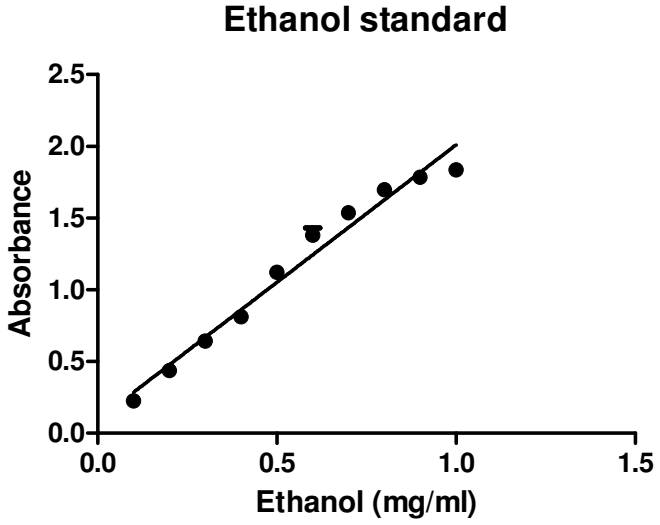


Figure14 Standard ethanol data from 0.1mg/ml to 1mg/ml.

3.10.5) X/G preference ratio

Xylose grown each transformed and untransformed cells of same OD was again inoclulated in YEP media with 20g/l glucose and 20g/l xylose by incubating at 28˚C, 250

rpm up to 48 hours. 1ml of the sample was taken out at 3 hour interval, up to 36 hour. The sample was tested for the remaining glucose and xylose in the media by DNS

method and phloroglucinol assay respectively. DNS method was done by boiling the mixture of 1 ml sample and 3ml DNS reagent for 5 min and adding 1ml of 40% potassium sodium tartarate solution was to stabilize the colour and the absorbance was taken reading at 575nm by Spectrophotometer (Genesys 10vis Thermoscientific). Phloroglucinol assay was performed by boiling the mixture of 200μl of sample and 5ml freshly prepared phloroglucinol reagent for 5-10 min and reading was taken at 554nm by Spectrophotometer (Genesys 10vis Thermoscientific). Glucose and xylose concentration was calculated using standard concentration of glucose and xylose. The X/G ratio was calculated by measuring the number of moles of xylose consumed by number of moles of glucose consumed in the medium during co- utilization of glucose/xylose.

$$\text{Preference ratio (X/G)} = \frac{\text{Number of moles of xylose decreased in medium}}{\text{Number of moles of glucose decreased in the medium}}$$

4. Results

4.1) Transformation of pGXF1 in *E. coli*

Transformation was done in *E coli DH5α* competent cells using heat shock method and selection was done in LBA with ampicillin plates.

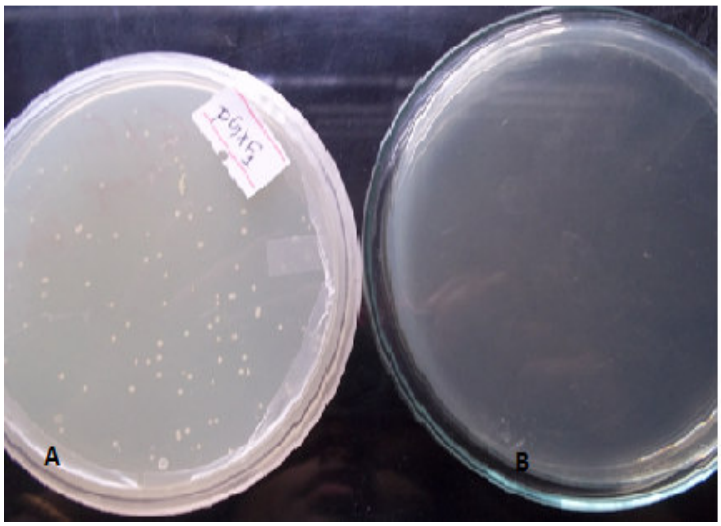


Figure 15 Transformation in *E coliDH5α* A) *E. coli* colonies transformed with GXF1 B) Negative control

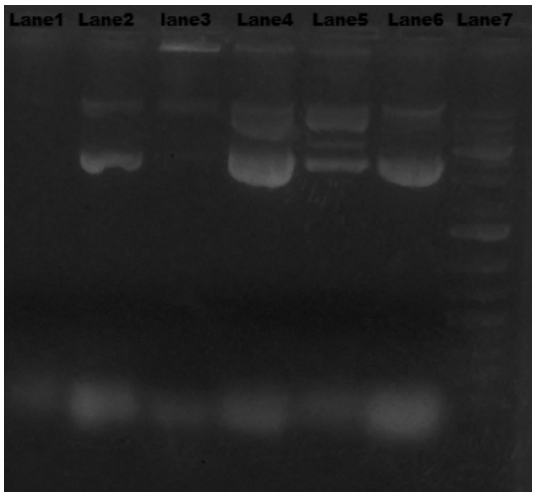
About 60 well grown colonies was seen for pGXF1 transformed cells in LBA ampicillin and no any growth was observed in negative control plate (untransformed *E coli* DH5 α in LBA ampicillin)

4.2) Confirmation of *E.coli* transformants

From the transformation plate, about five well grown far situated colonies were selected and grown in LB with ampicillin for plasmid isolation. A negative control was taken from the growth of untransformed *E coli* DH5α in LB. Confirmation of transformants was done by plasmid isolation and PCR method.

4.2.1) Plasmid isolation

Plasmid was extracted by using alkaline lysis method was confirmed by 1% agarose gel electrophoresis, and then visualized by UV transilluminator.



Lane 1- Control

Lane 2, 3, 4, 5, 6, – isolated plasmids

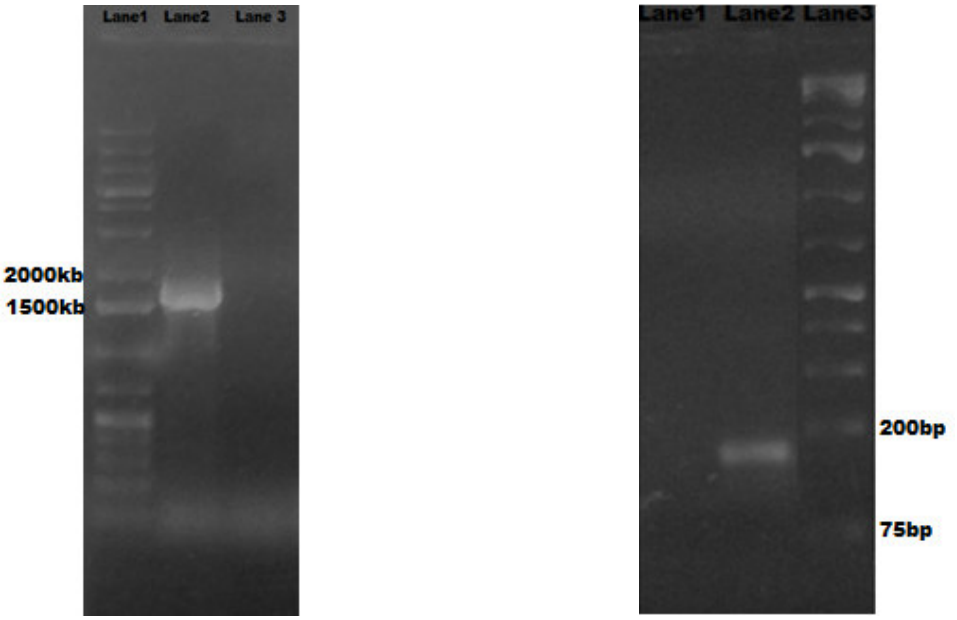
Lane7 – 1 Kb plus ladder

Figure16 Plasmid isolated from *E coli* transformants separated in gel.

Plasmid DNA bands was seen for all five colonies (Lane 2-6) while there was absence of band in the control (Lane 1).

4.2.2) Confirmation of *E coli* transformants (pGXF1) by PCR

Transformants were confirmed by PCR with two sets of primers. One set of primer (YTUF1F; YTUF1R) amplifies the the 1.6Kb fragment, and another set of primer (CiGXFL1; CiGXFR1) amplifies 163 bp of the GXF1 insert. Cloned plasmid DNA isolated from pGXF1 transformed and untransformed (control) *E coli* DH5α cells were used as template for PCR. PCR product was observed in 1% agarose gel electrophoresis with 1Kb plus geneuler (Thermoscientific) ladder and visualized by UV transilluminator.



Lane 1: 1Kb plus ladder

Lane 2: PCR product (pGXF1 transformed cell ,1.6Kb fragment)

Lane 3 : PCR product (control)

Lane 3: 1Kb plus ladder

Lane 2: PCR product (pGXF1 transformed cell ,163bp fragment)

Lane1: PCR product (Control)

Figure17 Confirmation of pGXF1 cloning in *E coli DH5α* by PCR.

PCR product when visualized by transilluminator, band was seen at 1.6Kb (Lane2) and 163bp (Lane 2) for two sets of primer (YTUF1F; YTUF1R) and (CiGXFL1; CiGXFR1) respectively. No bands were seen for control (lane 3, Lane 1).

4.3) Transformation in *S. cerevisiae*

Transformation in *S. cerevisiae* DTY165 was done by electroporation using electroporator and selection was done in YNB leucine selective media. Control cells when grown in leucine media showed growth; while in absence of leucine did not showed growth. The electroporated cells when grown in absence of leucine media showed growth.

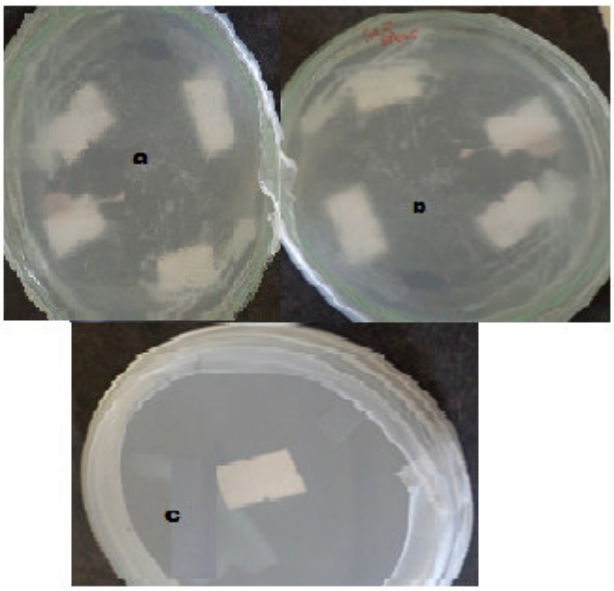


Figure18 a) Growth of Electroporated yeast DTY 165 without leucine b) Growth of control cells with leucine c) No growth of control cells without leucine.

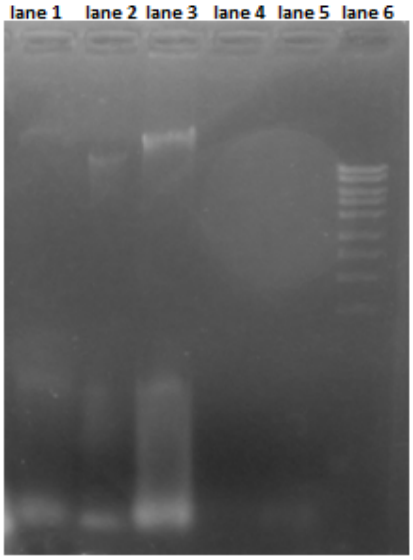
About 15-20 well grown colonies were seen for electroporated cells grown without leucine plate. No colonies were seen for control cells in the plate where leucine was not added. The control cells showed growth in the presence of leucine.

4.4) Confirmation of *S.cerevisiae* transformants

About 4 well grown transformed *S. cerevisiae* DTY165 colonies were selected and grown in YEPD media for the confirmation procedure. A control was taken from growing of the untransformed *S. cerevisiae* DTY165 in YEPD. Transformation in *S .cerevisiae* was confirmed by plasmid isolation and PCR.

4.4.1) Plasmid isolation

Four grown yeast colonies from transformation plate were picked up and a control was taken by the picking up the untransformed cells and growing in YEPD. Plasmid was isolated using lyticase.



Lane 1,Lane 2, Lane 3 - yeast plasmid

Lane5- Control

Lane 6: 10 kb plus ladder

Figure19 Plasmid extracted from transformed yeast cells separated in gel.

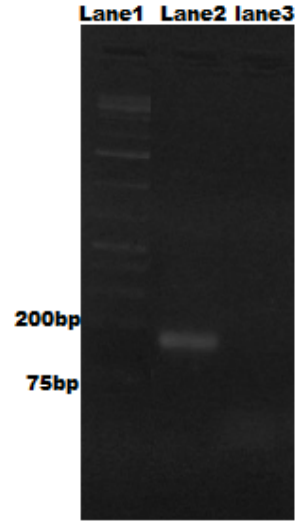
Among four yeast colonies, plasmid was seen in 3 colonies (Lane1-3) and no band was seen for control when run in 1% agarose gel. 1Kb plus thermoscientific ladder was used as marker. The gel was observed in UV transilluminator. The further confirmation of plasmid DNA for successful integration of GXF1 was done by PCR method.

4.4.2) Confirmation of *S. cerevisiae* transformants by PCR and Southern Blotting

Transformation of pGXF1 in *S. cerevisiae* was confirmed by PCR using a set of primer (F_w -CiGXFL1 and R_v -CiGXFR1). Confirmation of the pGXF1 transformation in *S. cerevisiae* was also performed by Southern Blotting using biotin labeled probe.

4.4.2.1) Confirmation of cloning in *S.cerevisiae* by PCR

The isolated plasmid was verified by PCR using a set of primer (F_w -CiGXFL1 and R_v -CiGXFR1) that amplifies 163bp of insert, GXF1 transformed cells and untranformed cells (control) was used as template for PCR.



Lane 1: 1 Kbplus ladder

Lane 2: PCR product, pGXF1 transformed cell (1.6Kb fragment)

Lane3: PCR product(Control without pGXF1)

Figure20 Amplification of 1.6kb band of pGXF1 by PCR.

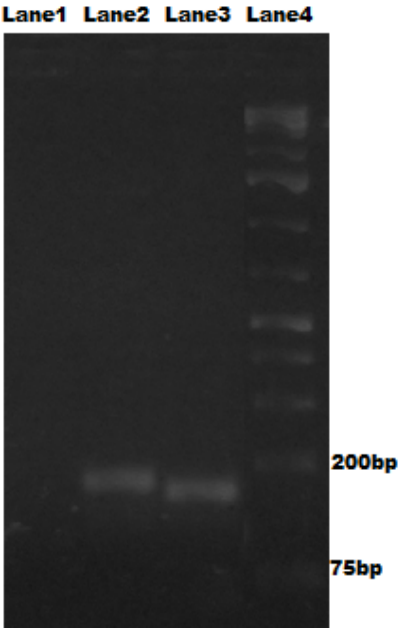
PCR product when run in 1% agarose gel with 1kb plus generuler (Thermoscientific) ladder and observed in UV transilluminator, a band of 163bp (lane 2) was seen for GXF1 transformed cells and no band was seen in control (lane 3).

4.4.2.2) Southern blotting of GXF1

The confirmation of GXF1 transformation in *Saccharomyces cerevisiae* was also done by Southern Blotting.

4.4.2.2.1) Probe preparation

Probe was prepared using PCR method using a set of primer (CiGXFL1 and CiGXFR1). Three PCR were done simultaneously; two with with GXF1 transformed plasmid DNA one with biotin labelled nucleotides and another with only normal nucleotides and a negative control was also taken with GXF1 untransformed DNA as template. PCR product was run in 2% agarose gel with 1kb plus generuler (Thermoscientific) ladder.



Lane1: PCR product (Control)

Lane 2: Product of PCR with biotin labelled nucleotides

Lane 3: Product of PCR with unlabelled nucleotides

Lane 4: 1Kb plus ladder

Figure21 Biotin labeled Probe preparation for Southern Blotting by PCR.

When the gel was visualized in UV transilluminator, biotin labeled PCR product (Lane 2) moved slightly slowly than the unlabelled PCR product(Lane 3). No bands was seen for control. This showed the successful preparation of probe.

4.4.2.2.2) Labelling efficiency of probe

Dilution of probe (15ng/µl) was done 100fold up to 1:100000000. 1µl of each unlabelled and diluted probes were used to hybridize the GXF1 gene from transformed plasmid DNA to check labelling efficiency. 1:100 was found to be the best concentration of probe for Southern blotting detection. So, for the southern blotting, 1/100 dilution was used.

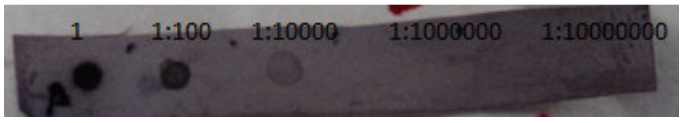


Figure22 Checking of the labelling efficiency of probe by probe dilution.

4.4.2.2.3) Detection of probe bound to GXF1 gene in membrane

The biotin labelled probe prepared by PCR was hybidrized to the positive transformed plasmid and the detection of probe was performed by Streptavidin –AP conjugate substrate.

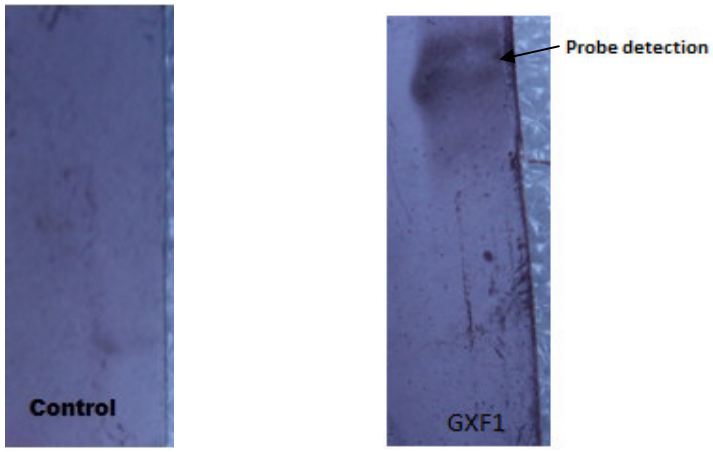


Figure23 Confirmation of transformation of *S. cerevisiae* by Southern Blotting.

Blue purple precipitate was visible in the GXF1 labelled hybridized probe, while no colouration was seen for negative control (GXF1 untransformed cells).

4.5) Functional Expression analysis

4.5.1) Growth Kinetics in glucose (20g/l) medium

Transformed and untransformed cells were grown in 20g/l glucose up to 36 hr and OD was checked at 3 hr time interval by spectrophotometer at 600nm.

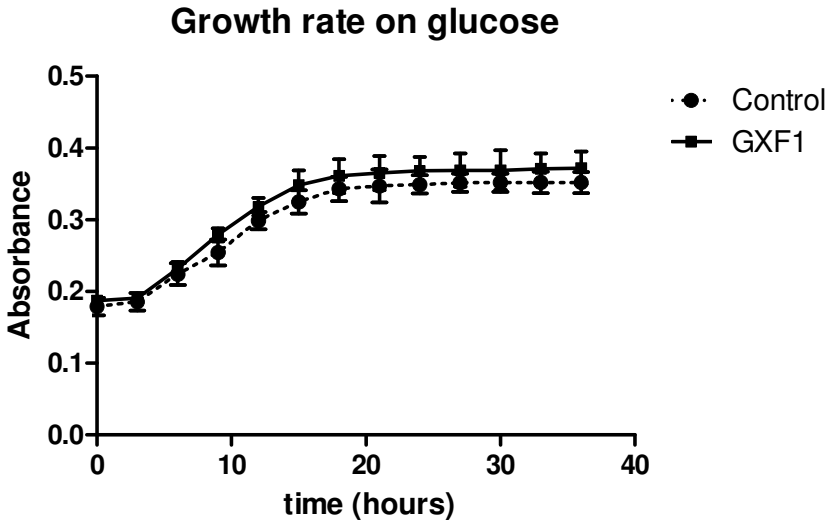


Figure24 Growth kinetics of GXF1 transformed *S. cerevisiae* (upper) and control (lower) growing on glucose. (OD measured at 600nm)

Specific growth rate on glucose20g/l is

Cells	Specific growth rate (hr^{-1})
Control	0.018
GXF1 transformed	0.01908

The result showed GXF1 transformed *S. cerevisiae* with slightly higher growth rate compared to GXF1 untransformed cells when grown in glucose medium. There was no significance difference in growth kinetics in GXF1 transformed cells and untransformed cells.

Both transformed and untransformed cells showed a good increase of growth rate on glucose up to 18 hours; however growth rate was seen slight elevated for GXF1 transformed cells compared to the control cells. T- Test was performed and P value was calculated to be 0.54 which stated there is no significant difference in growth for both control cells and GXF1 transformed cells at alpha value 0.05 (95% confidence interval). Specific growth rate observed was $0.018hr^{-1}$ and $0.01908\ hr^{-1}$ for control and GXF1 transformed cells. 6% increase in growth rate was seen for GXF1 transformed cells compared to control cells.

4.5.2) Growth kinetics in Xylose (20 g/l) medium.

Transformed and untransformed cells were grown in 20g/l xylose up to 36 hr and OD was measured at 3 hr time interval, absorbance was measured in spectrophotometer at 600nm.

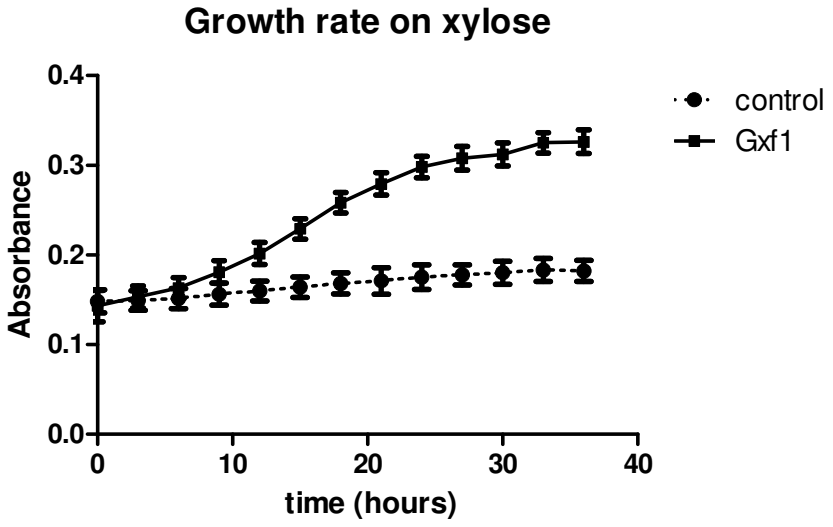


Figure25 Growth kinetics of control (lower) and GXF1 transformed (upper) *S. cerevisiae* growing on xylose containing media. (OD measured at 600nm)

Specific growth rate on 20g/l xylose

Cells	Specific growth rate (hr^{-1})
Control	0.00572
GXF1 transformed	0.022

The result of the present work showed the significantly higher growth rate of the GXF1 transformed *S. cerevisiae* compared to GXF1 untransformed cells when grown in xylose medium. There is significantly higher growth kinetics of GXF1 transformed cells compared to control cells.

Transformed *S. cerevisiae* cells showed a good growth rate on 20g/l xylose. Slight increase in growth rate was seen for both control cells and GXF1 transformed cells up to 9 hours. After 9 hours, GXF1 transformed yeast cells showed a high increase in growth rate up to 30 hours while control cells showed no significant increase in growth rate. T-test was performed and P value was calculated to be 0.0015 which stated there is significant difference in growth for control cells and GXF1 transformed cells at alpha value 0.05 (95% confidence interval). Specific growth rate was observed 0.00572hr^{-1} and 0.022hr^{-1} for control and transformed cells respectively. The GXF1 transformed cells showed 249.65 % increase in growth rate compared to control cells.

4.5.3) Carbohydrate utilization test

4.5.3.1) Extracellular xylose concentration

Extracellular xylose concentration was estimated by growth of transformed cells and untransformed cells on 20g/l xylose up to 30 hr taking out the sample at 3 hour time interval. The depletion of xylose in the media was estimated by the xylose remaining in the broth by phloroglucinol assay and the absorbance was measured at 554nm in Spectrophotometer.

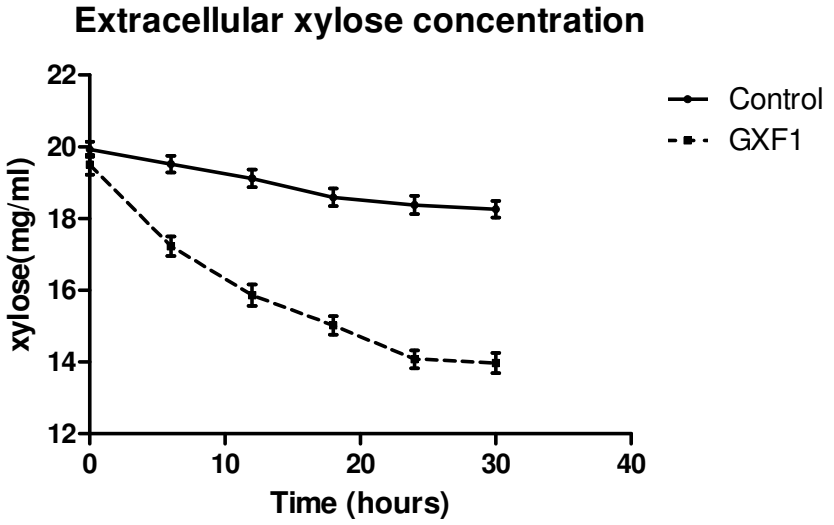


Figure26 Extracellular xylose concentration of control (upper) and pGXF1 transformed yeast (lower) growing in xylose medium by phloroglucinol test.

The result showed highly decreased extracellular xylose concentration decreased in pGXF1 transformed *S. cerevisiae* cells compared to untransformed cells when grown in

xylose. There was a significant difference for the decrease in extracellular xylose concentration between control cells and transformed cells.

Both transformed and untransformed cells showed a decrease in extracellular xylose concentration, where GXF1 transformed cells showed a more decrease in the xylose concentration compared to the control cells. T- Test was performed and P value was calculated to be 0.0158 which stated there is a significant difference between decrease in extracellular xylose concentration for control and GXF1 at alpha value 0.05 (95% confidence interval). Extracellular xylose concentration was seen to decrease from 19.73mg/ml to 18.263mg/ml in control cells; while GXF1 transformed cells showed decrease from 19.50mg/ml to 13.97mg/ml. Hence, control cells showed 12.5 % decrease of extracellular xylose concentration while GXF1 transformed cells showed 28.35 % decrease in extracellular xylose concentration.

4.5.3.2) Intracellular accumulation of xylose

The transportation of xylose inside the cells was confirmed by measuring the intracellular xylose concentration at different 3 hour interval up to 30 hours. The xylose test was performed by phloroglucinol assay and OD was measured at 554nm by Spectrophotometer.

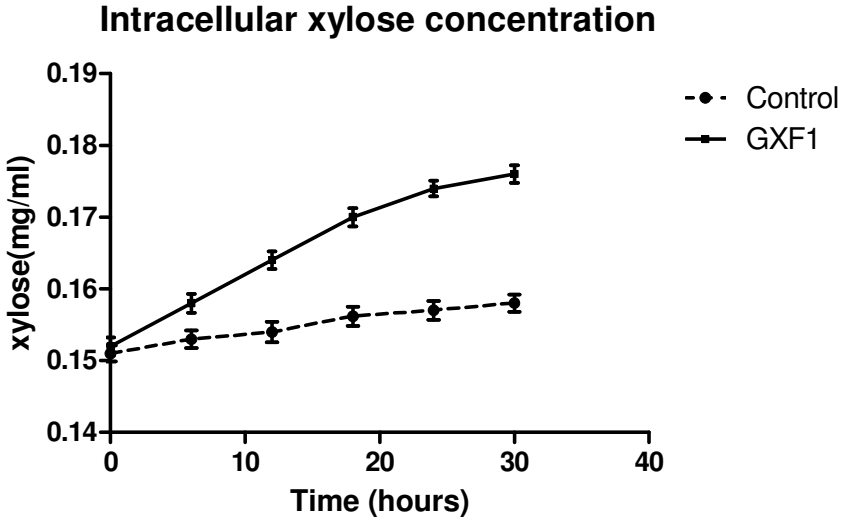


Figure27 Intracellular xylose concentration in control (lower) and pGXF1 transformed yeast (upper) measured by phloroglucinol assay at different time period.

The result showed the increase in intracellular xylose level for both control and transformed cells in which the pGXF1 transformed *S. cerevisiae* showed significant increment in intracellular xylose concentration compared to control cells.

The intracellular accumulation of xylose was seen more for the transformed cells; control cells showed slight lower increase in intracellular xylose concentration. T- Test was performed and P value was calculated to be 0.035 which stated there is a significant difference in the intracellular accumulation of xylose for control and GXF1 at alpha value 0.05 (95% confidence interval). Control cells showed increase of intracellular xylose concentration from 0.151mg/ml to 0.158mg/ml while, GXF1 transformed cells showed increase from 0.152mg/ml to 0.176mg/ml. Hence control cells showed 5.9% increase in intracellular xylose accumulation while, GXF1 transformed cells showed 15.78% increase in intracellular xylose accumulation.

Study of the decrease of extracellular xylose in the medium and the increase of intracellular xylose concentration inside the cells up to 30 hours was done for the confirmation of xylose transport kinetics. Control cells showed, 12.5 % decrease of xylose in the medium and GXF1 transformed cells showed 28.35% decrease in xylose concentration in the medium up to 30 hours. Similarly, intracellular xylose concentration for control cells showed 5.9% increase in xylose concentration while GXF1 transformed cells showed 15.78% increase in xylose concentration up to 30 hours. There does exist a strong negative correlation between the variables of intracellular xylose concentration and extracellular xylose concentration. The correlation coefficient (r) was calculated -0.994 and -0.983 for control cells and transformed cells respectively. This meant the increment of xylose concentration inside the cells is in correspondence with the decrease xylose concentration in the medium. Correlation of the three variables, growth rate on xylose, intracellular xylose concentration and extracellular xylose concentration was analysed by using multiple correlation coefficient which gave a value of 0.994 ± 0.228 and 0.987±0.732 for control and GXF1 respectively. The value is near to 1 which indicated the stronger linear association between the three variables for transformed and control cells.

4.5.4) Ethanol production test

Ethanol production by the transformed and untransformed *S .cerevisiae* was analyzed by anaerobically growing the cells for 7 days at 28˚C. Ethanol test was performed by dichromate test.

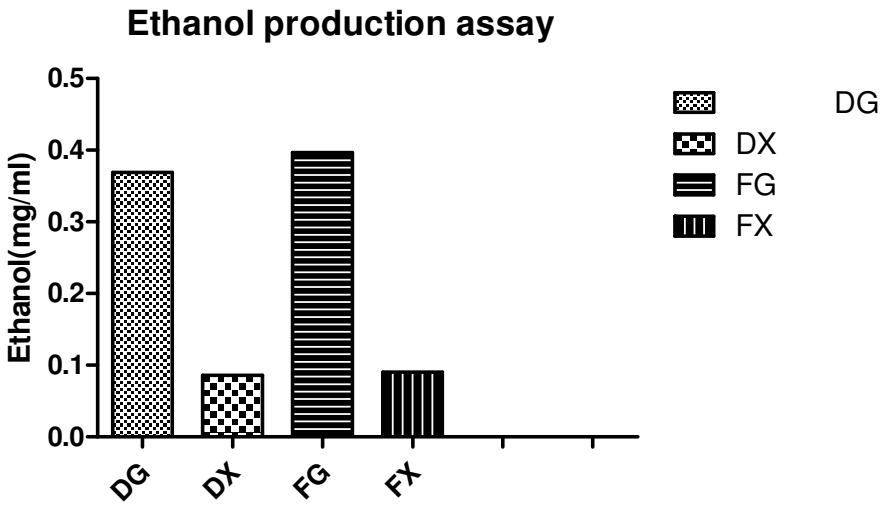


Figure28 Ethanol production by control and transformed yeast growing in xylose and glucose containing media.

Ethanol production test using dichromate method showed slightly higher ethanol production for GXF1 transformed cells compared to control cells when grown either in glucose or xylose. The ethanol production is high for both control and transformed cells when glucose was used in media compared to xylose medium.

Ethanol production for the control and transformed cells when grown on glucose and xylose each, transformed cells showed a slight high level of ethanol production compared to the control cells. Glucose grown cells of control and transformed cells showed ethanol production of 0.368 mg/ml and 0.396 mg/ml respectively. Xylose grown control and transformed cells showed ethanol production of 0.0863 mg/ml and 0.0902mg/ml respectively. GXF1 transformed cells showed increase in ethanol production of 7.6% and 4.5% for glucose grown and xylose grown cells respectively compared to control untransformed cells.

4.5.5) X/G preference ratio

Transformed and untransformed cells were grown in the mixture of 20g/l glucose and xylose up to 36 hrs. Samples were taken out at 3hr time interval. Co-utilization of glucose and xylose and the glucose/xylose transport kinetics was studied by measuring the depletion of glucose and xylose in the broth. The analysis of glucose and xylose was

done by DNS method and phloroglucinol assay respectively. The absorbance was measured by Spectrophotometer at 575nm and 554nm for DNS method and Phloroglucinol assay respectively and the xylose, glucose concentration was calculated from the absorbance data.

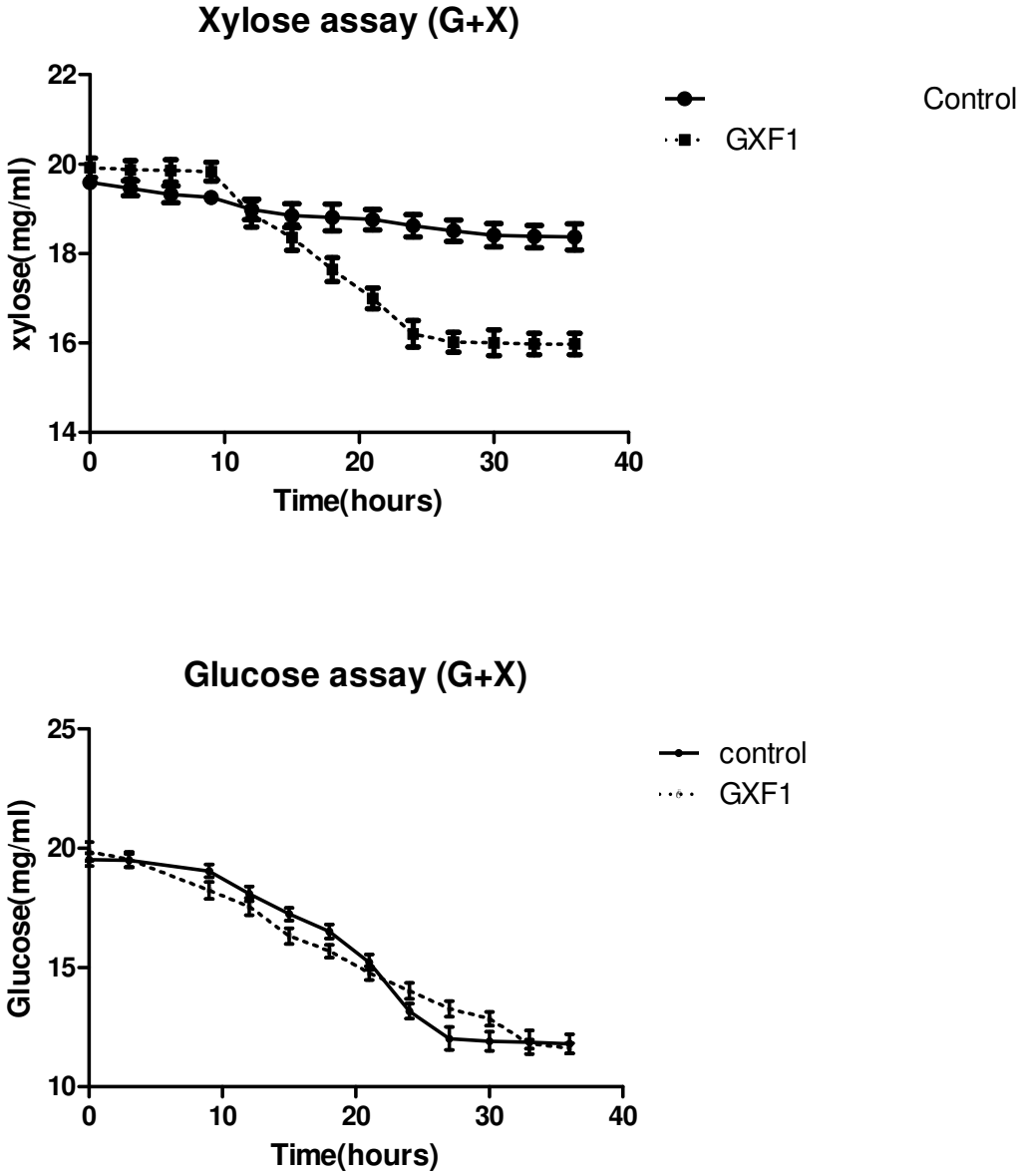


Figure29 Decrease of xylose (upper) and glucose (lower) in the media when *S. cerevisiae* with GXF1 and control were cultured in G+X medium for different time period.

The result concluded *S. cerevisiae* with GXF1 and control, both, did not show any significant difference in glucose transport when grown in glucose and xylose media. However, *S. cerevisiae* with GXF1 showed higher xylose transport after 10 hours when grown in glucose and xylose media, while no significant xylose reduction was seen for

control cells. Thus, pGXF1 transformed cells consumed significantly higher xylose compared to the control cell while no significant difference for glucose transport was seen for transformed and untransformed cells.

Xylose was transported very slowly by control whereas; good xylose transport kinetics was seen in transformed cells before the exhaustion of glucose. T- Test was performed and P value was calculated to be 0.048 which showed that there is a significant difference in decrease of extracellular xylose concentration between control and pGXF1 at alpha value 0.05 (95% confidence interval).

Glucose was utilized by both control and GXF1 from initial hours, although slightly high glucose was consumed by control cells. T- Test was performed and P value was calculated to be 0.97 which stated there is no significant difference in decrease of extracellular glucose concentration for both control and GXF1 at alpha value 0.05 (95% confidence interval).

The concentration of xylose in the media with control cells and transformed cells were found to be 18.37g/l and 15.97g/l; and the concentration of glucose in the media was found to be 11.78g/l and 11.54g/l respectively after 36 hours growth of *S. cerevisiae*. The number of moles of xylose consumed by GXF1 transformed cells and control cells were 0.0263 and 0.0081 respectively when measured after 36 hour. Also, the number of moles of glucose consumed by GXF1 transformed cells and control cells were 0.046 and 0.043 respectively when measured after 36 hour.

Hence, the molar consumption ratio (X/G preference ratio) for control and transformed *S .cerevisiae* is 0.18 and 0.571 respectively. Thus, it can be said that GXF1 transports near about 6 xylose molecules per 10 glucose molecules and the control transports near about 2 xylose molecules per 10 glucose molecules.

4.6) CONCLUDING RESULT

Successful transformation of pGXF1 in *E coli* by heat shock method was confirmed by PCR (Du *et al.,* 2010). The further transformation of pGXF1 in yeast was done by electroporation (Manivasakam & Schiestl 1993). Confirmation of transformation in *Saccharomyces cerevisiae* was confirmed by PCR (Makimura *et al.,* 1994) and Southern blotting (Baudin 1993).

Growth on glucose as a substrate showed slight high rate of growth for transformed compared to control, there was no significant difference in growth between transformed and control cells. When xylose sugar was used as a substrate, transformed cells showed significantly higher growth for transformed cells compared to control cells.

Xylose transport kinetics was measured using depletion of xylose in media and increment in intracellular xylose concentration inside cells. The depletion of xylose on the media was much more for GXF1 transformed cells compared to control cells. Similarly, the increment of intracellular xylose concentration was seen high for transformed cells compared to control cells. Hence, GXF1 transformed cells showed more xylose depletion from the media and more increment in intracellular xylose concentration than the control cells. Ethanol production by the control and transformed cells was high when used glucose as a substrate and very low when used xylose as a substrate but the ethanol production seemed slightly high for transformed compared to the control in both cases. X/G preference ratio was high for transformed cells (0.571) compared to control cells (0.18).

5.Discussion

5) DISCUSSION

The rising cost of petroleum and global warming has always been suggesting the search of alternative renewable fuel (Ragauskas *et al*., 2006). The production of bioethanol when prompted from second generation biomass i.e, lignocellulosic biomass is of more value for bioethanol production because of being economic, renewable and a non food material (Gomez *et al.,* 2008; Zabaniotou *et al.,* 2008). But the use of lignocellulosic biomass is naturally not much preffered for *Saccharomyces cerevisiae* because of its high /xylose content (Glazer & Nikaido, 2007), and the inability of baker's yeast to utilize xylose (Subtil and Boles 2011). The pentose utilization by yeast is a concerning problem for xylose utilization by *Saccharomyces cerevisiae* because of its inability to transport D-xylose inside the cell (Eliasson *et al.,* 2000a).

Wild *S. cerevisiae* lacks the potentiality to ferment pentose sugars (Subtil and Boles 2011), and also unable to utilize pentose until glucose is completely consumed (Kim *et al.,* 2012). This is because *S. cerevisiae* lacks specific pentose transporter and the pentose transport occurs through hexose transporter and galactose transporter system (Sedlak and Ho 2004) which are not high affinity transporter and not specific for xylose as well. Another problem associated with xylose utilization by *S. cerevisiae* is the xylose metabolism. Although, researches have shown *S. cerevisiae* cannot metabolize D-xylose (Van Maris *et al.,* 2007), but the gene encoding the reductive- oxidative xylose pathway enzymes XR, XDH, XK are present in its genome (Deng and Ho 1990; Kuhn *et al.,* 1995; Kuhn *et al.,* 1995; Richard *et al.,* 1999).

The importance of different xylose transporter for the utilization of lignocellulosic biomass for bioethanol production has been highlighted in many researches (Hector *et al.,* 2008, Leandro *et al.,* 2006; Saloheimo *et al.,* 2007, Hamacher *et al.,* 2002). In present work, a well characterized xylose transporter GXF1 (Leandro *et al.,* 2006) glucose xylose facilitator protein from *Candida intermedia* is expressed in *Saccharomyces cerevisiae* for enhancement of D- xylose transportation inside cell.

Results of the present work have shown the cloning of GXF1 on *Saccharomyces cerevisiae DTY165* improved its growth rate on glucose and xylose as well. Xylose uptake was confirmed by correspondence decrease of xylose in the media (28.35%) with increase in the intracellular xylose concentration inside the cell (15.78%). Ethanol production when analysed, ethanol production was slight high for transformed cells than control with the use of any of each substrate glucose or xylose. Ethanolic fermentation using glucose as a substrate led to high ethanol production (0.39g/l) while very low ethanol was produced from xylose substrate (0.09g/l) by transformed cells. X/G preference ratio was 0.57 for GXF1 recombinant *S. cerevisiae* whereas the control with only 0.18. GXF1 introduced *S. cererviviae* transported about 6 xylose molecules per 10

glucose molecules which is high preference to xylose compared with control which transported only 2 xylose molecules per 10 glucose molecules.

5.1) Growth kinetic measurement analysis

The growth on glucose was slightly higher for GXF1 transformed cells (μ =0.019 hr^{-1}) in comparision to control cells (μ =0.018 hr^{-1}). This might be attributed because GXF1 being a xylose and hexose transporter (Leandro *et al.,* 2006) and thus aids in transportation of hexoses too.

GXF1 transformed and control cells when grown on xylose media, the transformed cell showed a good increase in growth rate (μ =0.022hr^{-1}), while poor growth by control cells (μ= 0.00572 hr^{-1}). This highlights the functional expression of GXF1 in *S .cerevisiae*. The slight increase in growth rate in xylose by untransformed cells might be because of the role of different hexose transporters and galactose transporter playing the role of xylose transporter (Sedlak and Ho 2004; Young *et al.,* 2011; Hamacher *et al.,* 2002) with different affinity.

Resarches have shown the high specific growth rate by expression of GXF1 (0.04hr^{-1} (Runquist *et al.,* 2010) and μ= 0.081hr^{-1} (Parachin *et al.,* 2011) compared to the present work (μ=0.022). The higher growth rate of their results might be due to the expression of GXF1 in recombinant strain with overexpressed non- oxidative PPP genes in Runquist *et al.,* 2010 and XI overexpressing strain Parachin *et al.,* 2011 whereas in the present work, simple haploid laboratory yeast strain was used. Diao *et al.,* observed the *S. cerevisiae* strain with integrated xylose isomerase (XYLA) gene, endogenous non-oxidative PPP genes, XKS1 gene and GXF1 gene when led to adaptive evolution showed increased growth rate in xylose from μ= 0.096hr^{-1} to μ=0.187hr^{-1}.

Blast search of the GXF1 gene has showed high percentage similarity from 55% to 65% with different hexose transportes (shown in Appendix 8), which is the further evidence for possibility of hexose transporters acting also as xylose transporters; the uniprot result supports the fact of evolutionary compatibility of GXF1 gene from *Candida intermedia* with several hexose transporters present in *Saccharomyces cerevisiae.* This supports that GXF1 can be functionally expressed in *Saccharomyces cerevisiae*. High efficiency of GXF1 as xylose transporter in *Saccharomyces cerevisiae* was reported by comparision with different xylose transporters (Young *et al.,* 2011).

5.2) Extracellular and Intracellular xylose assay

In present work, xylose transport was determined by depletion of xylose in the media in correspondence to the similar increase in xylose intracellularly. In the result, the xylose concentration in the media decreased by 12.5 % in control cells and 28.35 % in the GXF1

transformed cells. Similarly, when intracellular xylose concentration were measured, control cells showed 5.9 % increment in the intracellular xylose concentration and GXF1 transformed cells showed 15.78% increment in intracellular xylose concentration. This , result confirms the GXF1 transformed *S. cerevisiae* transported more xylose inside the cell which is showed by higher xylose inside the cells and higher decrease in xylose in the medium for *S. cerevisiae* with GXF1 compared to the control cells without GXF1.

5.3) X/G preference ratio

Utilization of glucose and xylose was studied by growing *S. cerevisiae* in medium with both glucose and xylose. *S. cerevisiae* when grown in glucose/xylose media, GXF1 transformed cells showed increase in uptake of both glucose and xylose sugar from initial hours, similar results was also reported by Sonderegger and Sauer 2003. The control cells showed only a slight uptake of xylose along with high glucose uptake, this might be because of hexose and galactose transporters that worked as low affinity xylose transporter (Hamacher *et al.,* 2002, Lagunas *et al.,* 1982). Calculation of X/G ratio from the glucose/xylose substrates, in the present result it showed transformed cells to have high preference to xylose compared to control cells i.e. X/G preference ratio was found to be 0.18 and 0.57 for control and GXF1 respectively. High preferability of GXF1 transformed cells for xylose might be due to the role of GXF1 as a good xylose transporter. X/G ratio of GXF1 gene is quite high 0.57 compared to the X/G ratio of GXF1 result published in paper of Young *et al.,* 2011 (X/G ratio 0.10).This may be due to the use of hexose transporter null strain for GXF1 transformation by Young *et al.,* 2011 and the several hexose transporters present in the *S. cerevisiae DTY165* used in present study may have attributed for the increased xylose preference.

5.4) Ethanol production

Although, GXF1 transformed cells showed a good xylose uptake, but the ethanol production seemed relatively very low on xylose. It might be because D-xylose after entering inside cells needs to be converted to D-xylulose for entering in PPP to produce ethanol, and the conversion is done by 2 ways one oxidoreductive pathway (Kotter *et al.,* 1990) and other is xylose isomerase pathway (Walfridsson *et al.,* 1996; Brat *et al.,* 2009; Kuyper *et al.,* 2005) which are absent in wild *S. cerevisiae*.

Expression of xylose transporter does not necessarily conclude for increment in the ethanol production rate; this is predicted because of the excess formation of by product xylitol (Runquist *et al.,* 2009). Runquist *et al.,* 2009 concluded although more xylose was transported through GXF1 in recombinant *S.cerevisiae*, the ethanol production was not enhanced due to the excretion of by product as xylitol in higher amounts. Hence, xylose transport does not necessarily increase ethanol yield. In another research, the

expression of GXF1 in xylose metabolizing *S. cerevisiae* did not enhanced ethanol production although increased xylose consumption, this is due to the excessive byproduct glycerol formation (Parachin *et al .,*2011). Diao *et al.,* 2013 observed the increment of ethanol yield by GXF1 in *S. cerevisiae* adaptive evolved cells from 0.443g/l to 0.454g/l when grown in glucose/ xylose mixtures whereas, no increase in ethanol yield when grown in xylose only media. High efficiency *Arabidopsis thailiana* At5g59250 xylose transporter also showed 1.5g/l ethanol production from xylose used as substrate (Hector *et al.,* 2008). The high ethanol production can be attributed to the expression of the transporter done in recombinant *Saccharomyces cerevisiae* strain with integrated XYL1, XYL2, and XKS1 genes. Studies when performed regarding xylose metabolism in *S. cerevisiae*, ethanol production was found to be 0.9g/l when random mutagenesis was used for selection of ethanol producing mutated strains (Wahlbom *et al.,* 2003). About 3.2 g/l ethanol has been achieved by expression of XYL1 and XYL2 genes in *S. cerevisiae* (Kotter and Ciriacy 1992). Increment in ethanol production has been shown up to 0.43g/l by overexpression of XYLA, XR, XDH, XK and several non oxidative PPP (Karhumaa *et al* ., 2007b ,Madhavan *et al .,* 2008).

Blast search result of xylose isomerase gene (A9KN98) from *Clostridium phytofermentas* showed similarity in active site with *Saccharomyces cerevisiae* gene (Gal10, Uniprot id- P04397 and GCY1, Uniprot id- P14065) shown in appendix 8.1 and 8.2. Gal 10 is gene involved in galactose metabolism and hexose metabolism, while GCY1 is involved in Glycerol metabolism and also involved in D-xylose catabolic process. Uniprot alignment result showed the matching in active site for these genes with xylose isomerase encoding gene XylA from *Clostridium phytofermentans* which is a native xylose metabolizing enzyme. Hence, ethanol production from xylose may be attributed to the active site match of the *S. cerevisiae* genes Gal10 and GCY1 with XylA however, these genes are not native xylose metabolizing genes, and hence ethanol is not produced in high amount using xylose as a substrate. The introduction of xylose isomerase is believed to produce ethanol in large titres (Diao *et al.,* 2013; Moon *et al.,* 2013; Hector *et al.,* 2008). Hence it is concluded in this research that the low formation of ethanol although high xylose transport by the transporter is due to lack of xylose metabolizing pathways.

5.5) Concluding discussion

Growth on glucose substrate showed slight high growth rate for GXF1 transformed cells compared to control cells , which might be due to the GXF1 acting as a glucose as well as xylose transporter (Leandro *et al.,* 2006). When xylose was used as a substrate, GXF1 transformed *S. cerevisiae* cells showed high growth rate compared to control cells. The

low growth rate of untransformed cells can be attributed to the presence of hexose transporter which might act as low effficiency xylose transporter (Hamacher *et al.,* 2002, Lagunas *et al.,* 1982). The xylose transport was supported by the analysis of the extracellular and intracellular xylose concentration. Extracellular xylose in the media is decreased by 12.5% and 28.35% for control cells and GXF1 transformed cells respectively during 30 hours culture; whereas intracellular xylose concentration seemed to increase by 5.9% and 15.78 % for control cells and GXF1 transformed cells respectively during 30 hours culture. Therefore, the depletion of xylose in the media as well the increment in the intracellular xylose inside the cells was more for the GXF1 transformed cells compared to the control cells, this showed the good function of GXF1 as a xylose transporter. Glucose and xylose utilization when done, showed simultaneous glucose and xylose uptake by GXF1 cells, while in control cells showed initial glucose uptake more than xylose. GXF1 transfomed cells showed high preference to xylose (X/G = 5.7) than the control cells (X/G= 1.8). The high preference of GXF1 for xylose can be attributed for the good role of GXF1 as a xylose transporter. Ethanol production test showed high ethanol yield when glucose was used as a carbon source, while very low ethanol yield when xylose was used as a substrate, this might be because of some defect in xylose conversion to ethanol, so the D-xylose after being transported inside cannot go inside the metabolism that leads to ethanol production. However, slight ethanol was produced from xylose sugar , this is supported by the blast search result of xylose isomerase gene (A9KN98), a xylose metabolizing crucial gene, shows similarity in active site with *Saccharomyces cerevisiae* gene Gal10 (Uniprot id- P04397) and GCY1 (Uniprot id- P14065).

6) Conclusion

Production of fuel ethanol from the cheap renewable substrates has been limited mainly because of inability of the *Saccharomyces cerevisiae* to ferment the abundant sugar present in lignocellulosic biomass. Glucose is easily transported and metabolized by wild *S. cerevisiae* but xylose sugar is not utilized may be due to the lack of xylose transporter. Consumption of glucose alone would produce low level of ethanol in comparision if xylose could also be utilized when lignocellulosic substrate is used for fermentation. In present study, xylose transporter encoding gene GXF1 from *Candida intermedia* has been expressed in *S. cerevisiae* for xylose transportation.

In this research, the transformation of xylose transporter GXF1, glucose/xylose faciliator has been done in yeast *S. cerevisiae* and the functional parameters have been analysed for the xylose uptake and ethanol production. GXF1 transformed yeast cells has been shown for good xylose transport kinetics while very low ethanol production observed in this resarch might be due to the lack of xylose metabolizing pathways leading to ethanol production. Further development of xylose metabolizing pathways would probably lead to the development of *S. cerevisiae* for utilization of lignocellulosic biomass to give high titres of bioethanol.

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Appendix 1: Basic information of oligonucleotides

Appendix 1.1: Primer used for the amplification of 1.6Kb ORF

Fw: GACTA ***GAGCTCCATATG*** TCACAAGATTCGCATTC

Rv: TACTGT ***GGATCC*** CAT ***TCTAGA*** TTAAACCTGTTCGTCGGTG

ATGTCACAAGATTCGCATTCTTCTGGTGCCGCTACACCAGTCAATGGTTCCATCCTTGAAAAGGAAAAAG

AAGACTCTCCAGTTCTTCAAGTTGATGCCCCACAAAAGGGTTTCAAGGACTACATTGTCATTTCTATCTT

CTGTTTTATGGTTGCCTTCGGTGGTTTCGTCTTCGGTTTCGACACTGGTACCATTTCCGGTTTCGTGAAC

ATGTCTGACTTTAAAGACAGATTCGGTCAACACCACGCTGATGGTACTCCTTACTTGTCCGACGTTAGAG

TTGGTTTGATGATTTCTATTTTCAACGTTGGTTGCGCTGTCGGTGGTATTTTCCTCTGCAAGGTCGCTGA

TGTCTGGGGTAGAAGAATTGGTCTTATGTTCTCCATGGCTGTCTACGTTGTTGGTATTATTATTCAGATC

TCTTCATCCACCAAGTGGTACCAGTTCTTCATTGGTCGTCTTATTGCTGGTTTGGCTGTTGGTACCGTTT

CTGTCGTTTCCCCACTTTTCATCTCTGAGGTTTCTCCAAAGCAAATTAGAGGTACTTTAGTGTGCTGCTT

CCAGTTGTGTATCACCTTGGGTATCTTCTTGGGTTACTGTACTACTTACGGTACTAAGACCTACACTGAC

TCTAGACAGTGGAGAATTCCTTTGGGTTTGTGTTTCGCTTGGGCTATCTTGTTGGTTGTCGGTATGTTGA

ACATGCCAGAGTCTCCAAGATACTTGGTTGAGAAGCACAGAATTGATGAGGCCAAGAGATCCATTGCCAG

ATCCAACAAGATCCCTGAGGAGGACCCATTCGTCTACACTGAGGTTCAGCTTATTCAGGCCGGTATTGAG

AGAGAAGCTTTGGCTGGTCAGGCATCTTGGAAGGAGTTGATCACTGGTAAGCCAAAGATCTTCAGAAGAG

TTATCATGGGTATTATGCTTCAGTCCTTGCAACAGTTGACCGGTGACAACTACTTCTTCTACTACGGTAC

TACCATTTTCCAGGCTGTCGGTTTGAAGGATTCTTTCCAGACTTCTATCATTTTGGGTATTGTCAACTTT

GCTTCCACCTTCGTTGGTATCTATGTCATTGAGAGATTGGGTAGAAGATTGTGTCTTTTGACCGGTTCCG

CTGCTATGTTCATCTGTTTCATCATCTACTCTTTGATTGGTACTCAGCACTTGTACAAGCAAGGTTACTC

CAACGAGACCTCCAACACTTACAAGGCTTCTGGTAACGCTATGATCTTCATCACTTGTCTTTACATTTTC

TTCTTTGCTTCTACCTGGGCTGGTGGTGTTTACTGTATCATTTCCGAGTCCTACCCATTGAGAATTAGAT

CCAAGGCCATGTCTATTGCTACCGCTGCTAACTGGTTGTGGGGTTTCTTGATTTCCTTCTTCACTCCATT

CATCACCAGTGCCATCCACTTCTACTACGGTTTCGTTTTCACTGGTTGTTTGGCTTTCTCTTTCTTCTAC

GTCTACTTCTTCGTCTACGAAACCAAGGGTCTTTCTTTGGAGGAGGTTGATGAGATGTACGCTTCCGGTG

TTCTTCCACTCAAGTCTGCCAGCTGGGTTCCACCAAATCTTGAGCACATGGCTCACTCTGCCGGTTACGC

TGGTGCTGACAAGGC**CACCGACGAACAGGTTTAA**

S.No	Specifications		Forward Primer	Reverse Primer
Oligo Calculator Results				
1	Annealing sequence	T_m	54	49
		G + C	40	47
		length	20	19
2	T_m	Basic	63	62
		Salt adjusted	72	71
		Nearest neighbor	64	62
3	G + C content		44	40
4	No. of base pairs		34	35
5	Secondary structure			
	T_m		26	22.6
	dG		0.99	0.91
	3` end		Free	Free
DNA FOLD Results				
6	Secondary structure			
	Tm		35	36.4
	dG		0.14	0.03
	3` end		Free	Free
Oligo Analyzer Results				
7	Self dimer: with restriction site		-9.49	-10.76
	without restriction site		-7.82	-7.31
8	Hetero dimer		-10.51	
9	Amplicon size		1644bp	

Appendix 1.2: Primer amplifying 163bp of insert

Fw: 5’ GACTA ***GAGCTCCATATG*** TCACAAGATTCGCATTC 3’ (NdeI/SacI) (YTUF1F)

Rv: 5’ TACTGT ***GGATCC*** CAT***TCTAGA*** TTAAACCTGTTCGTCGGTG 3’ (XbaI/BamHI) (YTUF1R)

ATGTCACAAGATTCGCATTCTTCTGGTGCCGCTACACCAGTCAATGGTTCCATCCTTGAAAAGGAAAAAG

AAGACTCTCCAGTTCTTCAAGTTGATGCCCCACAAAAGGGTTTCAAGGACTACATTGTCATTTCTATCTT

CTGTTTTATGGTTGCCTTCGGTGGTTTCGTCTTCGGTTTCGACACTGGTACCATTTCCGGTTTCGTGAAC

ATGTCTGACTTTAAAGACAGATTCGGTCAACACCACGCTGATGGTACTCCTTACTTGTCCGACGTTAGAG

TTGGTTTGATGATTTCTATTTTCAACGTTGGTTGCGCTGTCGGTGGTATTTTCCTCTGCAAGGTCGCTGA

TGTCTGGGGTAGAAGAATTGGTCTTATGTTCTCCATGGCTGTCTACGTTGTTGGTATTATTATTCAGATC

TCTTCATCCACCAAGTGGTACCAGTTCTTCATTGGTCGTCTTATTGCTGGTTTGGCTGTTGGTACCGTTT

CTGTCGTTTCCCCACTTTTCATCTCTGAGGTTTCTCCAAAGCAAATTAGAGGTACTTTAGTGTGCTGCTT

CCAGTTGTGTATCACCTTGGGTATCTTCTTGGGTTACTGTACTACTTACGGTACTAAGACCTACACTGAC

TCTAGACAGTGGAGAATTCCTTTGGGTTTGTGTTTCGCTTGGGCTATCTTGTTGGTTGTCGGTATGTTGA

ACATGCCAGAGTCTCCAAGATACTTGGTTGAGAAGCACAGAATTGATGAGGCCAAGAGATCCATTGCCAG

ATCCAACAAGATCCCTGAGGAGGACCCATTCGTCTACACTGAGGTTCAGCTTATTCAGGCCGGTATTGAG

AGAGAAGCTTTGGCTGGTCAGGCATCTTGGAAGGAGTTGATCACTGGTAAGCCAAAGATCTTCAGAAGAG

TTATCATGGGTATTATGCTTCAGTCCTTGCAACAGTTGACCGGTGACAACTACTTCTTCTACTACGGTAC

TACCATTTTCCAGGCTGTCGGTTTGAAGGATTCTTTCCAGACTTCTATCATTTTGGGTATTGTCAACTTT

GCTTCCACCTTCGTTGGTATCTATGTCATTGAGAGATTGGGTAGAAGATTGTGTCTTTTGACCGGTTCCG

CTGCTATGTTCATCTGTTTCATCATCTACTCTTTGATTGGTACTCAGCACTTGTACAAGCAAGGTTACTC

CAACGAGACCTCCAACACTTACAAGGCTTCTGGTAACGCTATGATCTTCATCACTTGTCTTTACATTTTC

TTCTTTGCTTCTACCTGGGCTGGTGGTGTTTACTGTATCATTTCCGAGTCCTACCCATTGAGAATTAGAT

CCAAGGCCATGTCTATTGCTACCGCTGCTAACTGGTTGTGGGGTTTCTTGATTTCCTTCTTCACTCCATT

CATCACCAGTGCCATCCACTTCTACTACGGTTTCGTTTTCACTGGTTGTTTGGCTTTCTCTTTCTTCTAC

GTCTACTTCTTCGTCTACGAAACCAAGGGTCTTTCTTTGGAGGAGGTTGATGAGATGTACGCTTCCGGTG

TTCTTCCACTCAAGTCTGCCAGCTGGGTTCCACCAAATCTTGAGCACATGGCTCACTCTGCCGGTTACGC

TGGTGCTGACAAGGCCACCGACGAACAGGTTTAA

S.No	Specifications		Forward Primer	Reverse Primer
	Oligo Calculator Results			
1	Annealing sequence	T_m	58.4˚C	60.5˚C
		G + C	50	55
		length	20	20
2	T_m	Basic	51.8˚C	53.8˚C
		Salt adjusted	58.4˚C	60.5˚C
		Nearest neighbor	52.59˚C	53.11˚C
3	G + C content		50	55
4	No. of base pairs		20	20
5	Secondary structure			
	T_m		-16.13˚C	21.1˚C
	dG		1.58 Kcal/mol	1.15 Kcal/mol\
	3` end		Not free	Not Free
	DNA FOLD Results			
6	Secondary structure			
	Tm		4.1˚C	28˚C
	dG		0.90 Kcal/mol	0.63 Kcal/mol
	3` end		Free	Free
	Oligo Analyzer Results			
7	Self dimer: with restriction site		-3.61Kcal/mol	-3.61 Kcal/mol
8	Hetero dimer		-6.6 Kcal/mol	
9	Amplicon size		163bp	

Appendix 2: Bioinformatics Tools used for Primer Designing and other purposes

S.N	Bioinformatics tools	URL link
1	NCBI	http://www.ncbi.nlm.nih.gov/
2	Oligocalc	http://www.basic.northwestern.edu/biotools/oligocalc.html
3	Mfold	http://mfold.rit.albany.edu/?q=mfold
4	IDT	http://sg.idtdna.com/site

Appendix 3: Preparation of Stock solutions, Buffers and Reagents commonly used in Molecular Biology

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

Tris-Cl Buffer was prepared by adding 12.11g of Tris base in 80ml of ADW and pH was adjusted to 7.5 or 8 by adding concentrated HCl. Then final volume was maintained to 100ml. The solution was sterilized by autoclaving and stored at 4˚C.

EDTA (0.5M, pH 8.0)

18.61g of disodium EDTA.2H2O was added to 80ml of NFW, stirred vigorously on magnetic stirrer and pH was adjusted to 8.0 with NAOH. The final volume of the solution was made up to 100ml and sterilized by autoclaving and stored at 4˚C.

Tris EDTA (TE) buffer (pH 8.0)

10 mM Tris-Cl (pH 8.0)

1mM EDTA (pH 8.0)

Preparation of solutions for plasmid DNA extraction by Alkaline-lysis method.

Alkaline Lysis Solution I

50 mM glucose

25mM Tris-Cl (pH 8.0)

10 mM EDTA (pH 8.0)

The solution was sterilized by autoclaving and stored at 4˚C.

Alkaline Lysis solution II (Freshly prepared)

0.2N NAOH

1% w/v SDS

Freshly mixing of equal volume the above component will give lysis solution II.

Alkaline Lysis solution III

5 M Potassium acetate	60.0ml
Glacial acetic acid	11.5ml
H_2O	28.5ml

The solution was sterilized by autoclaving and stored at 4˚C.

Sodium acetate (3M, pH 5.2)

40.83 g of Sodium acetate.3 H_2O was dissolved in 80ml of deionized water and pH was adjusted to 5.2 with glacial acetic acid and final volume was made up to 100ml. The solution was sterilized by autoclaving and stored at 4˚C.

Preparation of solutions for Agarose Gel Electrophoresis

10× TAE Buffer

Tris base	4.84g
Glacial acetic acid	1.142ml
0.5 M EDTA (pH 8.0)	2ml
Deionized water	up to 100ml

The working solution (1X) of TAE buffer was prepared by diluting 10 ml of 10 X TAE with 90 ml of deionized water to make up the final volume 100ml.

10 X Gel loading Buffer

Bromophenol Blue	10.5mg
0.5M EDTA (pH 8.0)	2ml
98% Glycerol	2.5ml
Deionized water	up to 5ml

Ethidium Bromide (10mg/ml)

100mg of Ethidium Bromide was weighed and dissolved in 10 ml of deionized water. The solution was protected from sun light by wrapping with aluminium foil and stored at room temperature.

Antibiotics solution

Antibiotics	Stock Solution (-20˚C)	Working Solution	
		LB broth	LB agar plate
Ampicillin	100mg/ml in H_2O Filter sterilized	100μg/ml	100μg/ml

Appendix 4: List of media used in study

Luria Bertani (LB) media

2.5 g of LB media was weighed and dissolved in 100ml of ADW and sterilized by autoclaving. Once the medium is cooled (25˚C), appropriate antibiotics were supplemented if required and stored at 4˚C.

LB/Agar plate

LB medium 2.5g

Agar 1.5g

The volume was adjusted to 100ml with ADW and sterilized by autoclaving. Once the medium is cooled (60˚C), then appropriate antibiotics were added if required to make the desired concentration of antibiotics (as mentioned above) and the plates were stored at 4˚C.

YEPD media

Yeast Extract 5g

Peptone 10g

Dextrose 10g

The mixture was dissolved to make the final volume 500ml and the solution was sterilized by autoclaving and stored at 4˚C.

YEPX media

Yeast Extract 5g

Peptone 10g

Xylose 10g

The mixture was dissolved to make the final volume 500ml and the solution was sterilized by autoclaving and stored at 4˚C.

1M sorbitol

18.2 gm of Sorbitol was dissolved in 100ml of ADW.

DNS reagent

Dinitrosalicylic acid: 10 g

Phenol: 2 g

Sodium sulfite: 0.5 g

Sodium hydroxide: 10 g

Add water to: 1 liter

Potassium sodium Tartarate reagent

40g of Potassium sodium tartarate was added to 100ml of ADW.

Phloroglucinol reagent

Phloroglucinol 0.5g

Glacial acetic acid 100ml

Concentrated HCl 10ml

Potassium Dichromate Reagent Solution

Potassium dichromate: 1 g

Concentrated (6N) sulfuric acid to: 100 ml

Dissolved by Shaking.

Appendix5: PCR reaction mixture

Appendix5.1: PCR reaction mixture for confirmation

PCR reaction mixture	
Template	1μl
Unlabelled dNTPs mix(10mM each)	0.5μl
Taq buffer	5μl
Forward primer	1μl
Reverse primer	1μl
Taq polymerase(1U/μl	0.5μl
Nuclease free water	39.5μl
Total volume	50 μl

Appendix 5.2: Preparation of probe for southern blotting

PCR of 163bp insert

PCR reaction mixture with unlabelled nucleotides		PCR reaction mixture with biotin labelled nucleotides	
Template	1μl	Template	1μl
Unlabelled dNTPs mix(10mM each)	0.5μl	Biotin labelled dNTPs mix(10mM each)	0.5μl
Taq buffer	5μl	Taq buffer	5μl
Forward primer	1μl	Forward primer	1μl
Reverse primer	1μl	Reverse primer	1μl
Taq polymerase(1U/μl	0.5μl	Taq polymerase(1U/μl	0.5μl
Nuclease free water	39.5μl	Nuclease free water	39.5μl
Total volume	50 μl	Total volume	50 μl

Appendix6: Thermo Cycling Condition

Appendix 6.1: PCR amplification of 163bp fragment of GXF1

Step	Cycle	Temperature (˚C)	Time
Initial denaturation	1	95	5 min
Denaturation		95	30 sec
Annealing	30	59	30 sec
Extension		68	1 min 30 sec
Final extension	1	68	5 min
Hold	1	4	∞

Appendix 6.2: PCR amplification of 1.6Kb fragment GXF1

Step	cycle	Temperature(˚C)	Time
First denaturation	1	95	5 min
Second denaturation	5	95	30 sec
Annealing		49	30 sec
Extension		72	2 min
Denaturation	25	95	30 sec
Annealing		65	30 sec
Extension		72	2 min
Final Extension	1	72	5 min
Hold	1	4	∞

Appendix 7: Reagents used for Southern Blotting

Depurination solution 250 mM HCl

20.8 ml of concentrated HCl in 1 L of H_2O

Denaturation Solution: 0.5 M NaOH, 1.5 M NaCl

20g of NaOH, 58.4g of NaCl in liter of water

Neutralization solution: 0.5 M Tris-HCl (pH 7.5), 1.5 M NaCl

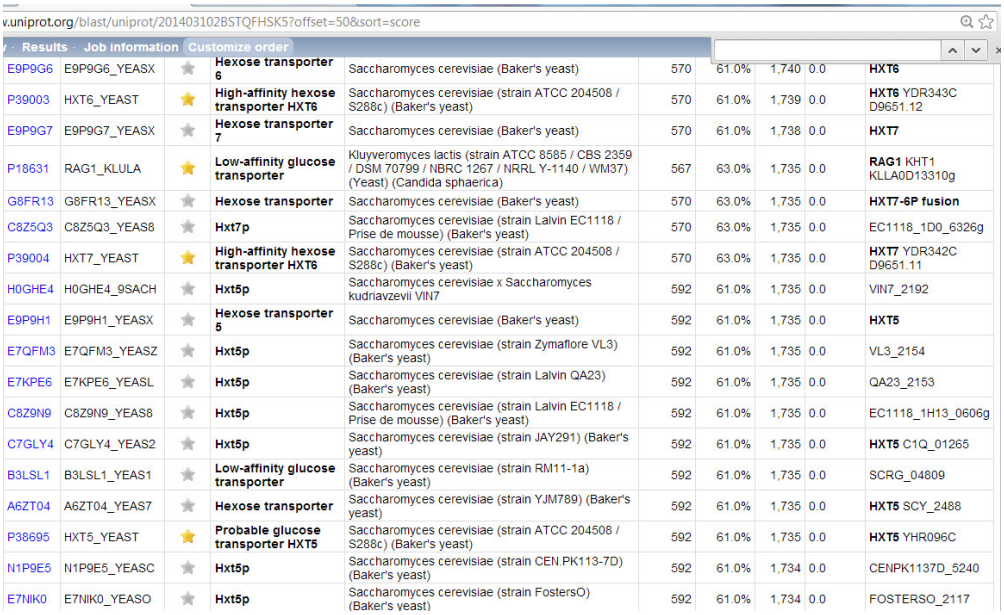
78.8g of Tris base, 87.6 g NaCl in 800ml of water

20×SSC

175.3 g of NaCl and 88.2 g of Sodium Citrate was dissolved in 800 ml water.The pH was adjusted to 7.0 with a few drops of 14 N solution of HCl. The volume was maintained to 1 liter.

Appendix 8: Uniprot database

Appendix 8.1 Evolutionary relationship of GXF1 with different hexose transporters of *Saccharomyces cerevisiae.*



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E9P9G6	E9P9G6_YEASX		**Hexose transporter 6**	Saccharomyces cerevisiae (Baker's yeast)	570	61.0%	1,740	0.0	**HXT6**
P39003	HXT6_YEAST	★	**High-affinity hexose transporter HXT6**	Saccharomyces cerevisiae (strain ATCC 204508 / S288c) (Baker's yeast)	570	61.0%	1,739	0.0	**HXT6** YDR343C D9651.12
E9P9G7	E9P9G7_YEASX		**Hexose transporter 7**	Saccharomyces cerevisiae (Baker's yeast)	570	61.0%	1,738	0.0	**HXT7**
P18631	RAG1_KLULA	★	**Low-affinity glucose transporter**	Kluyveromyces lactis (strain ATCC 8585 / CBS 2359 / DSM 70799 / NBRC 1267 / NRRL Y-1140 / WM37) (Yeast) (Candida sphaerica)	567	63.0%	1,735	0.0	**RAG1** KHT1 KLLA0D13310g
G8FR13	G8FR13_YEASX		**Hexose transporter**	Saccharomyces cerevisiae (Baker's yeast)	570	63.0%	1,735	0.0	**HXT7-6P fusion**
C8Z5Q3	C8Z5Q3_YEAS8		**Hxt7p**	Saccharomyces cerevisiae (strain Lalvin EC1118 / Prise de mousse) (Baker's yeast)	570	63.0%	1,735	0.0	EC1118_1D0_6326g
P39004	HXT7_YEAST	★	**High-affinity hexose transporter HXT6**	Saccharomyces cerevisiae (strain ATCC 204508 / S288c) (Baker's yeast)	570	63.0%	1,735	0.0	**HXT7** YDR342C D9651.11
H0GHE4	H0GHE4_9SACH		**Hxt5p**	Saccharomyces cerevisiae x Saccharomyces kudriavzevii VIN7	592	61.0%	1,735	0.0	VIN7_2192
E9P9H1	E9P9H1_YEASX		**Hexose transporter 5**	Saccharomyces cerevisiae (Baker's yeast)	592	61.0%	1,735	0.0	**HXT5**
E7QFM3	E7QFM3_YEASZ		**Hxt5p**	Saccharomyces cerevisiae (strain Zymaflore VL3) (Baker's yeast)	592	61.0%	1,735	0.0	VL3_2154
E7KPE6	E7KPE6_YEASL		**Hxt5p**	Saccharomyces cerevisiae (strain Lalvin QA23) (Baker's yeast)	592	61.0%	1,735	0.0	QA23_2153
C8Z9N9	C8Z9N9_YEAS8		**Hxt5p**	Saccharomyces cerevisiae (strain Lalvin EC1118 / Prise de mousse) (Baker's yeast)	592	61.0%	1,735	0.0	EC1118_1H13_0606g
C7GLY4	C7GLY4_YEAS2		**Hxt5p**	Saccharomyces cerevisiae (strain JAY291) (Baker's yeast)	592	61.0%	1,735	0.0	**HXT5** C1Q_01265
B3LSL1	B3LSL1_YEAS1		**Low-affinity glucose transporter**	Saccharomyces cerevisiae (strain RM11-1a) (Baker's yeast)	592	61.0%	1,735	0.0	SCRG_04809
A6ZT04	A6ZT04_YEAS7		**Hexose transporter**	Saccharomyces cerevisiae (strain YJM789) (Baker's yeast)	592	61.0%	1,735	0.0	**HXT5** SCY_2488
P38695	HXT5_YEAST	★	**Probable glucose transporter HXT5**	Saccharomyces cerevisiae (strain ATCC 204508 / S288c) (Baker's yeast)	592	61.0%	1,735	0.0	**HXT5** YHR096C
N1P9E5	N1P9E5_YEASC		**Hxt5p**	Saccharomyces cerevisiae (strain CEN.PK113-7D) (Baker's yeast)	592	61.0%	1,734	0.0	CENPK1137D_5240
E7NIK0	E7NIK0_YEASO		**Hxt5p**	Saccharomyces cerevisiae (strain FostersO) (Baker's yeast)	592	61.0%	1,734	0.0	FOSTERSO_2117

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H0GVU1	H0GVU1_9SACH	☆	**Hxt5p**	Saccharomyces cerevisiae x Saccharomyces kudriavzevii VIN7	592	62.0%	1,733	0.0	VIN7_7559
B5VK26	B5VK26_YEAS6	☆	**YHR096Cp-like protein**	Saccharomyces cerevisiae (strain AWRI1631) (Baker's yeast)	520	63.0%	1,732	0.0	AWRI1631_81470
C5DDE9	C5DDE9_LACTC	☆	**KLTH0C00462p**	Lachancea thermotolerans (strain ATCC 56472 / CBS 6340 / NRRL Y-8284) (Yeast) (Kluyveromyces thermotolerans)	570	63.0%	1,732	0.0	KLTH0C00462g
C8Z5Q4	C8Z5Q4_YEAS8	☆	**Hxt6p**	Saccharomyces cerevisiae (strain Lalvin EC1118 / Prise de mousse) (Baker's yeast)	570	62.0%	1,730	0.0	EC1118_1D0_6337g
E9P9G9	E9P9G9_YEASX	☆	**Hexose transporter 5**	Saccharomyces cerevisiae (Baker's yeast)	592	61.0%	1,730	0.0	**HXT5**
G8JN88	G8JN88_ERECY	☆	**Uncharacterized protein**	Eremothecium cymbalariae (strain CBS 270.75 / DBVPG 7215 / KCTC 17166 / NRRL Y-17582) (Yeast)	546	61.0%	1,726	0.0	Eqym_1317
J4U468	J4U468_SACK1	☆	**HXT6-like protein**	Saccharomyces kudriavzevii (strain ATCC MYA-4449 / AS 2.2408 / CBS 8840 / NBRC 1802 / NCYC 2889)	570	62.0%	1,726	0.0	**YDR343C** SKUD_164902
G8JN90	G8JN90_ERECY	☆	**Uncharacterized protein**	Eremothecium cymbalariae (strain CBS 270.75 / DBVPG 7215 / KCTC 17166 / NRRL Y-17582) (Yeast)	535	62.0%	1,725	0.0	Eqym_1319
Q6FW63	Q6FW63_CANGA	☆	**Strain CBS138 chromosome D complete sequence**	Candida glabrata (strain ATCC 2001 / CBS 138 / JCM 3761 / NBRC 0622 / NRRL Y-65) (Yeast) (Torulopsis glabrata)	564	63.0%	1,725	0.0	CAGL0D02640g CAGL0D02662g
G0VJ20	G0VJ20_NAUCC	☆	**Uncharacterized protein**	Naumovozyma castellii (strain ATCC 76901 / CBS 4309 / NBRC 1992 / NRRL Y-12630) (Yeast) (Saccharomyces castellii)	573	59.0%	1,724	0.0	**NCAS0H01880** NCAS_0H01880
Q6FY15	Q6FY15_CANGA	☆	**Strain CBS138 chromosome A complete sequence**	Candida glabrata (strain ATCC 2001 / CBS 138 / JCM 3761 / NBRC 0622 / NRRL Y-65) (Yeast) (Torulopsis glabrata)	588	62.0%	1,723	0.0	CAGL0A01826g
H0H2U6	H0H2U6_9SACH	☆	**Hxt11p**	Saccharomyces cerevisiae x Saccharomyces kudriavzevii VIN7	567	60.0%	1,722	0.0	VIN7_8183
H0GW34	H0GW34_9SACH	☆	**Hxt11p**	Saccharomyces cerevisiae x Saccharomyces kudriavzevii VIN7	567	63.0%	1,721	0.0	VIN7_7670
H0GRR1	H0GRR1_9SACH	☆	**Hxt11p**	Saccharomyces cerevisiae x Saccharomyces kudriavzevii VIN7	567	63.0%	1,721	0.0	VIN7_5797
H0GEG8	H0GEG8_9SACH	☆	**Hxt7p**	Saccharomyces cerevisiae x Saccharomyces kudriavzevii VIN7	570	63.0%	1,720	0.0	VIN7_1043
J7R449	J7R449_KAZNA	☆	**Uncharacterized protein**	Kazachstania naganishii (strain ATCC MYA-139 / BCRC 22969 / CBS 8797 / CCRC 22969 / KCTC 17520 / NBRC 10181 / NCYC 3082) (Yeast) (Saccharomyces naganishii)	564	60.0%	1,719	0.0	**KNAG0C05190** KNAG_0C05190
G8BX59	G8BX59_TETPH	☆	**Uncharacterized protein**	Tetrapisispora phaffii (strain ATCC 24235 / CBS 4417 / NBRC 1672 / NRRL Y-8282 / UCD 70-5) (Yeast) (Fabospora phaffii)	643	59.0%	1,718	0.0	**TPHA0H01570** TPHA_0H01570
G2WD77	G2WD77_YEASK	☆	**K7_Hxt9-2p**	Saccharomyces cerevisiae (strain Kyokai no. 7 / NBRC 101557) (Baker's yeast)	567	63.0%	1,717	0.0	**K7_HXT9-2** SYK7_022051
J7RJC6	J7RJC6_KAZNA	☆	**Uncharacterized protein**	Kazachstania naganishii (strain ATCC MYA-139 / BCRC 22969 / CBS 8797 / CCRC 22969 / KCTC 17520 / NBRC 10181 / NCYC 3082) (Yeast) (Saccharomyces naganishii)	564	60.0%	1,716	0.0	**KNAG0C05250** KNAG_0C05250
P32467	HXT4_YEAST	★	**Low-affinity glucose transporter HXT4**	Saccharomyces cerevisiae (strain ATCC 204508 / S288c) (Baker's yeast)	576	63.0%	1,714	0.0	**HXT4** LGT1 RAG1 YHR092C
G8ZV27	G8ZV27_TORDC	☆	**Uncharacterized protein**	Torulaspora delbrueckii (strain ATCC 10662 / CBS 1146 / NBRC 0425 / NCYC 2629 / NRRL Y-866) (Yeast) (Candida colliculosa)	587	62.0%	1,714	0.0	**TDEL0E02280** TDEL_0E02280
B3LFV6	B3LFV6_YEAS1	☆	**Putative uncharacterized protein**	Saccharomyces cerevisiae (strain RM11-1a) (Baker's yeast)	570	62.0%	1,713	0.0	SCRG_00188
P40885	HXT9_YEAST	★	**Hexose transporter HXT9**	Saccharomyces cerevisiae (strain ATCC 204508 / S288c) (Baker's yeast)	567	63.0%	1,712	0.0	**HXT9** YJL219W HRC667_J0222
Q6FY17	Q6FY17_CANGA	☆	**Strain CBS138 chromosome A complete sequence**	Candida glabrata (strain ATCC 2001 / CBS 138 / JCM 3761 / NBRC 0622 / NRRL Y-65) (Yeast) (Torulopsis glabrata)	568	60.0%	1,712	0.0	CAGL0A01782g
G2WFG7	G2WFG7_YEASK	☆	**K7_Hxt4p**	Saccharomyces cerevisiae (strain Kyokai no. 7 / NBRC 101557) (Baker's yeast)	576	63.0%	1,712	0.0	**K7_HXT4** SYK7_032121

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Entry	Entry name	Status	Protein names	Organism	Length	Identity	Score	E-value	Gene names
G2WFG7	G2WFG7_YEASK	☆	**K7_Hxt4p**	Saccharomyces cerevisiae (strain Kyokai no. 7 / NBRC 101557) (Baker's yeast)	576	63.0%	1,712	0.0	**K7_HXT4** SYK7_032121
C7GWV6	HXT4_YEAS2	★	**Low-affinity glucose transporter HXT4**	Saccharomyces cerevisiae (strain JAY291) (Baker's yeast)	576	63.0%	1,712	0.0	**HXT4** LGT1 RAG1 C1Q_04971
E7KDE7	E7KDE7_YEASA	☆	**Hxt4p**	Saccharomyces cerevisiae (strain AWRI796) (Baker's yeast)	576	63.0%	1,711	0.0	AWRI796_2159
A6ZT02	HXT4_YEAS7	★	**Low-affinity glucose transporter HXT4**	Saccharomyces cerevisiae (strain YJM789) (Baker's yeast)	576	63.0%	1,711	0.0	**HXT4** LGT1 RAG1 SCY_2486
E7KU07	E7KU07_YEASL	☆	**Hxt11p**	Saccharomyces cerevisiae (strain Lalvin QA23) (Baker's yeast)	567	63.0%	1,709	0.0	QA23_4239
C8ZHG1	C8ZHG1_YEAS8	☆	**Hxt11p**	Saccharomyces cerevisiae (strain Lalvin EC1118 / Prise de mousse) (Baker's yeast)	567	63.0%	1,709	0.0	EC1118_1O4_0056g
Q6FY41	Q6FY41_CANGA	☆	**Strain CBS138 chromosome A complete sequence**	Candida glabrata (strain ATCC 2001 / CBS 138 / JCM 3761 / NBRC 0622 / NRRL Y-65) (Yeast) (Torulopsis glabrata)	549	59.0%	1,708	0.0	CAGL0A02233g
C8ZB02	C8ZB02_YEAS8	☆	**Hxt9p**	Saccharomyces cerevisiae (strain Lalvin EC1118 / Prise de mousse) (Baker's yeast)	567	63.0%	1,708	0.0	EC1118_1J11_0111g
A6ZN55	A6ZN55_YEAS7	☆	**Hexose transporter**	Saccharomyces cerevisiae (strain YJM789) (Baker's yeast)	567	63.0%	1,708	0.0	**HXT11** SCY_4923
P54862	HXT11_YEAST	★	**Hexose transporter HXT11**	Saccharomyces cerevisiae (strain ATCC 204508 / S288c) (Baker's yeast)	567	63.0%	1,708	0.0	**HXT11** LGT3 YOL156W AOB567 O0414
G8ZMB3	G8ZMB3_TORDC	☆	**Uncharacterized protein**	Torulaspora delbrueckii (strain ATCC 10662 / CBS 1146 / NBRC 0425 / NCYC 2629 / NRRL Y-866) (Yeast) (Candida colliculosa)	573	62.0%	1,707	0.0	**TDEL0A04250** TDEL_0A04250
N1P268	N1P268_YEASC	☆	**Hxt4p**	Saccharomyces cerevisiae (strain CEN.PK113-7D) (Baker's yeast)	576	63.0%	1,706	0.0	CENPK1137D_5238
A7TN42	A7TN42_VANPO	☆	**Putative uncharacterized protein**	Vanderwaltozyma polyspora (strain ATCC 22028 / DSM 70294) (Kluyveromyces polysporus)	563	59.0%	1,705	0.0	Kpol_1059p39
G2WB83	G2WB83_YEASK	☆	**K7_Hxt3p**	Saccharomyces cerevisiae (strain Kyokai no. 7 / NBRC 101557) (Baker's yeast)	567	59.0%	1,705	0.0	**K7_HXT3** SYK7_015581
G0VH23	G0VH23_NAUCC	☆	**Uncharacterized protein**	Naumovozyma castellii (strain ATCC 76901 / CBS 4309 / NBRC 1992 / NRRL Y-12630) (Yeast) (Saccharomyces castellii)	568	58.0%	1,705	0.0	**NCAS0F03110** NCAS_0F03110
E7QFM1	E7QFM1_YEASZ	☆	**Hxt4p**	Saccharomyces cerevisiae (strain Zymaflore VL3) (Baker's yeast)	576	63.0%	1,705	0.0	VL3_2152
H0GZ52	H0GZ52_9SACH	☆	**Hxt2p**	Saccharomyces cerevisiae x Saccharomyces kudriavzevii VIN7	547	62.0%	1,704	0.0	VIN7_9079
Q755M1	Q755M1_ASHGO	☆	**AFL207Cp**	Ashbya gossypii (strain ATCC 10895 / CBS 109.51 / FGSC 9923 / NRRL Y-1056) (Yeast) (Eremothecium gossypii)	539	62.0%	1,703	0.0	AFL207C AGOS_AFL207C
M9MY35	M9MY35_ASHG1	☆	**FAFL207Cp**	Ashbya gossypii (strain FDAG1) (Yeast) (Eremothecium gossypii)	539	62.0%	1,703	0.0	FAGOS_FAFL207C
J5S3S1	J5S3S1_SACK1	☆	**HXT2-like protein**	Saccharomyces kudriavzevii (strain ATCC MYA-4449 / AS 2.2408 / CBS 8840 / NBRC 1802 / NCYC 2889)	547	62.0%	1,703	0.0	**YMR011W** SKUD_205404
Q6FR79	Q6FR79_CANGA	☆	**Strain CBS138 chromosome I complete sequence**	Candida glabrata (strain ATCC 2001 / CBS 138 / JCM 3761 / NBRC 0622 / NRRL Y-65) (Yeast) (Torulopsis glabrata)	555	59.0%	1,703	0.0	CAGL0I00286g
N1P8P4	N1P8P4_YEASC	☆	**Hxt3p**	Saccharomyces cerevisiae (strain CEN.PK113-7D) (Baker's yeast)	567	59.0%	1,703	0.0	CENPK1137D_4170
E7QD93	E7QD93_YEASZ	☆	**Hxt3p**	Saccharomyces cerevisiae (strain Zymaflore VL3) (Baker's yeast)	567	59.0%	1,703	0.0	VL3_1049
B3LFV5	B3LFV5_YEAS1	☆	**Low affinity glucose transporter**	Saccharomyces cerevisiae (strain RM11-1a) (Baker's yeast)	567	59.0%	1,703	0.0	SCRG_00186
A6ZYU5	A6ZYU5_YEAS7	☆	**Hexose transporter**	Saccharomyces cerevisiae (strain YJM789) (Baker's yeast)	567	59.0%	1,703	0.0	**HXT3** SCY_1233
P32466	HXT3_YEAST	★	**Low-affinity glucose transporter HXT3**	Saccharomyces cerevisiae (strain ATCC 204508 / S288c) (Baker's yeast)	567	59.0%	1,703	0.0	**HXT3** YDR345C D9651.14

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Entry	Entry name	Status	Protein names	Organism	Length	Identity	Score	E-value	Gene names
P32466	HXT3_YEAST	★	**Low-affinity glucose transporter HXT3**	Saccharomyces cerevisiae (strain ATCC 204508 / S288c) (Baker's yeast)	567	59.0%	1,703	0.0	**HXT3** YDR345C D9651.14
A6ZYU3	A6ZYU3_YEAS7	☆	**Hexose transporter**	Saccharomyces cerevisiae (strain YJM789) (Baker's yeast)	570	62.0%	1,703	0.0	**HXT7** SCY_1231
N1P133	N1P133_YEASC	☆	**Hxt1p**	Saccharomyces cerevisiae (strain CEN.PK113-7D) (Baker's yeast)	570	59.0%	1,702	0.0	CENPK1137D_5239
G2WFG8	G2WFG8_YEASK	☆	**K7_Hxt1p**	Saccharomyces cerevisiae (strain Kyokai no. 7 / NBRC 101557) (Baker's yeast)	570	58.0%	1,702	0.0	**K7_HXT1** SYK7_032151
R9XKC9	R9XKC9_ASHAC	☆	**AaceriAFL207Cp**	Ashbya aceri (Yeast)	539	62.0%	1,701	0.0	AACERI_AaceriAFL207C
J5RLE8	J5RLE8_SACK1	☆	**HXT1-like protein**	Saccharomyces kudriavzevii (strain ATCC MYA-4449 / AS 2.2408 / CBS 8840 / NBRC 1802 / NCYC 2889)	570	58.0%	1,700	0.0	**YHR094C** SKUD_150601
E9P9G4	E9P9G4_YEASX	☆	**Hexose transporter 1**	Saccharomyces cerevisiae (Baker's yeast)	570	58.0%	1,700	0.0	**HXT1**
E9P9G8	E9P9G8_YEASX	☆	**Hexose transporter 3**	Saccharomyces cerevisiae (Baker's yeast)	567	58.0%	1,699	0.0	**HXT3**
H0GHE3	H0GHE3_9SACH	☆	**Hxt1p**	Saccharomyces cerevisiae x Saccharomyces kudriavzevii VIN7	570	58.0%	1,699	0.0	VIN7_2191
C8Z9N8	C8Z9N8_YEAS8	☆	**Hxt1p**	Saccharomyces cerevisiae (strain Lalvin EC1118 / Prise de mousse) (Baker's yeast)	570	58.0%	1,699	0.0	EC1118_1H13_0584g
H2AW13	H2AW13_KAZAF	☆	**Uncharacterized protein**	Kazachstania africana (strain ATCC 22294 / BCRC 22016 / CBS 2517 / CECT 1963 / NBRC 1671 / NRRL Y-8276) (Yeast) (Kluyveromyces africanus)	563	60.0%	1,698	0.0	**KAFR0E04120** KAFR_0E04120
H0GT72	H0GT72_9SACH	☆	**Hxt3p**	Saccharomyces cerevisiae x Saccharomyces kudriavzevii VIN7	567	59.0%	1,698	0.0	VIN7_6424
E7Q4Q0	E7Q4Q0_YEASB	☆	**Hxt1p**	Saccharomyces cerevisiae (strain FostersB) (Baker's yeast)	570	58.0%	1,698	0.0	FOSTERSB_2125
G2WK62	G2WK62_YEASK	☆	**K7_Hxt2p**	Saccharomyces cerevisiae (strain Kyokai no. 7 / NBRC 101557) (Baker's yeast)	541	61.0%	1,697	0.0	**K7_HXT2** SYK7_052931
H0GLX8	H0GLX8_9SACH	☆	**Hxt2p**	Saccharomyces cerevisiae x Saccharomyces kudriavzevii VIN7	541	61.0%	1,696	0.0	VIN7_3666
E9P9G5	E9P9G5_YEASX	☆	**Hexose transporter 2**	Saccharomyces cerevisiae (Baker's yeast)	541	61.0%	1,696	0.0	**HXT2**
E7LY94	E7LY94_YEASV	☆	**Hxt2p**	Saccharomyces cerevisiae (strain VIN 13) (Baker's yeast)	541	61.0%	1,696	0.0	VIN13_3698
E7KSG2	E7KSG2_YEASL	☆	**Hxt2p**	Saccharomyces cerevisiae (strain Lalvin QA23) (Baker's yeast)	541	61.0%	1,696	0.0	QA23_3606
E7KGC9	E7KGC9_YEASA	☆	**Hxt2p**	Saccharomyces cerevisiae (strain AWRI796) (Baker's yeast)	541	61.0%	1,696	0.0	AWRI796_3611
C8ZEL7	C8ZEL7_YEAS8	☆	**Hxt2p**	Saccharomyces cerevisiae (strain Lalvin EC1118 / Prise de mousse) (Baker's yeast)	541	61.0%	1,696	0.0	EC1118_1M3_1695g
C7GLA8	C7GLA8_YEAS2	☆	**Hxt2p**	Saccharomyces cerevisiae (strain JAY291) (Baker's yeast)	541	61.0%	1,696	0.0	**HXT2** C1Q_01030
B5VPD7	B5VPD7_YEAS6	☆	**YMR011Wp-like protein**	Saccharomyces cerevisiae (strain AWRI1631) (Baker's yeast)	541	61.0%	1,696	0.0	AWRI1631_131500
B3LLR2	B3LLR2_YEAS1	☆	**High affinity hexose transporter-2**	Saccharomyces cerevisiae (strain RM11-1a) (Baker's yeast)	541	61.0%	1,696	0.0	SCRG_01906
A6ZM72	A6ZM72_YEAS7	☆	**Hexose transporter**	Saccharomyces cerevisiae (strain YJM789) (Baker's yeast)	541	61.0%	1,696	0.0	**HXT2** SCY_4184
G0VH22	G0VH22_NAUCC	☆	**Uncharacterized protein**	Naumovozyma castellii (strain ATCC 76901 / CBS 4309 / NBRC 1992 / NRRL Y-12630) (Yeast) (Saccharomyces castellii)	570	60.0%	1,696	0.0	**NCAS0F03100** NCAS_0F03100
Q6FY42	Q6FY42_CANGA	☆	**Strain CBS138 chromosome A complete sequence**	Candida glabrata (strain ATCC 2001 / CBS 138 / JCM 3761 / NBRC 0622 / NRRL Y-65) (Yeast) (Torulopsis glabrata)	552	59.0%	1,694	0.0	CAGL0A02211g

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Entry	Entry name	Status	Protein names	Organism	Length	Identity	Score	E-value	Gene names
Q6FY42	Q6FY42_CANGA	☆	**Strain CBS138 chromosome A complete sequence**	Candida glabrata (strain ATCC 2001 / CBS 138 / JCM 3761 / NBRC 0622 / NRRL Y-65) (Yeast) (Torulopsis glabrata)	552	59.0%	1,694	0.0	CAGL0A02211g
E7NG44	E7NG44_YEASO	☆	**Hxt3p**	Saccharomyces cerevisiae (strain FostersO) (Baker's yeast)	567	58.0%	1,694	0.0	FOSTERSO_0877
C7GUT1	C7GUT1_YEAS2	☆	**Hxt11p**	Saccharomyces cerevisiae (strain JAY291) (Baker's yeast)	567	62.0%	1,693	0.0	**HXT11** C1Q_04190
H8U0Q4	H8U0Q4_YEASX	☆	**Hexose transporter 2**	Saccharomyces cerevisiae (Baker's yeast)	532	61.0%	1,692	0.0	**HXT2**
N1P5B7	N1P5B7_YEASC	☆	**Hxt2p**	Saccharomyces cerevisiae (strain CEN.PK113-7D) (Baker's yeast)	541	61.0%	1,692	0.0	CENPK1137D_40
P23585	HXT2_YEAST	★	**High-affinity glucose transporter HXT2**	Saccharomyces cerevisiae (strain ATCC 204508 / S288c) (Baker's yeast)	541	61.0%	1,692	0.0	**HXT2** YMR011W YM8270.15
J8QG70	J8QG70_SACK1	☆	**HXT3-like protein**	Saccharomyces kudriavzevii (strain ATCC MYA-4449 / AS 2.2408 / CBS 8840 / NBRC 1802 / NCYC 2889)	567	58.0%	1,692	0.0	**YDR345C** SKUD_164903
C8Z5Q6	C8Z5Q6_YEAS8	☆	**Hxt3p**	Saccharomyces cerevisiae (strain Lalvin EC1118 / Prise de mousse) (Baker's yeast)	567	58.0%	1,692	0.0	EC1118_1D0_6359g
H0GVT9	H0GVT9_9SACH	☆	**Hxt4p**	Saccharomyces cerevisiae x Saccharomyces kudriavzevii VIN7	576	57.0%	1,692	0.0	VIN7_7567
H0GEG9	H0GEG9_9SACH	☆	**Hxt3p**	Saccharomyces cerevisiae x Saccharomyces kudriavzevii VIN7	550	57.0%	1,691	0.0	VIN7_1044
G8C1Z2	G8C1Z2_TETPH	☆	**Uncharacterized protein**	Tetrapisispora phaffii (strain ATCC 24235 / CBS 4417 / NBRC 1672 / NRRL Y-8282 / UCD 70-5) (Yeast) (Fabospora phaffii)	558	59.0%	1,690	0.0	**TPHA0P00120** TPHA_0P00120
E9P9H0	E9P9H0_YEASX	☆	**Hexose transporter 3**	Saccharomyces cerevisiae (Baker's yeast)	567	58.0%	1,690	0.0	**HXT3**
I9KWR6	I9KWR6_YEASC	☆	**Uncharacterized protein**	Saccharomyces cerevisiae (strain CEN.PK113-7D) (Baker's yeast)	535	62.0%	1,689	0.0	CENPK1137D_2820
J6EEH2	J6EEH2_SACK1	☆	**HXT4-like protein**	Saccharomyces kudriavzevii (strain ATCC MYA-4449 / AS 2.2408 / CBS 8840 / NBRC 1802 / NCYC 2889)	576	57.0%	1,689	0.0	**YHR092C** SKUD_150603
E7Q2L0	E7Q2L0_YEASB	☆	**Hxt3p**	Saccharomyces cerevisiae (strain FostersB) (Baker's yeast)	567	58.0%	1,688	0.0	FOSTERSB_1032
C5DHA7	C5DHA7_LACTC	☆	**KLTH0E02750p**	Lachancea thermotolerans (strain ATCC 56472 / CBS 6340 / NRRL Y-8284) (Yeast) (Kluyveromyces thermotolerans)	567	57.0%	1,688	0.0	KLTH0E02750g
E7KDE9	E7KDE9_YEASA	☆	**Hxt1p**	Saccharomyces cerevisiae (strain AWRI796) (Baker's yeast)	570	58.0%	1,688	0.0	AWRI796_2160
A6ZT03	A6ZT03_YEAS7	☆	**Hexose transporter**	Saccharomyces cerevisiae (strain YJM789) (Baker's yeast)	570	58.0%	1,688	0.0	**HXT1** SCY_2487
P32465	HXT1_YEAST	★	**Low-affinity glucose transporter HXT1**	Saccharomyces cerevisiae (strain ATCC 204508 / S288c) (Baker's yeast)	570	58.0%	1,688	0.0	**HXT1** HOR4 YHR094C
R9XIY2	R9XIY2_ASHAC	☆	**AaceriAFL205Cp**	Ashbya aceri (Yeast)	545	62.0%	1,687	0.0	AACERI_AaceriAFL205C
H2ATM1	H2ATM1_KAZAF	☆	**Uncharacterized protein**	Kazachstania africana (strain ATCC 22294 / BCRC 22015 / CBS 2517 / CECT 1963 / NBRC 1671 / NRRL Y-8276) (Yeast) (Kluyveromyces africanus)	568	61.0%	1,687	0.0	**KAFR0D00740** KAFR_0D00740
Q6FY37	Q6FY37_CANGA	☆	**Strain CBS138 chromosome A complete sequence**	Candida glabrata (strain ATCC 2001 / CBS 138 / JCM 3761 / NBRC 0622 / NRRL Y-65) (Yeast) (Torulopsis glabrata)	556	59.0%	1,686	0.0	CAGL0A02321g
E7Q7S7	E7Q7S7_YEASB	☆	**Hxt2p**	Saccharomyces cerevisiae (strain FostersB) (Baker's yeast)	541	60.0%	1,684	0.0	FOSTERSB_3561
B5VK25	B5VK25_YEAS6	☆	**YHR094Cp-like protein**	Saccharomyces cerevisiae (strain AWRI1631) (Baker's yeast)	507	60.0%	1,683	0.0	AWRI1631_81460
C5DC63	C5DC63_LACTC	☆	**KLTH0B00330p**	Lachancea thermotolerans (strain ATCC 56472 / CBS 6340 / NRRL Y-8284) (Yeast) (Kluyveromyces thermotolerans)	566	62.0%	1,683	0.0	KLTH0B00330g
G0VJ21	G0VJ21_NAUCC	☆	**Uncharacterized protein**	Naumovozyma castellii (strain ATCC 76901 / CBS 4309 / NBRC 1992 / NRRL Y-12630) (Yeast) (Saccharomyces castellii)	591	59.0%	1,682	0.0	**NCAS0H01890** NCAS_0H01890

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Entry	Entry name	Status	Protein names	Organism	Length	Identity	Score	E-value	Gene names
G0VJ21	G0VJ21_NAUCC	☆	**Uncharacterized protein**	Naumovozyma castellii (strain ATCC 76901 / CBS 4309 / NBRC 1992 / NRRL Y-12630) (Yeast) (Saccharomyces castellii)	591	59.0%	1,682	0.0	**NCAS0H01890** NCAS_0H01890
J8Q1C7	J8Q1C7_SACAR	☆	**Hxt1p**	Saccharomyces arboricola (strain H-6 / AS 2.3317 / CBS 10644) (Yeast)	570	59.0%	1,681	0.0	SU7_1470
C5DPW5	C5DPW5_ZYGRC	☆	**ZYRO0A06688p**	Zygosaccharomyces rouxii (strain ATCC 2623 / CBS 732 / NBRC 1130 / NCYC 568 / NRRL Y-229) (Candida mogii)	571	60.0%	1,681	0.0	ZYRO0A06688g
S6EKH1	S6EKH1_ZYGBA	☆	**BN860_03114g1_1**	Zygosaccharomyces bailii CLIB 213	569	58.0%	1,679	0.0	BN860_03114g
H2ASL7	H2ASL7_KAZAF	☆	**Uncharacterized protein**	Kazachstania africana (strain ATCC 22294 / BCRC 22015 / CBS 2517 / CECT 1963 / NBRC 1671 / NRRL Y-8276) (Yeast) (Kluyveromyces africanus)	580	60.0%	1,679	0.0	**KAFR0C03750** KAFR_0C03750
Q6FXX9	Q6FXX9_CANGA	☆	**Strain CBS138 chromosome A complete sequence**	Candida glabrata (strain ATCC 2001 / CBS 138 / JCM 3761 / NBRC 0622 / NRRL Y-65) (Yeast) (Torulopsis glabrata)	549	58.0%	1,678	0.0	CAGL0A00737g
J7RHU6	J7RHU6_KAZNA	☆	**Uncharacterized protein**	Kazachstania naganishii (strain ATCC MYA-139 / BCRC 22969 / CBS 8797 / CCRC 22969 / KCTC 17620 / NBRC 10181 / NCYC 3082) (Yeast) (Saccharomyces naganishii)	560	59.0%	1,676	0.0	**KNAG0B06940** KNAG_0B06940
Q6FY16	Q6FY16_CANGA	☆	**Strain CBS138 chromosome A complete sequence**	Candida glabrata (strain ATCC 2001 / CBS 138 / JCM 3761 / NBRC 0622 / NRRL Y-65) (Yeast) (Torulopsis glabrata)	563	59.0%	1,675	0.0	CAGL0A01804g
K0KEE9	K0KEE9_WICCF	☆	**Hexose transporter 2**	Wickerhamomyces ciferrii (strain F-60-10 / ATCC 14091 / CBS 111 / JCM 3599 / NBRC 0793 / NRRL Y-1031) (Yeast) (Pichia ciferrii)	566	59.0%	1,675	0.0	**KHT5** BN7_808
J8PXZ4	J8PXZ4_SACAR	☆	**Hxt3p**	Saccharomyces arboricola (strain H-6 / AS 2.3317 / CBS 10644) (Yeast)	567	58.0%	1,675	0.0	SU7_2694
G8ZV28	G8ZV28_TORDC	☆	**Uncharacterized protein**	Torulaspora delbrueckii (strain ATCC 10662 / CBS 1146 / NBRC 0425 / NCYC 2629 / NRRL Y-866) (Yeast) (Candida colliculosa)	571	62.0%	1,675	0.0	**TDEL0E02290** TDEL_0E02290
S6DY01	S6DY01_ZYGBA	☆	**BN860_03092g1_1**	Zygosaccharomyces bailii CLIB 213	580	62.0%	1,674	0.0	BN860_03092g
G0W7S4	G0W7S4_NAUDC	☆	**Uncharacterized protein**	Naumovozyma dairenensis (strain ATCC 10597 / BCRC 20456 / CBS 421 / NBRC 0211 / NRRL Y-12639) (Saccharomyces dairenensis)	607	58.0%	1,673	0.0	**NDAI0C01740** NDAI_0C01740
A6ZYU4	A6ZYU4_YEAS7	☆	**Hexose transporter**	Saccharomyces cerevisiae (strain YJM789) (Baker's yeast)	544	63.0%	1,671	0.0	**HXT6** SCY_1232
G0W8K6	G0W8K6_NAUDC	☆	**Uncharacterized protein**	Naumovozyma dairenensis (strain ATCC 10597 / BCRC 20456 / CBS 421 / NBRC 0211 / NRRL Y-12639) (Saccharomyces dairenensis)	568	60.0%	1,669	0.0	**NDAI0C04570** NDAI_0C04570
Q5XQN7	Q5XQN7_SACMI	☆	**GAL2**	Saccharomyces mikatae IFO 1815	573	56.0%	1,669	0.0	
E7KAX2	E7KAX2_YEASA	☆	**Hxt7p**	Saccharomyces cerevisiae (strain AWRI796) (Baker's yeast)	503	64.0%	1,666	0.0	AWRI796_1051
E7NIJ9	E7NIJ9_YEASO	☆	**Hxt1p**	Saccharomyces cerevisiae (strain FostersO) (Baker's yeast)	500	61.0%	1,663	0.0	FOSTERSO_2116
B3LIR4	B3LIR4_YEAS1	☆	**Glucose permease**	Saccharomyces cerevisiae (strain RM11-1a) (Baker's yeast)	501	64.0%	1,663	0.0	SCRG_01252
G8ZUG9	G8ZUG9_TORDC	☆	**Uncharacterized protein**	Torulaspora delbrueckii (strain ATCC 10662 / CBS 1146 / NBRC 0425 / NCYC 2629 / NRRL Y-866) (Yeast) (Candida colliculosa)	568	58.0%	1,663	0.0	**TDEL0E00200** TDEL_0E00200
E1U7Y1	E1U7Y1_TORDE	☆	**Intermediate affinity glucose transporter**	Torulaspora delbrueckii (Yeast) (Candida colliculosa)	568	60.0%	1,663	0.0	**IGT1**
G2WIS2	G2WIS2_YEASK	☆	**K7_Gal2p**	Saccharomyces cerevisiae (strain Kyokai no. 7 / NBRC 101557) (Baker's yeast)	574	57.0%	1,663	0.0	**K7_GAL2** SYK7_046461
E7QHV0	E7QHV0_YEASZ	☆	**Gal2p**	Saccharomyces cerevisiae (strain Zymaflore VL3) (Baker's yeast)	574	57.0%	1,663	0.0	VL3_3177
C8ZD52	C8ZD52_YEAS8	☆	**Gal2p**	Saccharomyces cerevisiae (strain Lalvin EC1118 / Prise de mousse) (Baker's yeast)	574	57.0%	1,663	0.0	EC1118_1L10_1585g
B5VN13	B5VN13_YEAS6	☆	**YLR081Wp-like protein**	Saccharomyces cerevisiae (strain AWRI1631) (Baker's yeast)	574	57.0%	1,663	0.0	AWRI1631_121250
B3LT49	B3LT49_YEAS1	☆	**Galactose permease**	Saccharomyces cerevisiae (strain RM11-1a) (Baker's yeast)	574	57.0%	1,663	0.0	SCRG_05065

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B3LT49	B3LT49_YEAS1	☆	**Galactose permease**	Saccharomyces cerevisiae (strain RM11-1a) (Baker's yeast)	574	57.0%	1,663	0.0	SCRG_05065
E7LXG5	E7LXG5_YEASV	☆	**Gal2p**	Saccharomyces cerevisiae (strain VIN 13) (Baker's yeast)	574	57.0%	1,662	0.0	VIN13_3157
E7M1Z4	E7M1Z4_YEASV	☆	**Hxt7p**	Saccharomyces cerevisiae (strain VIN 13) (Baker's yeast)	500	64.0%	1,661	0.0	VIN13_5179
J5RE25	J5RE25_SACK1	☆	**GAL2-like protein**	Saccharomyces kudriavzevii (strain ATCC MYA-4449 / AS 2.2408 / CBS 8840 / NBRC 1802 / NCYC 2889)	573	57.0%	1,660	0.0	**YLR081W** SKUD_900201
D3XDC4	D3XDC4_SACKU	☆	**GAL2p**	Saccharomyces kudriavzevii (Yeast)	573	57.0%	1,660	0.0	**GAL2**
C7GQQ5	C7GQQ5_YEAS2	☆	**Gal2p**	Saccharomyces cerevisiae (strain JAY291) (Baker's yeast)	574	57.0%	1,659	0.0	**GAL2** C1Q_02666
P13181	GAL2_YEAST	★	**Galactose transporter**	Saccharomyces cerevisiae (strain ATCC 204508 / S288c) (Baker's yeast)	574	57.0%	1,659	0.0	**GAL2** IMP1 YLR081W L9449.6
I2GVJ8	I2GVJ8_TETBL	☆	**Uncharacterized protein**	Tetrapisispora blattae (strain ATCC 34711 / CBS 6284 / DSM 70876 / NBRC 10599 / NRRL Y-10934 / UCD 77-7) (Yeast) (Kluyveromyces blattae)	570	58.0%	1,658	0.0	**TBLA0A03510** TBLA_0A03510
E7Q4P9	E7Q4P9_YEASB	☆	**Hxt4p**	Saccharomyces cerevisiae (strain FostersB) (Baker's yeast)	500	64.0%	1,657	0.0	FOSTERSB_2124
E7NKD9	E7NKD9_YEASO	☆	**Gal2p**	Saccharomyces cerevisiae (strain FostersO) (Baker's yeast)	574	57.0%	1,657	0.0	FOSTERSO_3121
N1P121	N1P121_YEASC	☆	**Gal2p**	Saccharomyces cerevisiae (strain CEN.PK113-7D) (Baker's yeast)	574	57.0%	1,656	0.0	CENPK1137D_467
B5VRA2	B5VRA2_YEAS6	☆	**YOL156W-like protein**	Saccharomyces cerevisiae (strain AWRI1631) (Baker's yeast)	501	63.0%	1,655	0.0	AWRI1631_150040
Q755L9	Q755L9_ASHGO	☆	**AFL205Cp**	Ashbya gossypii (strain ATCC 10895 / CBS 109.51 / FGSC 9923 / NRRL Y-1056) (Yeast) (Eremothecium gossypii)	546	60.0%	1,654	0.0	AFL205C AGOS_AFL205C
M9N2A9	M9N2A9_ASHG1	☆	**FAFL205Cp**	Ashbya gossypii (strain FDAG1) (Yeast) (Eremothecium gossypii)	546	60.0%	1,654	0.0	FAGOS_FAFL205C
E7Q6K0	E7Q6K0_YEASB	☆	**Gal2p**	Saccharomyces cerevisiae (strain FostersB) (Baker's yeast)	574	57.0%	1,654	0.0	FOSTERSB_3131
A7A0Y5	A7A0Y5_YEAS7	☆	**Galactose transporter**	Saccharomyces cerevisiae (strain YJM789) (Baker's yeast)	574	57.0%	1,649	0.0	**GAL2** SCY_3659
J8Q2M4	J8Q2M4_SACAR	☆	**Hxt10p**	Saccharomyces arboricola (strain H-6 / AS 2.3317 / CBS 10644) (Yeast)	546	57.0%	1,644	0.0	SU7_0992
A7TS07	A7TS07_VANPO	☆	**Putative uncharacterized protein**	Vanderwaltozyma polyspora (strain ATCC 22028 / DSM 70294) (Kluyveromyces polysporus)	633	57.0%	1,644	0.0	Kpol_365p10
H0GVH3	H0GVH3_9SACH	☆	**Hxt8p**	Saccharomyces cerevisiae x Saccharomyces kudriavzevii VIN7	566	58.0%	1,640	0.0	VIN7_7418
H2ATM2	H2ATM2_KAZAF	☆	**Uncharacterized protein**	Kazachstania africana (strain ATCC 22294 / BCRC 22015 / CBS 2517 / CECT 1963 / NBRC 1671 / NRRL Y-8276) (Yeast) (Kluyveromyces africanus)	557	59.0%	1,634	0.0	**KAFR0D00750** KAFR_0D00750
H2ATM7	H2ATM7_KAZAF	☆	**Uncharacterized protein**	Kazachstania africana (strain ATCC 22294 / BCRC 22015 / CBS 2517 / CECT 1963 / NBRC 1671 / NRRL Y-8276) (Yeast) (Kluyveromyces africanus)	558	58.0%	1,633	0.0	**KAFR0D00800** KAFR_0D00800
N1P0E9	N1P0E9_YEASC	☆	**Hxt8p**	Saccharomyces cerevisiae (strain CEN.PK113-7D) (Baker's yeast)	569	58.0%	1,632	0.0	CENPK1137D_1469
P40886	HXT8_YEAST	★	**Hexose transporter HXT8**	Saccharomyces cerevisiae (strain ATCC 204508 / S288c) (Baker's yeast)	569	58.0%	1,632	0.0	**HXT8** YJL214W HRA569 J0232
J7S523	J7S523_KAZNA	☆	**Uncharacterized protein**	Kazachstania naganishii (strain ATCC MYA-139 / BCRC 22969 / CBS 8797 / CCRC 22969 / KCTC 17520 / NBRC 10181 / NCYC 3082) (Yeast) (Saccharomyces naganishii)	555	57.0%	1,630	0.0	**KNAG0C05180** KNAG_0C05180
G2WD81	G2WD81_YEASK	☆	**K7_Hxt8p**	Saccharomyces cerevisiae (strain Kyokai no. 7 / NBRC 101557) (Baker's yeast)	569	58.0%	1,630	0.0	**K7_HXT8** SYK7_022111
J6ECW9	J6ECW9_SACK1	☆	**HXT10-like protein**	Saccharomyces kudriavzevii (strain ATCC MYA-4449 / AS 2.2408 / CBS 8840 / NBRC 1802 / NCYC 2889)	546	58.0%	1,628	0.0	**YFL011W** SKUD_191005

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Entry	Entry name	Status	Protein names	Organism	Length	Identity	Score	E-value	Gene names
J6ECW9	J6ECW9_SACK1	☆	**HXT10-like protein**	Saccharomyces kudriavzevii (strain ATCC MYA-4449 / AS 2.2408 / CBS 8840 / NBRC 1802 / NCYC 2889)	546	58.0%	1,628	0.0	**YFL011W** SKUD_191005
G2WDC9	G2WDC9_YEASK	☆	**K7_Hxt10p**	Saccharomyces cerevisiae (strain Kyokai no. 7 / NBRC 101557) (Baker's yeast)	546	58.0%	1,628	0.0	**K7_HXT10** SYK7_022661
S6E3R6	S6E3R6_ZYGBA	☆	**ZYBA0S05-03048g1_1**	Zygosaccharomyces bailii CLIB 213	558	61.0%	1,628	0.0	BN860_03048g
J6EDD0	J6EDD0_SACK1	☆	**HXT8-like protein**	Saccharomyces kudriavzevii (strain ATCC MYA-4449 / AS 2.2408 / CBS 8840 / NBRC 1802 / NCYC 2889)	568	58.0%	1,627	0.0	**YJL214W** SKUD_188603
H2ATM3	H2ATM3_KAZAF	☆	**Uncharacterized protein**	Kazachstania africana (strain ATCC 22294 / BCRC 22015 / CBS 2517 / CECT 1963 / NBRC 1671 / NRRL Y-8276) (Yeast) (Kluyveromyces africanus)	559	59.0%	1,625	0.0	**KAFR0D00760** KAFR_0D00760
C5E1P6	C5E1P6_ZYGRC	☆	**ZYRO0G00286p**	Zygosaccharomyces rouxii (strain ATCC 2623 / CBS 732 / NBRC 1130 / NCYC 568 / NRRL Y-229) (Candida mogii)	589	58.0%	1,623	0.0	ZYRO0G00286g
B1PM38	B1PM38_PICAN	☆	**Low affinity glucose transporter**	Pichia angusta (Yeast) (Hansenula polymorpha)	540	60.0%	1,621	0.0	**HXT1**
A7A227	A7A227_YEAS7	☆	**Hexose transporter**	Saccharomyces cerevisiae (strain YJM789) (Baker's yeast)	546	58.0%	1,620	0.0	**HXT10** SCY_1737
J7S4Y1	J7S4Y1_KAZNA	☆	**Uncharacterized protein**	Kazachstania naganishii (strain ATCC MYA-139 / BCRC 22969 / CBS 8797 / CCRC 22969 / KCTC 17520 / NBRC 10181 / NCYC 3082) (Yeast) (Saccharomyces naganishii)	557	57.0%	1,620	0.0	**KNAG0B04370** KNAG_0B04370
H2ATM6	H2ATM6_KAZAF	☆	**Uncharacterized protein**	Kazachstania africana (strain ATCC 22294 / BCRC 22015 / CBS 2517 / CECT 1963 / NBRC 1671 / NRRL Y-8276) (Yeast) (Kluyveromyces africanus)	561	58.0%	1,620	0.0	**KAFR0D00790** KAFR_0D00790
H0GFM8	H0GFM8_9SACH	☆	**Hxt10p**	Saccharomyces cerevisiae x Saccharomyces kudriavzevii VIN7	546	58.0%	1,619	0.0	VIN7_1510
C7GY98	C7GY98_YEAS2	☆	**Hxt10p**	Saccharomyces cerevisiae (strain JAY291) (Baker's yeast)	546	58.0%	1,619	0.0	**HXT10** C1Q_05526
B5VI16	B5VI16_YEAS6	☆	**YFL011Wp-like protein**	Saccharomyces cerevisiae (strain AWRI1631) (Baker's yeast)	546	58.0%	1,619	0.0	AWRI1631_60500
B3LUJ8	B3LUJ8_YEAS1	☆	**High affinity hexose transporter**	Saccharomyces cerevisiae (strain RM11-1a) (Baker's yeast)	546	58.0%	1,619	0.0	SCRG_05536
P43581	HXT10_YEAST	★	**Hexose transporter HXT10**	Saccharomyces cerevisiae (strain ATCC 204508 / S288c) (Baker's yeast)	546	58.0%	1,619	0.0	**HXT10** YFL011W
N1P4M3	N1P4M3_YEASC	☆	**Hxt10p**	Saccharomyces cerevisiae (strain CEN.PK113-7D) (Baker's yeast)	546	58.0%	1,618	0.0	CENPK1137D_3371
E7R0W8	E7R0W8_OGAPD	☆	**Low affinity glucose transporter**	Ogataea parapolymorpha (strain DL-1 / ATCC 26012 / NRRL Y-7560) (Yeast) (Hansenula polymorpha)	542	61.0%	1,617	0.0	HPODL_0455
E7NH79	E7NH79_YEASO	☆	**Hxt10p**	Saccharomyces cerevisiae (strain FostersO) (Baker's yeast)	546	58.0%	1,617	0.0	FOSTERSO_1455
C8Z7S5	C8Z7S5_YEAS8	☆	**Hxt10p**	Saccharomyces cerevisiae (strain Lalvin EC1118 / Prise de mousse) (Baker's yeast)	546	58.0%	1,615	0.0	EC1118_1F14_0782g

Appendix 8.2 Alignment results of *Clostridium phytofermentas* XI with GCY1 and Gal 10 of *Saccharomyces cerevisiae*.