FERMENTATION OF COCOA (*Theobroma cacao L.*) POD HUSK AND ITS HYDROLYSATE FOR ETHANOL PRODUCTION USING IMPROVED STARTER CULTURES

BY

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Abstract

Fossil fuel, a main but dwindling energy source for automobiles, causes emission of environment unfriendly oxides of carbon. These contribute substantially to greenhouse gases which bring about climate change. There is therefore the need for sustainable source of energy like ethanol an environmental friendly bioenergy. Hence this study was aimed at the fermentation of cocoa pod husk for ethanol production.

Isolates of yeast were obtained from sun-dried Cocoa Pod Husk (CPH), subjected to spontaneous submerged fermentation for 7 days. Five strains of *Saccharomyces sp.* (MX1, MX2, MX3, MX4 and MX5) with high frequency of occurrence were selected for further studies. The MX1 and MX2 were used for genetic modifications. Dried CPH was subjected to chemical analysis and pretreatment using particle size reduction and high pressure liquid hot water at 130^{o}C for 30 minutes. Acid and enzymatic hydrolysis of the pretreated CPH was carried out using standard method. Products of the hydrolysis were analysed with high performance liquid chromatography. Two genes XL1 (xylose reductase) and XL2 (xylitol dehydrogenase) encoding pentose utilization were obtained from genomic DNA of *Pichia stipitis* (CBS 6054) using basic local alignment search tool. Primers of these genes were designed with Saccharomyces genome database, amplified with Polymerase Chain Reaction (PCR) and purified. The amplicon (genes) were ligated into plasmid vectors (pGAPZA and pVT100-U). Strains MX1 and MX2 were transformed with these construct using lithium acetate method. Physiological characterization of the selected unmodified yeast strains and the two genetically-modified strains was done under different environmental conditions including temperatures, pH and varied concentrations of acetic acid. The CPH hydrolysates were fermented for 120 hours using the unmodified and genetically-modified yeast strains respectively and the ethanol yield determined. Data were analysed using ANOVA.

Twenty yeast isolates identified as *Saccharomyces cerevisiae* (80%) and *Saccharomyces uvarum* (20%) were obtained. Chemical composition of CPH included hemicellulose (13.9%) cellulose (18.6%) and lignin content (14.2%). Acid hydrolysis yielded 50.1% glucose, 11.97% xylose, 11.2% mannose while enzymatic hydrolysis gave

31.7% glucose, 4.8% mannose and 16.8% galactose. The inserted gene XL1 had 318 amino acids polypeptides while XL2 had 363 amino acid polypeptides. Restriction enzyme analysis and colony PCR confirmed the transformational integration of these constructs into *Saccharomyces cerevisiae* MX1 and MX2. The five isolates had optimal growth at 30 – 40^{o}C and pH of 4.0 – 5.5. However the genetically-modified yeast strains were able to utilize xylose and arabinose carbon sources better than the unmodified types and also tolerated low concentration of acetic acid than the unmodified types. Ethanol production was highly significant ($p<0.05$) in the modified starters (29.7g/L) than the unmodified strains (14.0g/L).

Genetically-modified organisms performed better in ethanol production than the non-modified organisms. The application of genetic modification of microorganisms will aid the potential use of waste biomass like cocoa pod husk for bioenergy production and this will contribute significantly to reducing greenhouse gases associated with climate change.

Keywords: Ethanol production, Cocoa pod husk, Genetic modification, *Saccharomyces cerevisiae.*

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Richard Osaretin Igbinadolor,

July, 2012.

Certification

I certify that the research work reported in this thesis was carried out under my supervision by **Richard Osaretin IGBINADOLOR** in the Department of Microbiology, University of Ibadan, Ibadan, Nigeria.

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Dedication

I dedicate this thesis to God Almighty,

My special family – who are my constant source of joy,

and

As a special tribute to my son

Osakpolor Success Igbinadolor,

Evidence to prove that our God liveth!

LISTS OF ABBREVIATIONS

1.	ADF	Acid detergent fibre
2.	BLAST	Basic Local Alignment Search Tool
3.	BSA	Bovine serum albumin
4.	CDS	Coding sequence
5.	CPH	Cocoa pod husk
6.	cfu	Colony forming unit
7.	CMC	Carboxymethyl cellulose
8.	gDNA	Genomic DNA
9.	EDTA	Ethylenediaminetetra acetic acid
10.	EtBr	Ethidium bromide
11.	FWD	Forward primer
12.	HMF	5-hydroxymethyl fufural
13.	IPTG	Isopropyl –B- D- thiogalactoside
14.	LB	Luria Bertani
15.	LiA	Lithim acetate
16.	LHW	Liquid hot water
17.	MCS	Multiple cloning site
18.	NCBI	National Centre for Biotechnological Information
19.	NDF	Neutral detergent fibre
20.	OFR	Open reading frame
21.	PCR	Polymerase Chain Reaction
22.	PEG	Poly ethylene Glycol

23.	pDNA	Plasmid DNA
24.	PPP	Pentose phosphate pathway
25.	PMS	Premium motor spirit
26.	RE	Restriction enzymes
27.	REW	Reverse primer
28.	SDS	Sodium dodecyl sulphate
29.	SGD	Saccharomyces Genome Database
30.	SHF	Separate hydrolysis and fermentation
31.	SSF	Simultaneous saccharification and fermentation
32.	TE buffer	Tris EDTA buffer
33.	XR	Xylose reductase gene
34.	XDH	Xylitol dehydrogenase gene
35.	MTBE	Methyl tertiary-butyl ether
36.	ETBE	Ethyl tertiary-butyl ether
37.	Ura 3	Orotidine-5'- phosphate decarboxylase
38.	VOCs	Volatile organic compounds
39.	YPD	Yeast peptone dextrose medium

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CHAPTER 1

1.0 INTRODUCTION

1.1 General Consideration

Climatic change induced by human activities and increasing oil demand with unsecure supply compels the search for alternative fuels, as it is the responsibility of the society to address these anthropogenic causes of climate change and the looming shortfall in fossil fuels. Energy utilization is a determining factor for the standards of living around the World; current estimates indicate that about 95% of the world's energy comes from burning of fossil fuels (Somerville, 2007) which is the leading contributor of carbon (iv) oxide - the main greenhouse gas emissions to the atmosphere (Wyman, 1996; McMillan, 1997). For this reason creating "carbon neutral" fuels should be an important part of global efforts to reducing carbon emissions. There is also wide spread prediction that the world population will likely increase by about 50% in the next 50 years and the standard of living worldwide is also increasing. These trends will result in heightened world demand for food and energy from petroleum. If the current production and consumption rates of petroleum resources continue, global oil reserves will be exhausted in less than 65 years as predicted by the United Nations Conference on Trade and Development (UNCTAD) (CTA Spore, 2006). Conservation of the earth's biodiversity and its natural resources are very important for the sustainability of the future generation. To meet this challenge is to focus and develop alternative fuel from biobased materials/biomass for the production of liquid fuel such as ethanol through fermentation processess that could ease the strain caused by diminishing petroleum resources.

One solution already in widespread use is bioethanol produced from fermented sugar cane in Brazil or cornstarch in USA by various strains of *Saccharomyces cerevisiae.* It is used as a major component or additive to liquid transportation fuels (Baso *et al*., 2008). The use of bioethanol can reduce our dependence on fossil fuels, while at the same time decreasing net emissions of carbon (iv) oxide into the atmosphere. However, large-scale production of bioethanol is being increasingly criticized for its use of food sources as raw material. Brazil's bioethanol production consumes large quantities of sugar cane, while in the USA corn suffers the same fate (Wheals *et al*., 1999). Other starch-rich grains, such as

wheat and barley, are mostly used in Europe (Galbe and Zacchi, 2002). The use of such sugar-rich feedstock and starch materials causes the escalation of food prices, owing to competition on the market (Catic and Sokele, 2008; Knocke and Vogt, 2009). Therefore, future expansion of biofuel production must be increasingly based on bioethanol from lignocellulosic materials, such as agricultural byproducts, forest residues, industrial waste streams or energy crops (Classen *et al.*, 1999; Solomon *et al*., 2007). These feedstocks, which are being used in second-generation (2G) bioethanol production, are abundant, have fewer competing uses and their cost is lower than that of food crops (Tan *et al.*, 2008; Chu and Lee, 2007).

For bioethanol to become a sustainable, economically viable commodity and not to compete with food sources, in third world countries like Nigeria, it is necessary to move away from sugarcane or corn feedstock toward lignocellulosic biomass source such as corn stover, cocoa pod husk and other agricultural wastes. Plant biomass, particularly when accruing as a waste product, is an attractive feedstock for bioethanol production (Angspannefòreningen, 1994; Brat *et al.*, 2009). It is renewable and diffusely distributed unlike petroleum that is localized and not renewable in the time frame over which we use them. Bioethanol produced by fermentation of lignocellulosic biomass (second generation bioethanol), from agricultural by-products, forest residues or energy crops, shows many potential advantages in comparison to sugar or starch-derived bioethanol (first generation bioethanol) (Farrell *et al.*, 2006).

Fermentation has been defined as the enzymatic oxidation of compounds by the action of microorganisms (Pelczar *et al*., 1993). Prescott *et al.* (1999) defined fermentation as an energy-yielding process in which organic molecules serves as both electrons donors and acceptors. Different types of fermentation exist, one of which is the ethanol fermentation. Ethanol is one of the most important products of biotechnology to man. Ethanol (ethyl alcohol, C_2H_5OH) arises as the main product in the fermentation of sugars. It is a colourless liquid with a burning and spirituous smell but is miscible with water in all proportions. Absolute alcohol is spirit with less than 0.2% of water and it is combustible (Drawert and Klisch, 1987).

Renewable fuels from biomass such as ethanol constitute energy sources that preserve the environment since the carbon dioxide released from their combustion can be integrated into the photosynthetic cycle, which does not participate in a net carbon (iv) oxide

buildup in to the atmosphere. Lignocellulose is a more-abundant and less-expensive raw material with the potential to give a high net energy gain (Hammerschlag, 2006; Lynd *et al.*, 2008)

The Federal Government of Nigeria initiated the ethanol programme in which ethanol would be produced from fermentable agricultural materials/residues e.g sugarcane, cassava and would be gradually introduced into Premium Motor Spirit (PMS) or petrol to a maximum of 10 percent as being practiced in countries like United States, Brazil and Thailand. The programme was introduced to diversify the depleting source of energy, provide employment opportunities for the unemployed, reduced environmental pressure and the adverse effect of fossil fuels like oil, coal and gas in the country.

However, the proposed utilization of cassava, maize or sugar cane for the production of bioethanol in Nigeria and in some countries of the world may become counter productive in the future especially in developing countries like Nigeria owing to the escalating cost of cassava based staple food like 10% cassava flour being incorporated into bread, it is equally used for the production of garri and fufu which are going beyond the reach of an average Nigerian. In 2005 Nigeria exported 40 metric tones of cassava chip to the Republic of China (www.fao.org/docrep/007/y5548e/y5578e09.htm).

In most countries of the world, bioethanol are produced from food-based materials. Malek (1989) showed that about 6 million tons of industrial ethanol is produced annually from sugar cane. However with increasing population and competitive demand for bioethanol, the food-based materials will not suffice as this will be counter productive in the future resulting in an undesirable increase in the prices of food, hunger and a decrease in the soil fertility of agricultural fields. This has led some to question the wisdom of embracing bioethanol and spawned a “food versus fuel” debate (Chu and Lee, 2007).

Thus to promote more balanced development of ethanol production, there is need for diversification of resources for its production, as this will lessen the pressure on a single raw material. Among different lignocellulosic raw materials, cocoa pod husk is an abundant source of biomass and are abundant in cocoa-growing area of Nigeria as agricultural residues or wastes in the farm with vast quantities of sugars occurring as structural polysaccharide – cellulose and hemicellulose. Biomass is solar energy stored in organic matter and a renewable energy source because the growth of new plants and trees

replenishes the supply (Balat and Havva, 2009), the main biomass energy conversion routes are shown in Plate 1.1.

Cocoa pod husk (CPH) consists of dry matter (DM) 84%, crude protein 10.16%, crude fibre 34.92%, ether extract 2.49%, potassium 3.64%, Theobromine 0.32% and gross energy 20.32MJ/kg (Barnes and Amega, 1998). It represents one of the most important Nigerian agricultural residues with the present initiative being given to cocoa rehabilitation at the moment through National Cocoa Development Committee (NCDC). The immediate past and present administration in Nigeria promised to revive agriculture and it was made clear that abandoning the farms was a distortion and an aberration that must be checked. Nigeria, it is realized, cannot afford to continue to depend solely on oil sector. Much attention has been given to the revival of agricultural sector with several initiatives put in place among which is the Cocoa Rehabilitation programme with the NCDC as the implementing agency. The NCDC is headed by the Minister of Agriculture and has the deputy governors of the fourteen cocoa-producing States of Abia, Adamawa, Akwa Ibom, Cross River, Delta, Edo, Ekiti, Kogi, Kwara, Ogun, Ondo, Osun, Oyo and Taraba as members. Each ton of dry cocoa seeds represents about 10 tons of cocoa husks (fresh weight) (Figueira *et al.,* 1993). At the present time, pod husk (lignocellulose) is a waste product of the cocoa industry, and poses a serious disposal problem. It becomes a significant source of disease inoculum when used as mulch inside the plantation.

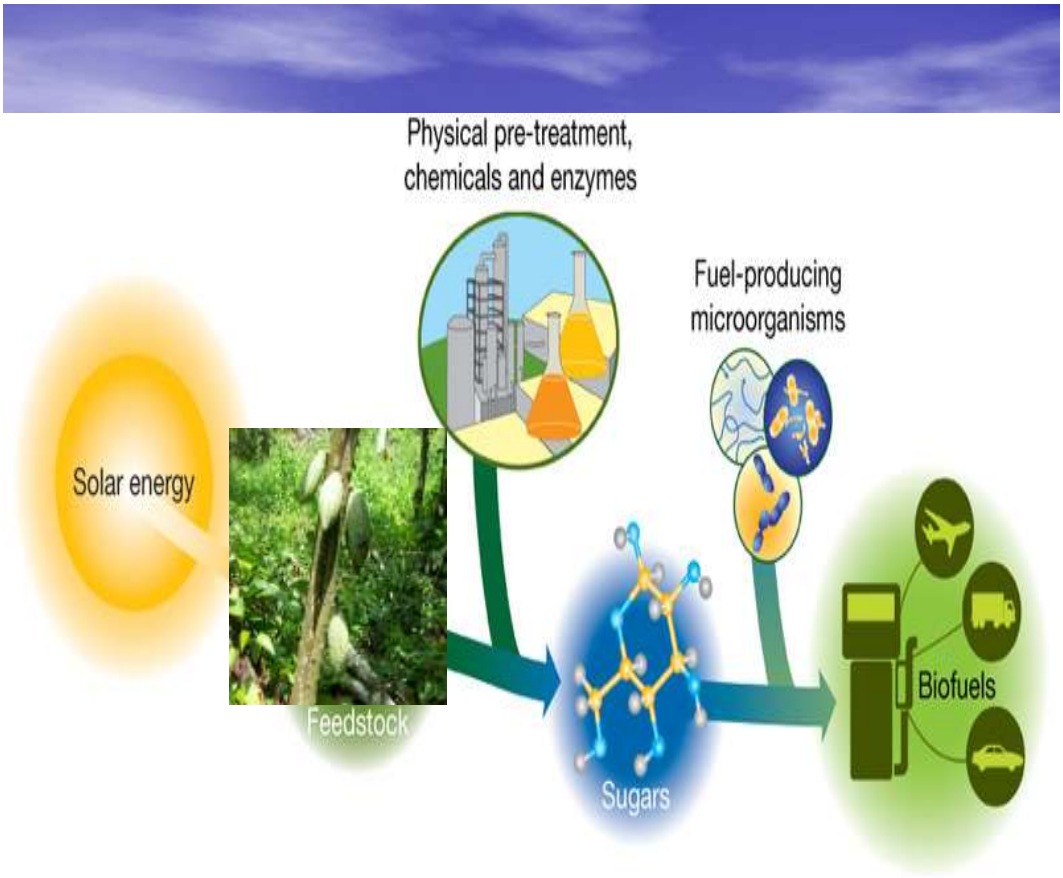


Plate 1.1 *Biological conversion of solar energy in plants into ethanol (Biofuel) (Adapted from Biofuel, 2007)*

For sugar cane and corn biomass, the predominant sugars are glucose and/or fructose, both of which are readily fermented to ethanol by various strains of *Saccharomyces cerevisiae* yeasts (Argueso *et al*., 2009; Stambuk *et al*., 2009). In lignocellulosic biomass sources, the second most abundant carbohydrate after glucose is xylose, the major pentose sugar of hemicellulose. The budding yeast *Saccharomyces cerevisiae* is the microorganism of choice for industrial fermentation for a variety of reasons, mainly due to its high ethanol productivity both aerobically and anaerobically, its high ethanol and low pH tolerance, and its resistance to many of the harmful compounds in typical biomass hydrolysate. Despite recent evidence that some natural *S. cerevisiae* can grow, albeit poorly, on xylose (Attfield and Bell, 2006), it has generally been reported that both natural and laboratory *S. cerevisiae* strains do not ferment xylose (Chiang *et al.*, 1981; Gong *et al.*, 1983) leading to the assumption that they can not, without recourse to genetic engineering, be utilized for efficient conversion of lignocellulose to ethanol.

1.2 Statement of Problem

Worldwide, considerable effort have been focused in the last few years on ethanol production to serve as alternative energy source due to the dwindling supply of petroleum resources and to reduce its consumption due to its associated air pollution and global warming effects. Large scale production of ethanol has focused on the use of food based materials like grains, cassava and sugar cane which has brought wide spread criticisms owning to the escalation of food prices due to the competitive use of these materials as feed stock for man and animals.

The proposed utilization of cassava, maize and sugar-cane for bioethanol production by the Federal government of Nigeria was seen as a venture that will be counter productive in future considering our population growth. This necessitated the need for this research work, inorder to find alternative feed stock that is more abundant than food crops and have fewer competing use. This research is therefore designed to investigate the use of cocoa pod husk – a lignocellulosic material that is readily available in cocoa-growing areas of Nigeria as a waste residue for the production of bioethanol.

1.3 Justification for the Current Work

In the context of rising demand of environmental concerns and with biomass well distributed all around the world, the option of extended use of plant biomass should be considered. In view of this the justification for research into fuel ethanol production from cocoa pod could be premised on the following reasons:

It is desirable with the aim to diversify Nigeria's energy sources and contribute towards energy security; this in turn will free more crude oil for the export market. The use of fuel ethanol will help to minimize air pollution, now that emphasis is on reducing pollution and making a contribution towards the 'Kyoto protocol' of December 1997 in Japan by limiting global warming level by year 2008 – 12.

It will help to reduce the environmental stress and the agitation of marginalization in the Niger Delta as a result of oil exploration causing youth restiveness. In addition, fuel ethanol production on a large scale has the potential to create more job opportunities and thereby reduces unemployment that has remained an intractable problem in the country.

Furthermore, agricultural residues like Cocoa Pod Husk (wastes) are available in considerable quantities in cocoa producing areas and using this residue for industrial production could be an additional source of revenue for cocoa farmers. This inturn will help to provide partial/total solution to the disposal of solid Agricultural wastes that has constituted a source of disease inoculum in the environment

Finally, it will help to promote one of the seven point agenda of the present government by ensuring food security

1.4 Aims and Objectives of the Study

The ultimate aim of any fermentation technology is to obtain products of economic importance at a very low cost. The proposed utilization of cassava and sugarcane in the production of ethanol by the Federal Government may become counter-productive in the future owing to the escalating cost of cassava-based staple food items like cassava flour being incorporated at 10% (w/w) into bread, garri and fufu which are going beyond the reach of an average Nigerian.

Sugar cane also is an expensive seasonal crop (De Moraes *et al.*, 1995). About 80% of the average Nigerian relies on cassava-, and sugarcane-based products. Thus there are

great economic advantages in extending the substrate range of ethanol fermenting organisms so that ethanol can be produced from other substrates. In order to get a competitive price compared to conventional fuels, production cost of ethanol need to be reduced. Cheap and abundant raw materials have to be used. In this context, the use of lignocellulosic materials (LM) is seen as a promising choice as a second generation bioethanol fuel.

Food security of the country and the welfare of the people in general should be given a priority. In view of this, this research work was designed to use locally available agricultural residues of Cocoa Pod Husk (CPH) – a misplaced asset of economic importance in the production of Ethanol. Cocoa pod husk is a waste, an inexpensive and abundant carbon source in Nigeria. No previous attempts to perform ethanolic fermentation using cocoa pod husks as the substrate have been found in the literature.

1.4.1 Specific Objectives

In evaluating the use and suitability of this waste (CPH) as a fermentative material for ethanol production, this study aims:

1. To isolate and identify microorganisms involved in a spontaneously-degrading cocoa pod husk.
2. To carry out genetic modification of the identified yeasts isolates to augment pentose utilization.
3. To carry out comparative physiological studies on the ethanol-producing yeasts from cocoa pod husk
4. To produce and characterize ethanol (biofuel) using these improved microorganisms.

CHAPTER 2

2.0 LITERATURE REVIEW

2.1 Fuel Ethanol

The idea of biofuel is as old as the idea of the vehicle with an engine, when Henry Ford first designed his model T automobile in 1908. Then, he expected ethanol, made from renewable resources to be fuel. About the same time in Germany, Rudolf Diesel thought that his compression ignition would be run on vegeble oils (Sims, 2002).

Fuel ethanol is currently made by large scale yeast fermentation of sugars that are extracted or prepared from crops followed by separation of the ethanol by distillation (Lyons *et al.*,1995). The conversion of glucose (C6) has been known for at least 6000years when Sumerians, Babylonians and Egyptians began to perfect and describe the process of making beer from grain (starch). The conversion of xylose (pentose) is more complicated and research has only lately (1980s) obtained success (Sims, 2002).

A variety of microorganisms, mostly bacteria, yeast or fungi ferment carbohydrates to ethanol under oxygen-free conditions. This is a natural reaction necessary to obtain energy and thereby grow. According to the reactions, the theoretical maximum yield is 0.51kg of ethanol and 0.49kg CO_2 per kg of xylose and glucose (Sims, 2002).

Pentose $3C_5H_{10}O_5 \rightarrow 5C_2H_5OH + 5CO_2$

Hexose $C_6H_{12}O_6 \rightarrow 2C_2H_5OH + 2CO_2$

The first major fuel ethanol programe (PROAICOOL) started in Brazil in 1975 (Lima, 1975), followed by programmes in U.S.A. in 1978 and more recently in Canada. Although many other countries produce ethanol for fuel and other purposes (http://www.distill.com/bergl), major production has only occurred in those countries with especially favourable agricultural and economic conditions.

Fuel ethanol is used in a variety of ways. Ethanol has a higher octane rating than petrol (Wheals *et al.*, 1999), enabling combustion engines to be run at a higher compression ratio and thus giving a net performance gain of approximately 15% (w/w) (Wheals *et al.*, 1999), even though a litre of pure ethanol contains only about two-third ($^2/_3$) of the calorific value of a litre of conventional gasoline. In the past, direct government intervention in Brazil ensured that hydrous ethanol was made available at all 26,000 gasoline stations.

Today, the major use of ethanol is as an oxygenated fuel additive that reduces emissions of carbon monoxide, nitrogen oxides and hydrocarbons. The European Union (EU) has recommended a 5% admixture of ethanol in all gasoline for internal combustion engines before 2010 (Wheals *et al.*, 1999). Accumulation of CO_2 in the atmosphere is long recognized as a major contributor to global warming and climate change (Revelle and Suess, 1957). Bioethanol used as a replacement for gasoline reduces vehicle CO_2 emissions by 90% (Tyson *et al.*, 1993). With respect to global warming, ethanol from biomass reduces net CO_2 emissions.

The main environmental advantages of ethanol over gasoline are that it is a renewable and in principle, a fully sustainable resource that is less polluting than gasoline. The most important aspect is that the CO_2 released by ethanol combustion has been fixed recently by growing plants and therefore this 'Greenhouse' gas makes no net contribution to global warming (Wheals *et al*., 1999)

Ethanol combustion does produce acetaldehyde and because it has a low vapour pressure (Gaffney *et al.*, 1997), ethanol releases Volatile Organic Compounds (VOCS). As a result of the USA Clean Air Act Amendments (1990), oxygenated gasoline additives (MTBE, ETBE or ethanol) were required to provide at least 2% by weight of O_2 to reduce the Ozone-forming potentials of gasoline by reducing CO, NO_2 and VOCs. This led to the Reformated Federal Gasoline (RFG) programme, which regulated the content and volatility of gasoline during summer months in specified areas and the Federal oxygenated fuel programme, which required gasoline to contain 2.7% oxygen in other designated areas of high risk of CO pollution in winter.

The RFG programme has been successful at reducing the Ozone-forming potentials of gasoline both directly and indirectly, although probably by less than anticipated (Committee on Ozone forming potentials, 1996). Collectively, biofuels work to restore the atmosphere's natural CO_2 balance by not only displacing fossil-fuel emissions, but by relying on feedstock that pulls from the atmosphere the CO_2 they need to grow. The Renewable Fuels Association says ethanol reduces tailpipe carbon monoxide emissions by as much as 30 percent, VOC emissions by 12 percent and toxic emissions by 30 percent (www.ethanolrfa.org/factfic_envir.html) and offers environmental advantages especially improved quality of the urban air and its association with the reduction of CO_2, heavy metals, nitrogen-oxides and hydrocarbon emissions (Dobereiner & Baldani, 1998).

Increased used of bioethanol could become a vital part of the long-term solution to climate change.

2.1.1. Fuel properties of Ethanol and its Applications

Bioethanol has much lower energy content than gasoline (about two-third of the energy content of the latter on a volume base). This means that, for mobility applications, for a given tank volume, the range of the vehicle is reduced in the same proportion. The octane number of ethanol is higher than that for petrol; hence ethanol has better antiknock characteristics. This better quality of the fuel can be exploited if the compression ratio of the engine is adjusted accordingly. This increases the fuel efficiency of the engine. The oxygen content of ethanol also leads to a higher efficiency, which results in a cleaner combustion process at relatively low temperatures (Chandel *et al.*, 2007); (http://www eubia.org/332.0html#832)

The Reid vapour pressure, a measure for the volatility of a fuel, is very low for ethanol. This indicates a slow evaporation, which has the advantage that the concentration of evaporative emissions in the air remains relatively low. This reduces the risk of explosions. However, the low vapour pressure of ethanol, together with its single boiling point, is disadvantageous with regard to engine start at low ambient temperatures. Without aids, engines using ethanol cannot be started at temperatures below 20ºC. Cold start difficulties are the most important problem with regard to the application of alcohols as automotive fuels (http://www.eubia.org/332.0html#832). With its ability to reduce ozone precursors by 20 – 30%, bioethanol can play a significant role in reducing the harmful gases in metro cities worldwide. (Subramanian *et al*., 2005; Chandel *et al*., 2007)

Ethanol can be used: as a transport fuel to replace gasoline, as a fuel for power generation by thermal combustion, as a fuel for fuel cells by thermochemical reaction, as a fuel in cogeneration systems and as a feedstock in the chemicals industry. Ethanol is best used in spark-ignition engines because of its high octane rating. Due to its poor ignition quality (low cetane number), it is less suitable for diesel engines.

Table 2.1 Fuel properties of gasoline, bioethanol and ETBE

Fuel properties	Gasoline	Bioethanol	ETBE
Molecular weight [kg/kmol]	111	46	102
Density [kg/l] at 15ºC	0.75	0.80-0.82	0.74
Oxygen content [wt-%]		34.8	
Lower Calorific Value [MJ/kg] at 15ºC	41.3	26.4	36
Lower Calorific Value [MJ/l] at 15ºC	31	21.2	26.7
Octane number (RON)	97	109	118
Octane number (MON)	86	92	105
Cetane number	8	11	-
Stoichiometric air/fuel ratio [kg air/kg fuel]	14.7	9.0	-
Boiling temperature [ºC]	30-190	78	72
Reid Vapour Pressure [kPa] at 15ºC	75	16.5	28

(Adapted from(http://www.baff.info*)*

It is generally impractical to use neat ethanol in spark-ignition engines due to its low vapour pressure and high latent heat of vaporisation which make cold start problematic. The most cost-effective aid is the blending of ethanol with a small proportion of a volatile fuel such as gasoline. Thus, various mixture of bioethanol with gasoline or diesel fuels had been used. The most well-known blends are (by volume) E5G to E26G (5-26% ethanol, 95-74% gasoline); E85G (85% ethanol, 15% gasoline), E15D (15% ethanol, 85% diesel), E95D (95% ethanol, 5% water, with ignition improver) (http://www.eubia.org/332.0html#832). Bioethanol has been extensively tested in light duty flexible fuel vehicles (FFV) as E85G. ETBE is also used in blends of 10-15 % with gasoline to enhance its octane rating and reduce emissions. Blends of gasoline with up to 22% ethanol (E22G) can be used in spark ignition engines without any material or operating problems. Blends of diesel with up to 15% ethanol (E15D) do not introduce any technical engine problem and require no ignition improver.

The introduction of E85G in Europe started in Sweden around the year 2000. Only in the last 2 years has the E85G infrastructure expanded to other countries in the EU such as Germany, France and Ireland (http://www.baff.info/).

2.1.2. Bioethanol in the world

Bioethanol is probably the most widely used alternative automotive fuel in the world, mainly due to Brazil's decision to produce fuel alcohol from sugar cane, but also due to its use in North America as octane enhancer of gasoline in small percentage. The world's largest ethanol producers are Brazil and the USA, which together account for more than 65% of global ethanol production; the figure for Europe is 13% (http://www.eubia.org/332.0.html#832). Fuel ethanol is produced in Brazil mainly from sugar cane and in the USA from corn, accounting for 11.9 and 7.6 million m^3 respectively in 2001.

In Brazil, 60% of the produced ethanol is sold in hydrated form (93% vol- ethanol and 7% vol- water), which completely replaces petrol in vehicle engines. The remaining 40% ethanol is applied in water-free form in a mixture with petrol up to 24%.

2.1.3. Bioethanol production in the EU

The European bioethanol production amounted to 309,500 tons in 2003 and 1,592m litres in 2006 (http://www.eubia.org/332.0.html#832). With 431 million litres, Germany is the leading producer in Europe. However Spain is a close second with 396 million litres. The sector's success in Spain can be explained by the fact that Spain does not collect tax on ethanol. France was the third largest European producer in 2006 with 293 m litres (http://www.eubia.org/332.0.html#832). Spain and France transform part of their bioethanol production into ETBE.

2.2 Feedstocks

Lignocellulosic materials containing cellulose, hemicellulose and lignin are the most abundant renewable organic resources on earth (Aristidou and Penttila, 2000). The chemical composition of biomass varies among species, but biomass consist of 25% lignin and 75% carbohydrate polymers (cellulose and hemicellulose) (Aristidou and Penttila, 2000).

2.2.1 Cellulose

Cellulose is an organic compound with the formula $(C_6H_{10}O_5)_n$, a polysaccharide consisting of a linear chain of several (1,4) linked D-glucose unit (Crawford, 1981). It is the main structural component that provides strength and stability to the plant cell walls and the fibre (Reddy and Yang, 2005). The amount of cellulose in a fibre influences the properties, economics of fibre production and the utility of the fibre for various applications. The cellulose in a plant consists of parts with a crystalline (organized) structure, and parts with a not well-organised, amorphous structure. The chains are bundled together and form so called cellulose fibrils or cellulose bundles. These cellulose fibrils are mostly independent and weakly bound through hydrogen bonding (Laureano-Perez *et al.*, 2005). Cellulose is a high-molecular weight linear glucose polysaccharide with the elementary formula $\left[C_6H_{10}O_5\right]_n$. It has a degree of polymerization (DP) in the range of 200-2000 kDa (4000-8000 glucose molecules connected with β-1, 4 glycosidic bonds). Some animals, particularly ruminants and termites can digest cellulose with the help of symbiotic microorganisms that live in their guts. Cellulose is not digestible by humans and is often referred to as "dietary fibres" or roughage, acting as hydrophilic bulking agent for faeces.

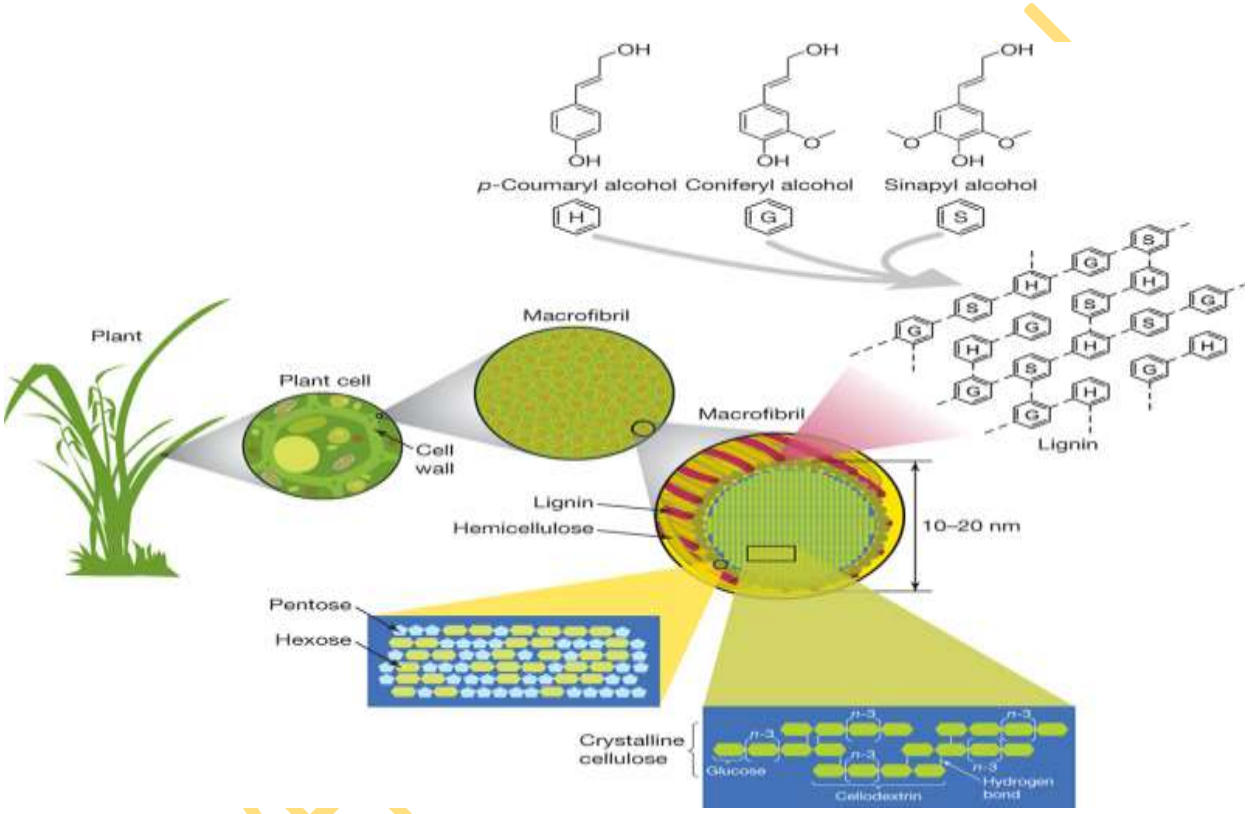


Fig 2.1 Structure of Lignocellulose (adapted from Genomics of cellulosic Biofuel)

(http://www.nature.com/nature/journal/v454/n7206/full/nature07190.html)

Cellulose is the structural component of the primary cell wall of green plants, many forms of algae. Some species of bacteria secrete it to form biofilm.

The major combustible component of non-food energy crops is cellulose, with lignin second. Cellulose has no taste, is odourless, hydrophilic, insoluble in water and most organic solvent, it is chiral and biodegradable (Klemm *et al.*, 2005). Cellulose is derived D-glucose units, which condensed through β(1→ 4) glycosidic bonds. This linkage motif contrasts with that of α(1→4) glycosidic bonds present in starch, glycogen and other carbohydrates. Cellulose is very strong and its links are broken by cellulase enzyme cleaving the molecule by the addition of water molecules (Hamelinck, 2005).

$$[C_6H_{10}O_5]_n + nH_2O \rightarrow nC_6H_{12}O_6$$

Cellulose is a straight chain polymer, unlike starch, no coiling occurs and the molecule adopts an extended and rather stiff rod-like conformation. The multiple hydroxyl groups on the glucose residues from one chain form hydrogen bonds with oxygen molecules on another chain holding the chains firmly together side by side and forming microfibrils with high tensile strength (*http://www.en.wikipedia.org/wiki/cellulose*). This strength is important in cell walls, where they are meshed into a carbohydrate matrix, conferring rigidity to plants cells.

Compared to starch, cellulose is also much more crystalline. Whereas starch undergoes a crystalline to amorphous transition when heated beyond 60 - 70^{o}C in water (as in cooking), cellulose requires a temperature of 320^{o}C and pressure of 25MPa to become amorphous in water (Deguchi *et al.*, 2006). Many properties of cellulose depend on its degree of polymerization or chain length, the number of glucose units that make up one polymer molecule. Molecules with very small chain length resulting from the breakdown of cellulose are known as cellodextrins; in contrast to long chain cellulose, cellodextrins are typically soluble in water and organic solvents. Plants derived cellulose is usually contaminated with hemicellulose, lignin, pectin and other substances, while microbial cellulose is quite pure, has much higher water content and consists of long chain (Klemm *et al.*, 2005).

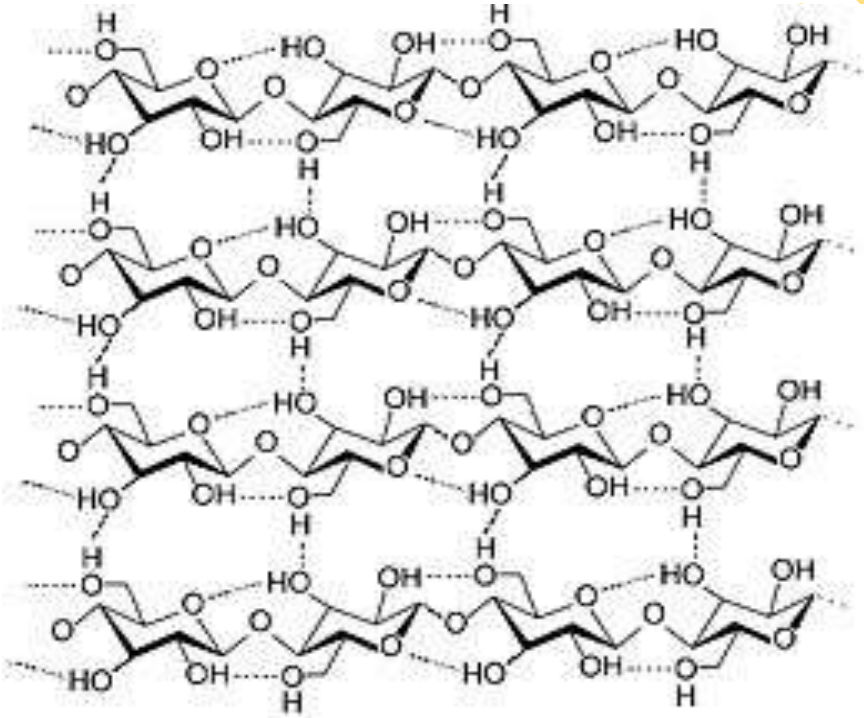


Fig 2.2 *A strand of cellulose, showing the hydrogen bonds (dashed) within and between cellulose molecules (adapted from http://www.en.wikipedia.org/wiki/cellulose)*

2.2.2. Hemicellulose

Hemicellulose in plants is slightly crosslinked and is composed of multiple polysaccharide polymers with a degree of polymerization and orientation less than that of cellulose (Reddy and Yang, 2005). Hemicellulose usually acts as filler between cellulose and lignin and consists of sugars including glucose, xylose, galactose, arabinose and mannose. Mechanically, hemicellulose contributes little to the stiffness and strength of fibers or individual cells (Thompson, 1993). Hemicellulose is more easily hydrolyzed into sugars than cellulose hence fibers containing a higher proportion of hemicellulose would be preferable for producing sugars, and eventually for fuels such as ethanol. Hemicellulose is also a low-molecular weight heteropolysaccharide (DP < 200, typically β-1,3 links), with a wide variation in both structure and composition. Commonly occurring hemicelluloses are xylans, arabinoxylan, gluco-mannan, galacto-glucomann, and so on. In contrast to cellulose, which is crystalline, strong, and resistant to hydrolysis, hemicellulose has a random, amorphous structure with little strength (Aristidou and Penttilà, 2000).

2.2.3. Lignin

Lignin on the other hand is a complex aromatic heteropolymer consisting of phenylpropane units (P –coumaryl, Coniferyl and Sinapyl alcohol) synthesized from phenylpropanoid precussors (Adler, 1977), Lignin are divided into two classes namely "Guaiacyl lignin" and guaiacyl-syringyl lignin", differing in the substituents of the phenylpropanoid skeleton. Guaiacyl-lignin has a methoxy group in the 3 carbon position, whereas guaiacyl-syringyl lignins have a methoxy group in both the 3-carbon and 5-carbon positions. Softwood and hardwood lignin belong to the second category respectively. Softwoods generally contain more lignin than hardwoods (Saka, 1991) Lignin are cross-linked to each other with a variety of different chemical bonds and acts as glue between individual cells and between the fibrils forming the cell wall (Mohanty, 2000).

Lignin is first formed between neighboring cells in a 'middle lamella' binding them tightly into a tissue, and then spreads into the cell wall penetrating the hemicelluloses and bonding the cellulose fibrils (Majumdar and Chanda, 2001). Lignin degradation is primarily an aerobic process and in an anaerobic environment, lignin can persist for very long periods (Van Soest, 1994). Because lignin is the most recalcitrant component of the plant cell wall, its presence lowers the bioavailability of cellulose and hemicellulose for enzymatic penetration and activity (Haug, 1993). With the advent of modern genetics and engineering tools the cost of producing sugars from these recalcitrant lignocellulosic fractions and converting them into products like ethanol has been significantly reduced.

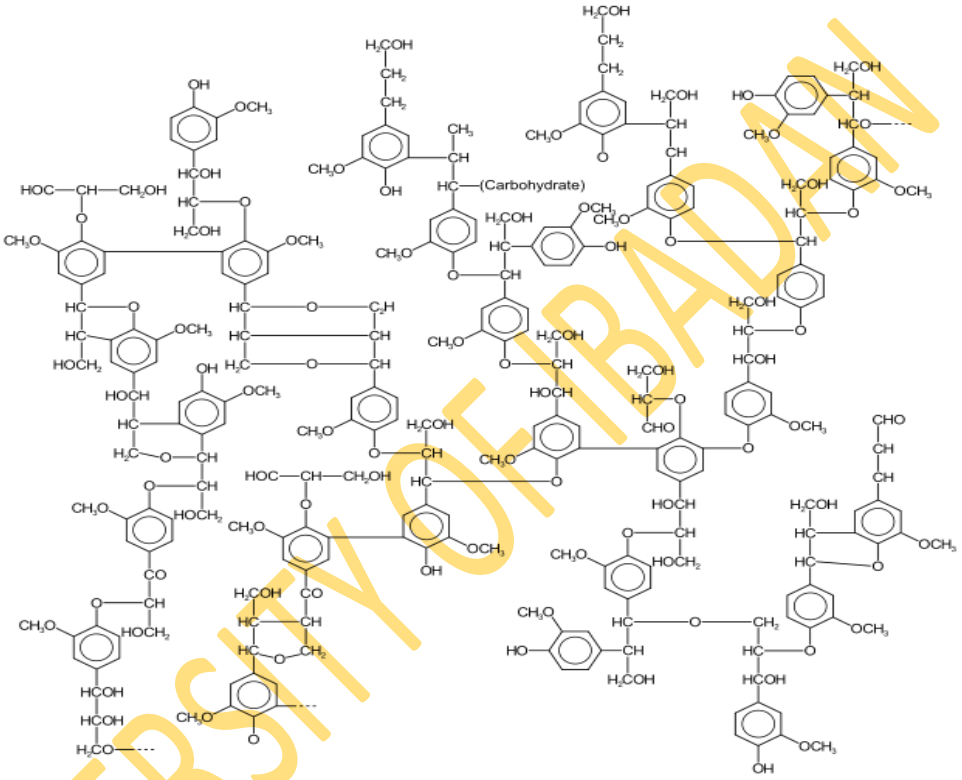


Fig. 2.3: ***Lignin structure (****adapted from htpp:// www.en.wikilpedia.org/wiki/lignin****)***

2.3 Degradation of Lignocellulosic Materials

Lignocellulosic materials are composed of sugars polymerized to cellulose and hemicellulose that can be liberated by hydrolysis and subsequently fermented to ethanol. The ethanol yield and productivity are decreased due to the presence of inhibitory compounds such as weak acids, furan derivatives and phenolic compounds generated during the hydrolysis of lignocellulose (Palmqvist and Hahn-Hagerdal, 2000)

Hydrolysis procedures which involves treatment of lignocellulose at high temperature under acidic conditions usually leads to the formation and liberation of a range of compounds. The main degradation pathways are schematically represented in figure 2.5 below.

When hemicellulose is degraded, xylose, mannose, acetic acid, galactose and glucose are liberated. Cellulose is hydrolysed to glucose. At high temperature and pressure pentoses (xyloses) undergo dehydration losing three molecules of water to become furfurals (Dunlop, 1948).

Similarly 5-hydroxymethyl furfural (HMF) is formed from hexose degradation (Ulbricht *et al.,* 1984). Formic acids are formed when furfurals and HMF are broken down (Dunlop, 1948; Ulbricht *et al.,* 1984). Levulinic acid is formed by HMF degradation (Ulbricht *et al.,* 1984). Phenolic compounds are generated from partial breakdown of lignin (Sears *et al.,* 1971; Lapierre *et al.,* 1983; Bardet *et al.,* 1985), and have also been reported to be formed during carbohydrate degradation (Popoff and Theander, 1976; Suortti, 1983). Vanillic and vanillin are formed by the degradation of the guaiacylpropane units of lignin. In hardwood hydrolysate, syringaldehyde and syringic acid, formed in the degradation of syringyl propane units have been reported (Jonsson *et al.,* 1998; Trans and Chambers, 1985). Hydroquinone (1,4-di-hydroxybenzene) has been identified in hydrolysate of spruce and catechol (1,2-di-hydroxybenzene) in the hydrolysates of willow (Jonsson *et al.,* 1998). 4-hydroxybenzoic acid constitutes a large fraction of lignin derived compounds in hydrolysates from hardwoods (Ando *et al.,* 1986).

Fig 2.4 *Reactions occurring during the hydrolysis of lignocellulosic materials (Adapted from* Palmqvist and Hahn-Hagerdal, 2000)

2.4 Degradation product of lignocellulose and mechanisms of Inhibition

2.4.1 Furfurals and HMF: Furfural is an aromatic aldehyde with a ring structure derived from a variety of agricultural byproducts like corncobs, wheat bran, cocoa pod husk, saw dust etc. In its pure state, it is a colourless oily liquid with odour of almonds, but on exposure to air it quickly becomes yellow (http://www.en.wikipedia.org/wiki/furfural). Its chemical formula is $C_5H_4O_2$. When plant materials containing hemicellulose are hydrolysed, it yields pentose sugars, principally xylose. Under the same condition of heat, xylose and other five carbon sugars undergo dehydration, losing three molecules of water to become furfural:

$$C_5H_{10}O_5 \longrightarrow C_5H_4O_2 + 3H_2O$$

Pentose sugar furfural

Furfural can be metabolized by *S. cerevisiae* under aerobic (Taherzadeh *et al.*, 1998), oxygen-limited (Fireoved and Mutharasan, 1986; Navarro, 1994) and anaerobic conditions (Palmqvist *et al.*, 1999). During fermentation furfural reduction to furfuryl alcohol occurs with high yields (Diaz de Villegas, 1992; Villa, 1992; Taherzadeh *et al.*, 1998; Palmqvist *et al.*, 1999). Inhibition of aerobic growth of *Pichia stipitis* by furfuryl alcohol has been reported (Weigert *et al.*, 1988), whereas only slight inhibition of anaerobic growth of *S.cerevisiae* has been detected (Palmqvist *et al.*, 1999).

Furfural oxidation to furoic acid by *S.cerevisiae* occurs to some extent, primarily under aerobic condition (Taherzadeh *et al.*, 1998; Palmqvist *et al.*, 1999). The furfural reduction rate has been shown to increase with increasing inoculum size (Chung and Lee, 1984; Boyer *et al.*, 1992; Navarro, 1994) and with increasing specific growth rate in chemostat and batch cultures (Taherzadeh *et al.*, 1998). The reduction rate in anaerobic batch fermentation has been reported to increase with increasing furfural concentration up to approximately 84mmol/g, and then decrease again, probably due to cell death at high furfural concentrations (Palmqvist *et al.*, 1999). Furfural has been shown to reduce the specific growth rate (Azhar *et al.*, 1981; Boyer *et al.*, 1992, Navarro, 1994), the volumetric (Azhar *et al.*, 1981; Navarro, 1994), and specific ethanol productivities (Taherzadeh *et al.*, 1998; Palmqvist *et al.*, 1999a). Growth is more sensitive to furfural than is ethanol production (Palmqvist *et al.*, 1999).

NADH-dependent yeast alcohol dehydrogenase (ADH) is believed to be responsible for furfural reduction (Weigert *et al.,* 1988; Diaz de Villegas *et al.,* 1992; Taherzadeh *et al.,* 1998). Under anaerobic conditions, glycerol is normally produced to regenerate excess NADH formed in biosynthesis (Oura, 1977). Glycerol production has been shown to be significantly reduced during furfural reduction, suggesting that furfural reduction regenerates NAD^{++} (Palmqvist *et al.,* 1999a). The fact that less carbon was consumed for glycerol production in the presence of furfural resulted in an increased ethanol yield in the presence of 29 mmol 1^{-1} furfural compared with fermentation in the absence of furfural. Elevated concentration of acetaldehyde was excreted in the beginning of the fermentation, which was suggested to be due to a decreased NADH concentration in the cell during furfural reduction (Palmqvist *et al.,* 1999a). Furfural inhibition of glycolytic enzymes in vivo has been reported (Banerjee *et al.,* 1981b), and direct inhibition of ADH might have contributed to acetaldehyde excretion. Intracellular acetaldehyde accumulation has been suggested to be the reason for the lag-phase in growth in the presence of furfural.

A mechanistic model describing the specific rates of growth, glucose consumption, ethanol and glycerol formation, and furfural reduction in batch fermentation has recently been developed (Palmqvist *et al.,* 1999a). The model was based on the following assumptions: (i) furfural reduction to furfuryle alcohol by NADH dependent dehydrogenases had a higher priority than reduction of dihydroxyacetone phosphate to glycerol, and (ii) furfural caused inactivation of cell replication.

Adaptation of S. *cerevisiae* to furfural has been reported in batch (Banerjee *et al.,* 1981), fed batch (Villa, 1992), and continuous culture (Chung and Lee, 1984; Fireoved and Mutharasan, 1986), leading to increased growth and volumetric ethanol productivity.

The adaptation might be due to the synthesis of new enzymes or co-enzymes for furfural reduction (Boyer *et al.,* 1992). Supporting this hypothesis, the ADH activity in anaerobic fermentation has been reported to increase by 78% after 48 h fermentation with an initial furfural concentration of 2 g 1^{-1} (Banerjee *et al.,* 1981).

HMF is also metabolized by *S. cerevisiae* (Larsson *et al.,* 1998; Sanchez and Bautista, 1988; Taher-zadeh *et al.,* 1999). HMF has been reported to be converted at a lower rate than furfural causing a longer lag-phase in growth which might be due to lower membrane permeability (Larsson *et al.,* 1998). The main conversion product was 5-hydroxymethyl

furfural alcohol (Taherzadeh *et al.,* 1999), suggesting similar mechanisms for HMF and furfural inhibition.

2.4.2. Phenolic Compounds

Phenolic compounds partition into biological membranes and cause loss of integrity, thereby affecting their ability to serve as selective barriers and enzymes matrices (Heipieper *et al.,* 1994). Phenolic compounds have been suggested to exert a considerable inhibitory effect in the fermentation of lignocellulosic hydrolysates, the low molecular weight phenolic compounds being most toxic (Clark and Mackie, 1984; Buchert *et al.,* 1989). However, the mechanism of the inhibiting effect has not been elucidated, largely due to a lack of accurate qualitative and quantitative analyses. Model studies of the inhibitory action of phenolic compounds have been performed using far higher concentrations than are actually present in the hydrolysates (Clark and Mackie, 1984; Mikulaasova *et al.,* 1990, Delgenes *et al.,* 1996). When the results of those studies are interpreted, it should be borne in mind that the water solubility of phenolic compounds is limited. The solubility depends on the composition of the liquid and can be different in hydrolysate and in defined medium. When a high concentration of a certain compound has been used, it is therefore possible that the concentration actually experience by the microorganism has been lower.

Inhibition of fermentation has been shown to decrease when phenolic monomers and phenolic acids were specifically removed from a willow hemicellulose hydrolysate by treatment with the lignin-oxidising enzyme laccase (Jonsson *et al.,* 1998). 4-Hydroxybenzoic acid, vanillin, and catechol were major constituents in the untreated hydrolysate. 4-Hydrozybenzoic acid has been used as a model compound to study the influence of phenolic compounds on fermentation (Palmqvist *et al.,* 1999b). The choice of 4-hydroxybenzoic acid was based on the abundance in hardwood hydrolysates (Bardet *et al.,* 1985; Ando *et al.,* 1986; Jonsson *et al.,* 1998) and its reported inhibitory effect on fermentation with *S. cerevisiae* (1 $g1^{-1}$ has been reported to cause a 30% decrease in ethanol yield compared to a reference fermentation) (Ando *et al.,* 1986).

However, no significant effects on either growth or volumetric ethanol productivity have been detected during fermentation with 2 g 1^{-1} 4-hydroxybenzoic acid (Palmqvist *et al.,* 1999b). Vanillin constitutes a large fraction of the phenolic monomers in hydrolysates of spruce (Nilvebrant *et al.,* 1997), pine (Clark and Mackie, 1984) and willow (Jonsson *et al.,*

1998). Vanillin has been found to be less toxic than 4-hydroxybenzoic acid 1 g 1^{-1} caused a 25% decrease in the ethanol yield), and vanillic acid had no effect at concentrations up to 1 g1^{-1} (Ando *et al.*, 1986). *S. cerevisiae* assimilated vanillin, hydroxybenzaldehyde, and syringaldehyde during fermentation (Delgenes *et al.*, 1996) and growth has been reported on catechol recorcinol, salicylic acid, and p-hydroxybenzoic acid (Mills *et al.*, 1971).

2.4.3. Interaction Effects

Acetic acid (10 g1^{-1}) and furfural (3 g 1^{-1}) have been shown to interact antagonistically on growth (i.e., the observed decrease in the specific growth rate, μ, in the presence of both compounds was greater than the sum of the decreases caused by the individual compounds (Myers and Montgonery, 1991) when 0.2 g 1^{-1} initial cell mass was used (Palmqvist *et al.*, 1999b). The effect on the volumetric ethanol productivity, on the other hand, was solely additive. When, instead an initial cell mass of 10 g 1^{-1} was used, the ethanol yield obtained in the presence of 5 g 1^{-1} acetic acid, 10 g 1^{-1} formic acid, 23 g 1^{-1} levulinic acid, 1.2 g 1^{-1} furfural, and 1.3 g 1^{-1} HMF has been reported to decrease only slightly compared with a reference fermentation (Larsson *et al.*, 1998). This result was consistent with the fact that these compounds inhibit cell growth more than ethanol formation, and virtually no net growth occurred when the initial cell mass was 10 g 1^{-1}, even in the absence of inhibitors.

Little ethanol production was observed in a spruce hydrolysate containing the same concentrations of weak acids and furans as the model fermentation, even at high initial cell-mass concentration, in the referred study. This indicates that other compounds possibly lignin degradation products; largely contribute to inhibition in lignocellulosic hydrolysates. In addition to the specific inhibitors discussed above, the fermenting organism will be exposed to an aqueous solution containing sugars, amino acids, ions and ethanol, resulting in decreased water activity. The osmolarity was estimated to be equivalent to a salt content of 200 g 1^{-1} in spent sulphite liquor, and 20 g 1^{-1} in an enzymatic hydrolysate (Olsson and Hahn-Hagerdal, 1993).

2.4.4. Weak acids:

Acids are are classified as either strong or weak depending on their dissociation constant Ka, the negative logarithm of which is denoted pKa (Atkins, 1990). The concentration of undissociated acid is a function of pH and pKa and increases with

decreasing pH (Henderson-Hasselbach equation) (Atkins, 1990). Weak acids have rather high pKa values, e.g., the values of pKa at zero ionic strength for acetic, formic, octanoic and levulinic acid are 4.75 (25^{o}C), 3.75 (20^{o}C), 4.89 (25^{o}C) (Weast, 1975 – 1976) and 4.66 (25^{o}C) (Soni *et al.,* 1982) respectively. The concentration of undissociated acids in lignocellulosic hydrolysates is very sensitive to small pH deviations around pH 5.5.

Weak acids inhibit cell growth and are therefore used as food preservatives (Brown and Booth, 1991). Undissociated weak acids are liposoluble and can diffuse across the plasma membrane. The growth inhibiting effect on microorganisms has been proposed to be due to the inflow of undissociated acid into the cytosol (Stouthamer, 1979; Warth, 1988; Verduyn *et al.,* 1990; Verduyn *et al.,* 1992; Axe and Bailey, 1995). In the cytosol, dissociation of the acid occurs due to the neutral intracellular pH, thus decreasing the cytosolic pH (Pampulha and Loureiro-Dias, 1989). The concentration of undissociated acids in lignocellulosic hydrolysates is very dependent on pH which is a crucial variable during fermentation. Low fermentation pH inhibits cell proliferation and viability in the absence of weak acids due to the increased proton gradient across the plasma membrane resulting in an increase in the passive proton uptake rate (Verduyn *et al.*, 1990). Maintaining a neutral intracellular pH is crucial for cell viability. The cell replicative viability has been found to decrease linearly with decreasing intracellular pH (Imai and Ohono, 1995). The optimal external pH ranges for growth of *S. cerevisiae* is 5.0 – 5.5 (Venduyn *et al.,* 1990). Growth has been detected at a pH as low as 2.5 in the absence of acetic acid in modern fermentations, while the minimum pH for growth increased to 4.5 in the presence of acetic acid (10g/l) (Taherzadeh *et al.*, 1997). The influence of acetic, formic and levulinic acid on ethanol yield has been studied on modern fermentation (Larsson *et al.,* 1998) and reported a clear difference in the toxicity between acetic, formic and levulinic acid at the same concentration of undissociation.

2.5 Pretreatment of lignocellulose:

To ensure successful biological conversion of lignocellulosic materials, the interaction between lignin and the polysaccharide components of the cell wall must be reduced through pre-treatment, a process that is considered to be one of the most important steps in the process (Wyman *et al.,* 2005). The purpose of the pretreatment is intended to alter or disorganise the crystalline structure of macro and microfibrils of lignocellulose in order to release the polymer chains of cellulose and hemicellulose, and modify the pores in

the material to allow enzymes to penetrate into the fibre to render it amenable to enzymatic hydrolysis (Galbe and Zacchi, 2002).

The pretreatment methods may be classified into

(1) physical pretreatment such as mechanical communition, pyrolysis and irradiation (McMillan 1994; Wyman 1996)

(2) physico-chemical pretreatment

The lignocellulosic nature of cocoa pod husk makes the pretreatment a crucial step due to the physical and chemical barriers caused by the close association of the main components; cellulose, hemicellulose and lignin. During the pretreatment step, the enzyme accessibility to cellulose is enhanced; therefore, the efficiency of cellulases to release fermentable sugars is increased. This should be done with a minimum formation of compounds, which inhibit the fermenting microorganisms (Aimeida *et al.*, 2007). The accessible surface area is regarded as one of the most important factors affecting the effectiveness of enzymatic cellulose degradation (Gharpuray *et al.*, 1983; Grethlein, 1985; Lin *et al.*, 1985; Wong *et al.*, 1988 and Thompson *et al.*, 1992). In native wood, only a small fraction of the cell wall capillaries are accessible to the enzymes (Cowling, 1975). Pretreatment, however, increases the available area in several ways (Grethlein, 1985; Puls *et al.*, 1985; Donaldson *et al.*, 1988, Zeng *et al.*, 2007)

i) fragments and cracks are formed yielding increased area (Donaldson *et al.*, 1988),

ii) the hemicellulose fraction is hydrolysed which diminishes shielding effects (Kerr and Goring, 1975; Donaldson *et al.*, 1988)

iii) the lignin also undergoes structural changes (Brownell and Saddler 1984; Wong *et al.*, 1988; Donaldson *et al.*, 1988; Ramos *et al.*, 1999) and the wood is delignified to various degrees, depending on the pretreatment technology (Mosier *et al.*, 2005). Thus, the shielding of microfibrils and occluding of pores, caused by lignin, can be removed. Other factors, believed to influence the digestibility in simultaneous saccharification and fermentation (SSF), are the substrate crystallinity (Thompson *et al.*, 1992; Josefsson *et al.*, 2001; Kim *et al.*, 2003) and the degree of polymerization (DP) (Zhang and Lynd 2004).

The pretreatment methods can be divided into physical and chemical methods, and combinations of these two are commonly used (Mosier *et al.*, 2005). The type of feedstock strongly affects the choice of pretreatment method. The hemicellulose is, for instance, acetylated to a high degree in xylan-rich materials (Olofsson *et al.*, 2008). Since acetate is liberated during hydrolysis, the pretreatment of these materials is to some extent autocatalytic and require less added acid and milder process conditions. However, the liberated acetate adds to the toxicity of the hemicellulose hydrolysates.

2.5.1 Physical pretreatment

1. Ammonia fiber/freeze explosion (AFEX)

Ammonia fiber/freeze explosion (AFEX) pretreatment is regarded as an attractive method for pretreatment of agricultural residues, yielding highly digestible cellulose (Dale and Moreira, 1982; Holtzapple *et al.*, 1991). AFEX depolymerizes the lignin, removes the hemicellulose and decrystallizes the cellulose (Iyer *et al.*, 1996; Sharma *et al.*, 2002). The moderate temperature and pH also minimize formation of sugar degradation products. However, the method suffers from high costs of ammonia and ammonia recovery (Holtzapple *et al.*, 1991). In this context the lime method, based on calcium (or sodium) hydroxide (MacDonald *et al.*, 1983; Chang and Holtzapple, 1997; Sharma *et al.*, 2002) comes under mention. Alkali pretreatments are run at lower temperatures for long residence times, and as for the AFEX method, a delignification of the biomass is obtained.

2. Steam Explosion

Steam explosion is an intensively studied pretreatment method (Mosier *et al.*, 2005). The effects of uncatalyzed steam explosion – and liquid hot water pretreatments – on the biomass are primarily attributed to the removal of hemicelluloses. By adding an acid catalyst, the hydrolysis can be further improved (Brownell and Saddler, 1984; Brownell *et al.*, 1986). Dilute acid pretreatments using H_2SO_4 (Nguyen *et al.*, 1998; Soderstrom *et al.*, 2003; Sassner *et al.*, 2008) or SO_2 (Clark and Mackie 1987; Clark *et al.*, 1989; Stenberg *et al.*, 1998; Soderstrom, 2002; Ohgren *et al.*, 2005) are the most investigated pretreatment methods because of their effectiveness and inexpensiveness. These methods have been applied in pilot plants and, hence, are close to commercialization (Ropars *et al.*, 1992; Schell and Duff, 1996). Acid catalyzed treatment improves the hemicellulose removal (Brownell and Saddler, 1984) gives a partial hydrolysis of cellulose (Clark and Mackie, 1987; Clark *et*

al., 1989; Nguyen *et al*., 1998) and alters the lignin structure (Wong *et al*., 1988; Donaldson *et al*., 1988; Ramos *et al*., 1999). The main drawbacks are related to the process equipment requirements (Galbe and Zacchi, 2002; Mosier *et al.*, 2005) and inhibitor formation (Palmqvist, 2000). So far, successful pretreatments with alkali, AFEX and liquid hot water have been limited to agricultural residues and herbaceous crops (Holtzapple *et al*., 1991; Van Walsum *et al*., 1996; Kim *et al*., 2000; Varga *et al*., 2002), whereas acid catalysed steam pretreatments have generated high sugar yields from these materials as well as from softwood feedstocks (Nguyen *et al.*, 1998; Soderstrom *et al*., 2002).

A simple quantification of the harshness of a steam pretreatment process is the so called Severity Factor, *log* (R_0). This factor combines the time and the temperature of a process into a single entity, $R_0 = t \cdot e^{\frac{T_r - 100}{14.75}}$ Overend and Chornet (1987).

For acid catalyzed pretreatments the Combined Severity Factor, log(*CS*), is sometime used. This takes also the pH into account;

$\log(CS) = \log(R_0) - pH$ (Chum *et al.*, 1990)

Typical values for acid catalyzed steam explosion pretreatment of softwood are in the range 2 to 4 (Soderstrom *et al*., 2002; 2003).

Optimal pretreatment conditions in a simultaneous saccharification and fermentation (SSF) process do not necessarily differ much from those of separate hydrolysis and fermentation (SHF) processes utilizing lignocellulosic biomass. However, several compounds present in pretreatment hydrolysates, which inhibit enzymatic hydrolysis are converted by the fermenting organisms. This is a probable explanation behind the higher reported ethanol yields in SSF compared to SHF (Tengborg *et al.*, 2001; Soderstrom *et al*., 2005). Inhibitor formation from the pretreatment may therefore be tolerated to a higher extent in an SSF process. Inhibitory compounds can be put into three major groups; furaldehydes, weak acids, and phenolics. The two most common furaldehydes, HMF (5-hydroxymethyl-2-furaldehyde) and furfural (2-furaldehyde), are formed at severe conditions from hexoses and pentoses, respectively (Ulbricht *et al*., 1984; Palmqvist and Hahn-Hagerdal, 2000). Weak acids from lignocellulosic materials, such as acetic, formic and levulinic acid are mainly formed by de-acetylation of hemicellulose or HMF breakdown (Ulbricht *et al.*, 1984). Phenolic

compounds are formed chiefly during lignin breakdown, and are to be found in numerous variants, depending on the type of lignin (Perez *et al*., 2002).

2.5.2 Chemical Treatment

2.5.2.1. Liquid Hot Water (LHW):

Liquid hot water pretreatment is one of the oldest methods applied for pretreatment of cellulosic materials. Autohydrolysis plays an important role in this process, where no chemical is added. It results in dissolution of hemicelluloses mostly as liquid-soluble oligosaccharides and separates them from insoluble cellulosic fractions. The pH, processing temperature and time should be controlled in LHW pretreatment in order to optimize the enzymatic digestibility of lignocellulosic materials (Wyman 1996; Mosier *et al*., 2005). LHW pretreatment of corn fibre at 60°C and a pH above 4.0 dissolved 50% of the fibre in 20 minutes (Mosier *et al*., 2005). LHW causes ultrastructural changes and formation of micron-sized pores that enlarge accessible and susceptible surface area and make the cellulose more accessible to hydrolytic enzymes (Zeng *et al*., 2007).

2.5.2.2 Acid hydrolysis

Hydrolysis of cellulose can be brought about by using mineral acids or cellulolytic enzymes. Acid hydrolysis is a well-established process (Parisi, 1989), which gives good yields within a short reaction time. It has, however, several drawbacks, such as for example the requirement of costly corrosive-resistant construction materials (Nguyen, 1993). Furthermore, acid hydrolysis gives rise to inhibitory compounds which might inhibit the ethanolic fermentation (Olsson and Hahn-Hagerdal 1996; Larsson *et al.*, 1999a, b). Therefore, enzymatic hydrolysis offers advantages. Lignocellulosic materials must be pretreated prior to enzymatic hydrolysis in order to make the cellulose macromolecules accessible for the cellulolytic enzymes. There are several advantages and disadvantages of dilute-acid and enzymatic hydrolyses, which are listed in Table 2.2. Enzymatic hydrolysis is carried out under mild conditions, whereas acid hydrolysis requires high temperature and low pH, which results in corrosive conditions. While it is possible to obtain cellulose hydrolysis of close to 100% by enzymatic hydrolysis (Ogier *et al.*, 1999), it is difficult to achieve such high yield with the acid hydrolyses. Furthermore, several inhibitory

compounds are formed during acid hydrolysis, whereas this problem is not so severe for enzymatic hydrolysis (Wyman 1996; Lee *et al.*, 1999; Taherzadeh 1999).

2.5.2.3 Enzymatic Hydrolysis

(1) General Consideration

Enzymatic hydrolysis of cellulose to glucose is carried out by cellulose enzymes that are highly specific catalysts. The hydrolysis is performed under mild conditions of pH 4.5 – 5.0 and temperature of 40 – 50°C. Therefore, one may expect low corrosion problems, low utility consumption and low toxicity of the hydrolysates as the main advantages of this process (Taherzadeh and Karimi, 2007). Enzymatic hydrolysis of cellulose consists of the cellulase adsorption unto the surface of the cellulose, the biodegradation of cellulose to sugars and desorption of the cellulose.

(2) Important Factors in Enzymatic Hydrolysis

Substrate concentration and quality, applied pretreatment method, cellulose activity, and hydrolysis conditions such as temperature, pH, and mixing are the main factors in enzymatic hydrolysis of lignocellulosic materials. The optimum temperature and pH are functions of the raw material, the enzyme source, and hydrolysis duration. The optimum temperatures and pH of different cellulases are usually reported to be in the range of 40°C to 50 °C and pH 4 to 5 (Olsson and Halm-Hagerdal, 1996). However, the optimum residence time and pH might affect each other. Tengborg *et al.* (2001b) showed an optimal temperature of 38°C and pH 4.9 within 144 h residence time for cellulose (Commercial enzyme solutions, Celluclast 2 L, Novo Nordisk A/S, Bagsvaerd, Denmark).

One of the main factors that affect the yield and initial rate of enzymatic hydrolysis is substrate (cellulose and/or hemicellulose) concentration in the slurry solution. High substrate concentration can cause substrate inhibition, which substantially lower the hydrolysis rate. The extent of the inhibition depends on the ratio of total enzymes to total substrate (Sun and Cheng, 2002). Problems in mixing and mass transfer also arise in working with high substrate concentration. The ratio of enzyme to substrate used is another factor in enzymatic hydrolysis. Obviously, application of more cellulose, up to a certain level, increases the rate and yield of hydrolysis. However, increase in cellulose level would

significantly increase the cost of the process. Cellulase loading is usually in the range of 5 to 35 FPU per gram of substrate.

Addition of surfactants during hydrolysis can modify the cellulose surface properties. An important effect of surfactant addition in a process for lignocellulose conversion is the possibility to lower the enzyme loading. A number of surfactants have been examined for their ability to improve enzymatic hydrolysis. Non-ionic surfactants were found to be the most effective. Fatty acid esters of sobitan polyethozylates (Tween-20 and Tween-80), and polyethylene glycol, are among the most effective surfactants reported for enzymatic hydrolysis (Alkasrawi *et al.*,, 2003; Kim *et al.*, 2006a; Borjesson *et al.*, 2007). Addition of polyethylene glycol to lignocellulose substrates increased the enzymatic conversion from 42% to 78% in 16 h (Borjesson *et al.*, 2007). One reason for this effect might be adsorption of surfactants to lignin, which prevents unproductive binding of enzymes to lignin and results in higher productivity of the enzymes (Eriksson *et al.*, 2002). However, the surfactant should be selected carefully, since it may have negative impact on the fermentation of the hydrolysate. For instance, addition of 2.5 g/l Tween 20 helped to reduce enzyme loading by 50%, while retaining cellulose conversion (Eriksson *et al.*, 2002). However, this surfactant is an inhibitor to *D. clausenii* even at low concentration of 1.0 g/l (Wu and Ju 1998).

The recycling of cellulose enzymes is one potential strategy for reducing the cost of the enzymatic hydrolysis during the bioconversion of lignocelluloses to ethanol (Tu *et al.*, 2007). However, presence of solid residuals (mainly lignin) and dissolution of the enzymes in the hydrolysates make the enzymes difficult to separate. Immobilization is an alternative approach that can be used to retain the enzymes in the reactor, but steric hindrance, freedom of movement and gradual reduction of the cellulases activity must be considered. In this regard, it should be kept in mind that endoglucanase and exoglucanase should diffuse into lignocelluloses and be adsorbed to the surface of the particles in order to initiate hydrolysis and convert the cellulose to cellobiose. However, cellobiose is in the aqueous phase, where it is converted to glucose by β-glucosidase. Therefore, immobilization of β-glucosidase might theoretically be possible and effective (Tu *et al.*, 2006). It is also possible to co-immobilize β-glucosidase and a fermenting microorganism in order to improve the overall conversion of cellulose to ethanol (Lee and Woodward 1983). One of the major problems in immobilization is to separate the immobilized support from the residual solid of the reactor. One possible solution could be immobilization of the enzymes in magnetic particles, such as

magnetic agarose composite microspheres (Qiu and Li 2000; Qiu and Li 2001), or magnetic chitosan microspheres (Feng *et al.*, 2006).

(3) Factors limiting enzymatic hydrolysis:

The enzymatic hydrolysis of lignocellulose is limited by several factors. Several researchers concluded that crystallinity of cellulose is just one of the factors. Other factors are degree of polymerization (DP), moisture content, available surface area and lignin content (Puri, 1984; Koullas *et al.*, 1992; Chang and Holtzapple, 2000; Laureano-Perez *et al.*, 2005). Chang and Holtzapple (2000), however, mentioned that crystallinity affects the 1-hour enzymatic hydrolysis, but not the 3-d enzymatic hydrolysis. Caulfied and Moore (1974) opined that decreased particle size and increased available surface rather than crystallinity affect the rate and extent of the hydrolysis. Zhang and Lynd (2004) reported that a slower conversion of crystalline cellulose as compared to armorphous cellulose would increase the percentage crystallinity of the hydrolysed biomass.

Other researchers like Grethlein (1985), Grous *et al.* (1986) and Thompson *et al.* (1992) conclude that pore size of the substrate in relation to the size of the enzymes is the main limiting factor in the enzymatic hydrolysis of lignocelllulosic biomass. Removal of hemicellulose increases the mean pore size of the substrate thereby increasing the probability of the cellulose to get hydrolyzed (Grethlein, 1985; Gregg and Saddler, 1986; Palonen *et al.*, 2004). Drying of pretreated lignocellulose can cause a collapse in the pore structure resulting in a decreased (enzymatic) hydrolysability (Grous *et al.*, 1986). Zhang and Lynd (2004) also reported cellulases getting trapped in the pores if the internal area is much larger than the external area, which is the case with many lignocellulosic biomasses. Lignin also limits the rate and extent of (enzymatic) hydrolysis by acting as a shield, preventing the digestible parts of the substrate to be hydrolyzed (Chang and Holtzapple, 2000).

Table 2.2: Comparison between Dilute-acid and Enzymatic Hydrolyses

Comparing variable	*Dilute-acid hydrolysis*	Enzymatic hydrolysis
Mild hydrolysis conditions	No	Yes
High yields of hydrolysis	No	Yes
Product inhibition during hydrolysis	No	Yes
Formation of inhibitory by-products	Yes	No
Low cost of catalyst	Yes	No
Short time of hydrolysis	Yes	No

Source; *Taherzadeh and Karimi (2007). "Enzyme-based ethanol", BioResources 2(4), 707-738.*

2.6. Enzymes used in Hydrolysis

2.6.1 Cellulases

Enzymes specialized in breaking up the β-1-4-glycosidic bonds of glucan are collectively called cellulases. Reese *et al*. (1950) presented a model of enzymatic cellulose hydrolysis based on multiple enzymes (C_1 and C_X). The C_1 enzyme was assumed to produce shorter polyanhydro-glucose chains, while the solubilization was attributed to the C_X enzyme. The cellulases are divided into three sub-categories, representing three types of activity: an endo-1,4-β-glucosidase (EC 3.2.1.4) (endoglucanases), an exo-1,4-β-glucanase (EC 3.2.1.91) exoglucanases (cellobiohydrolases) and β-glucosidases (EC 3.2.1.21). Endoglucanases significantly reduce the degree of polymerization of the substrate by randomly attacking the interior parts, mainly in the amorphous regions of cellulose to create free ends. Exoglucanases (or cellobiohydrolases), on the other hand, incrementally shorten the glucan molecules by binding to the glucan ends and releasing mainly cellobiose units. Finally, β-glucosidases split the disaccharide cellobiose into two units of glucose as represented in figure 2.6 below.

Synergism between these two types of enzymes is attributed to the *endo-exo* form of cooperativity and has been studied extensively between cellulases in the degradation of cellulose by *Trichoderma reesei* (Bothast and Saha, 1997). β-glucosidases hydrolyze cellobiose and in some cases cellooligosaccharides to glucose. This type of enzyme is generally responsible for kinetic regulation of the whole cellulolytic process and is a rate-limiting factor during enzymatic hydrolysis of cellulose, as both endoglucanase and cellobiohydrolase activities are often inhibited by cellobiose (Bothast and Saha, 1997).Thus, β-glucosidase not only produces glucose from cellobiose but also reduces cellobiose inhibition, allowing the cellulolytic enzymes to function more efficiently. However, like β-glucanases, most β-glucosidases are subject to end-product (glucose) inhibition (Saha *et al.*, 1995). Several types of microorganisms can produce cellulase systems including aerobic filamentous fungi, aerobic actinomycetes, anaerobic hyperthermophilic bacteria and anaerobic fungi (Lynd *et al*., 2002). Intensive research on the aerobic filamentous fungi *T. reesei* during the past decades has resulted in an efficient cellulase-producing organism, which is currently dominating the industrial cellulase production (Esterbauer *et al*., 1991; Lynd *et al.*, 2002).

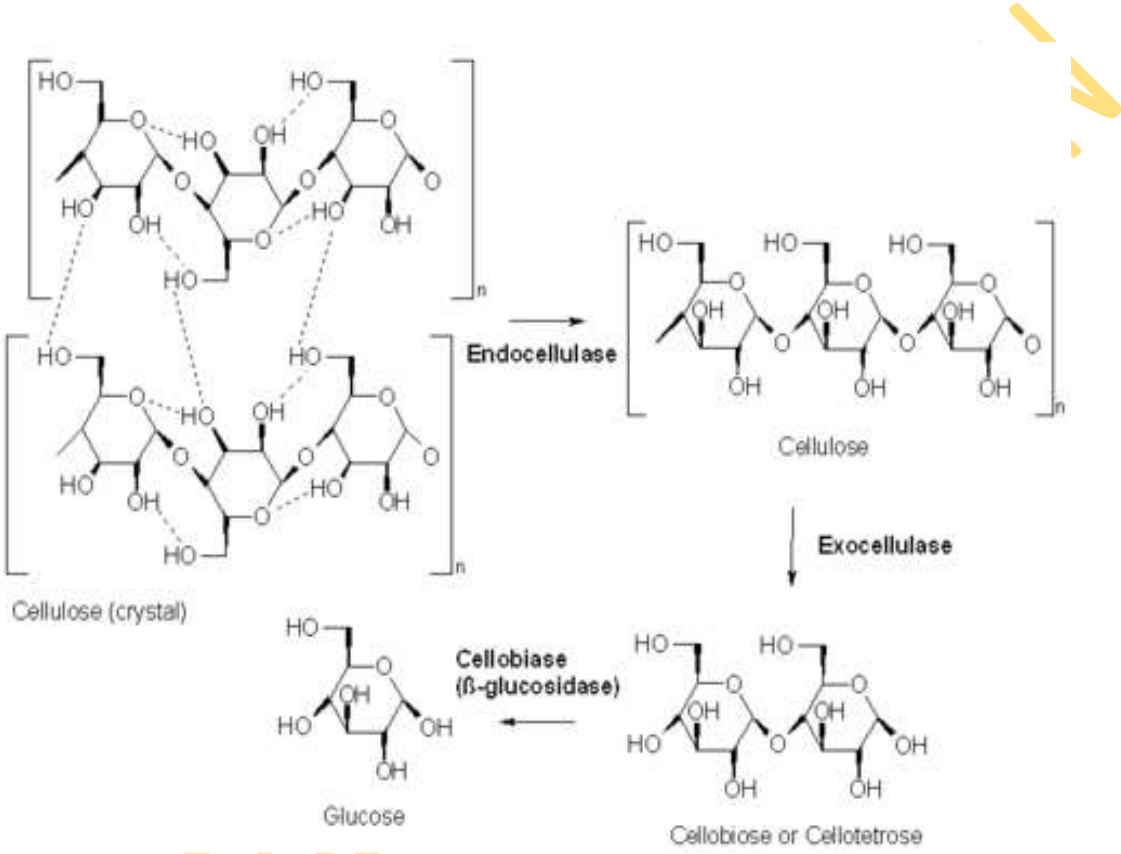


Fig. 2.5: *The 3 types of reaction catalysed by cellulases. Breakage of the non-covalent interactions present in the crystalline structure of cellulose (endo-cellulase) 2. Hydrolysis of the individual cellulose fibres to break into smaller sugars (exocellulases) 3. Hydrolysis of disaccharides and tetrasaccharides into glucose (beta-glucosidase) (adapted from http://www.en.wikilpedia.org/wiki/cellulose)*

2.6.2 Hemicellulases

Hemicellulases are mixture of enzymes which hydrolyse the indigestible component of the plant fibres. They work synergistically to completely degrade hemicelluloses into monosaccharide and disaccharides that can be used by organisms. Hemicellulases are frequently classified according to their action on distinct substrate (Perez *et al.*, 2002). Xylanases, the major component of hemicellulases have been isolated from many ecological niches where plants material is present. Xylanase (EC 3.2.1.8) hydrolyses the β -1,4 bonds in the xylan backbone yielding short xylo oligomers. β -mannase hydrolyses mannan based hemicelluloses and liberate short β-1,4- manna-oligomers which can further be hydrolyse to mannose by mannosidase (EC 3.2.1.25). β-xylosidase (EC 3.2.1.57) are exo type of glucosidase that hydrolyse short xylo oligomers into xylose unit.

2.6.3 Lignases/Laccases

Laccase (EC 1.10.3.2) is an exracellular copper-containing oxidase enzyme that plays an important role in the degradation of lignin; it is commonly found in the white-rot fungus *Trametes versicolor* (Kliskinen *et al.*, 2002)

Laccase have been used for the detoxification of lignocellulose hydrolysates prior to ethanolic fermentation. Treatment with laccase also represents a novel analytical tool for identification of fermentation inhibitors. Recently, bacterial laccases-like proteins have been found (Martin *et al.*, 2002). These enzymes polymerized a low-molecular-weight; water-soluble organic matter fraction isolated from compost into high-molecular weight products, suggesting the involvement of laccase in humification during composting (Chefetz *et al.*, 1998)

2.7. Detoxification of hydrolysate

The toxicity of lignocellulose hydrolysate can be removed by optimized "overliming" with $Ca(OH)_2$ (Martinez *et al.*, 2000). Toxicity of acid-hydrolyzed biomass is typically removed by addition of $Ca(OH)_2$ until the pH reaches 9-10, followed by pH neutralization (Hahn-Hagerdal, 1996). Sulphite and overliming treatment of hydrolyzate have been found best for fermentation by a recombinant *Escherichia coli* strain (Von Sivers *et al.*, 1994; Olsson *et al.*, 1995). Among the different detoxification methods on acid

hydrolysate, overliming produced the hydrolysate that was most completely fermented by *Pichia stipitis* and *Candida shehatae* (Eken-Sarcoglu and Arslan, 2000).

2.8 Ethanolic microorganisms

Ethanol has been derived from microbial fermentation for thousands of years. It is not only an important product of the alcoholic beverage industry, but also, it is one of the fastest growing fuel sources in the world. In 2004, the United States produced more than 12.5 x 10^9 liters of ethanol - a 17% increase over the amount generated in 2003 (http://www.ethanolrfa.org/pr041101.html). Keeping in step with this demand will require the engineering of new strains of fermentative microorganisms that can produce ethanol more efficiently, and more detailed information about the genetic circuits involved. Strains with saccharolytic/cellulolytic properties, which possess broader substrate specificity, are required. The general requirements for an organism to be used in ethanol production is that it should give a high ethanol yield, a high productivity and be able to withstand high ethanol concentrations in order to keep distillation costs low (von Sivers and Zacchi , 1996).

In addition to these general requirements, inhibitor tolerance, temperature tolerance and the ability to utilize multiple sugars are essential applications. Tolerance towards low pH-values will minimize the risk of contamination. The work-horse in starch or sucrose-based ethanol production is the common Bakers' yeast, *Saccharomyces cerevisiae*. This organism produces ethanol at a high yield (higher than 0.45 g g^{-1} at optimal conditions) and a high specific growth rate (up to 1.3 g g^{-1} cell mass h^{-1} (Verduyn *et al.*, 1990). It also has a very high ethanol tolerance, over 100 g L^{-1} has been reported for some strains and media (Casey and Ingledew 1986). In addition, the organism has proven to be robust to other inhibitors, and hence it is suitable for fermentation of lignocellulosic materials (Olsson and Hahn-Hàgerdal 1993; Hahn-Hàgerdal *et al*., 1994).

Xylose which is a five carbon sugar is abundant in hard woods and agricultural residues (Jefferies, 2006), so its fermentation is essential for the economic conversion of lignocellulose to ethanol (Saha *et al.*, 1998). The naturally xylose-fermenting yeasts, such as *Pichia stipitis, Pachysolen tannonophilus* and *Candida shehatae* (Toivola *et al.*, 1984; Du Preez *et al*., 1986; Prior *et al*., 1989) can efficiently ferment C_5 sugars and could be potentially be advantageous to use but, their tolerance to inhibitory compounds in undetoxified lignocellulose hydrolyzates is rather low (Van Zyl *et al.*, 1988; Roberto *et al.*,

1991) and their ethanol production rates are five times lower than those observed with *S. cerevisiae* (Hahn-Hagerdal *et al.*, 1994; Singh and Mishra, 1995; Lee, 1997). In addition these C_5-fermenting organisms require a very low and well-controlled supply of oxygen for efficient xylose fermentation (Ligthelm *et al.*, 1988; Grootjen *et al.*, 1990; Skoog and Hahn-Hàgerdal 1990) and are 3-4 times less alcohol tolerant than *S. cerevisiae* (Hinamn *et al.*, 1989; Picataggio and Zhang, 1996).

At the moment, the traditional microorganisms for the industrial scale production of ethanol is the yeast particularly *Saccharomyces cerevisiae* and *S. uvarum* since their fermentation capacities have been developed to near perfection by extensive research and development both on the yeast and on the process optimization as a result of their historical importance in the brewing and distillery industries. *Saccharomyces cerevisiae* is the most effective and safest organism for fermenting sugars to ethanol (Wantanabe *et al*., 2005; Chu and Lee, 2007). The advantages of *S. cerevisiae* as a host for ethanol production over other yeasts include tolerance to aerobic and anaerobic conditions, metabolic versatility, capacity to produce ethanol under anaerobic and aerobic (with excess carbon) conditions, and high ethanol tolerance (150 g/liter).

The main "competitors" to the yeast have been the bacterium *Zymomonas mobilis* and genetically engineered *Escherichia coli*. *Z. mobilis* is an obligately anaerobic bacterium, which lacks a functional system for oxidative phosphorylation, produces ethanol and carbon dioxide as principal fermentation products. Interestingly, *Z. mobilis* utilizes the Entner-Duodoroff pathway which gives a lower ATP production per catabolized glucose (Swings and De Ley 1977). This in turn gives a lower biomass yield and a higher ethanol yield on glucose compared to *S. cerevisiae* (Rogers *et al*., 1979). However, wild-type *Z. mobilis* lacks the ability to ferment pentose sugars and is also not a very robust organism. In general, bacteria appear to be less tolerant to lignocellulose-derived inhibitors (Hahn-Hàgerdal *et al*., 2007), and a detoxification step may be needed prior to the fermentation. In contrast to Bakers' yeast and *Z. mobilis*, *E. coli* is capable of metabolizing a wide variety of substrates (including hexoses, pentoses and lactose), but the wild-type organism has a mixed fermentative pathway, and is thus a poor ethanol producer. While the yeast can ferment only glucose, *Zymomonas* ferments glucose, fructose and sucrose, in addition to these advantages, the anaerobic nature of *Zymomonas* negates the requirement for oxygen necessary for the

growth of yeasts, its higher sugar uptake and ethanol yield, its lower biomass production, higher ethanol tolerance and its amenability to genetic manipulations (Jeffries, 2005).

Over the years *Zymomonas mobilis* has been shunned because it can spoil fermentation of cider and beer with sulfurous flavors and rotten odours. However, in this rapidly changing industry, *Z. mobilis* has gain popularity. Off-flavors are not a concern in the production of fuel ethanol, so the faster fermentation kinetics and higher product yields of *Z. mobilis* could give it an advantage (Jeffries, 2005).

Zymomonas mobilis is distinctive in that it is one of the few facultative anaerobic bacteria that use the Entner-Doudoroff pathway for glucose metabolism rather than the more familiar Embden-Meyerhoff-Parnas glycolytic pathway used by *S. cerevisiae* and other aerobic organisms. Although the Entner-Doudoroff pathway is widely distributed among pseudomonads, it is normally part of aerobic metabolism. Unlike glycolysis, which can theoretically generate two moles of ATP for each mole of glucose fermented to ethanol, the Entner-Doudoroff pathway has a net yield of only one ATP per mole of glucose. This low yield results in low cell mass and allows higher ethanol yields (Jeffries, 2005).

Deficiencies in glycolysis and the pentose phosphate pathway greatly constrain the ability of *Z. mobilis* to assimilate other sugars. Indeed, it was precisely the objective of adding the capacity for *xylose* and *arabinose* metabolism that led researchers at the National Renewable Energy Laboratory to engineer genes for xylulokinase, transketolase, transaldolase, xylose isomerase and three other enzymes into this organism (Deanda, 1996). Further improvements in substrate utilization can be expected to flow from additional manipulations of the genome to produce ethanol from lignocellulose. In general, bacteria appear to be less tolerant to lignocellulose-derived inhibitors (Hahn-Hàgerdal *et al.*, 2007), and a detoxification step may be needed prior to the fermentation.

2.8.1. *Pichia stipitis*

Pichia stipitis is haploid, homothallic, hemiascomycetous yeast (Kurtzman, 1990; Melake *et al.*, 1996) that has the highest native capacity for xylose fermentation for any known microbe (Van Dijken *et al.*, 1986). It forms yeast-like buds during exponential growth, has a hat-shaped spores and pseudomycelia, uses all the sugars found in hardwood (Lee *et al.*, 1986) and transforms low molecular weight lignin moieties (Targonski, 1992). *P. stipitis* Pignal (1967) is closely related to yeast endosymbionts of passalid beetles (Nardi,

2006) that inhabit and degrade white-rotted hardwood (Suh *et al.*, 2003). *P. stipitis* have been isolated from the guts of wood-inhabiting passalid beetles suggesting that this family of yeasts has evolved to inhabit an oxygen-limited environment rich in partially digested wood. The presence of numerous genes for endoglucanases and β-glucosidases, along with xylanase, mannanase and chitinase activities indicates that it could metabolize polysaccharides in the beetle gut. Expressed sequence tags (ESTs) supports 40% of the predicted genes with 84% showing strong similarity to proteins in other fungi (Jefferies *et al.*, 2007).

Sequenced genes of *P.stipitis* has revealed a better understanding of its biology, metabolism and regulations with genome size of 15.4Mbp It is made up of numerous genes and physiological features for lignocellulosic bioconversion including a high capacity for using cellobiose and other oligomers (http://www.jgi.doegov/pichia). It has eight chromosomes which range in size from 3.5 to 0.9Mbp. The finished chromosomes have only one gap in the centromere region of chromosome 1 (Passoth *et al.*, 1992). *P. stipitis* uses alternative genetic code in which CUG codes for serine rather than leucine. Unlike *Saccharomyces cerevisiae* which induces fermentation by sensing the presence of fermentable sugars such as glucose, *Pichia stipitis* induces fermentative ability in response to oxygen limitation (Passoth *et al.*, 2003; Klinner *et al.*, 2005). *P. stipitis* genes have been used to engineer xylose metabolism in *S. cerevisiae.*The naturally-occurring, xylose-fermenting yeasts metabolize xylose by relying on xylose reductase (XR) to convert xylose to xylitol, on xylitol dehydrogenase (XDH) to convert xylitol to xylulose, and on xylulokinase (XK) to convert xylulose to xylulose 5-phosphate, (Jeffries, 1983). The synthesis of these xylose-metabolizing enzymes in such yeasts as *P. stipitis* requires the presence of xylose for induction and is also totally or at least partially repressed by the presence of glucose (Bolen and Detroy, 1985).

2.8.2. Initial Steps of Xylose Metabolism in Yeasts

For yeasts able to grow on xylose, the metabolism of xylose is induced following its transport into the cell. In xylose-fermenting yeast such as *Pichia stipitis*, xylose is converted into xylulose by the sequential action of two oxidoreductases. First, xylose reductase (alditol: $NADP^+$ 1-oxidoreductase, EC 1.1.1.21) catalyzes reduction of the C1 carbonyl group of xylose, yielding xylitol as the product. Xylitol is then oxidized by xylitol dehydrogenase (XDH; EC 1.1.1.9) to give xylulose (Fig. 2.6). Xylulose is phosphorylated at the C5 –OH

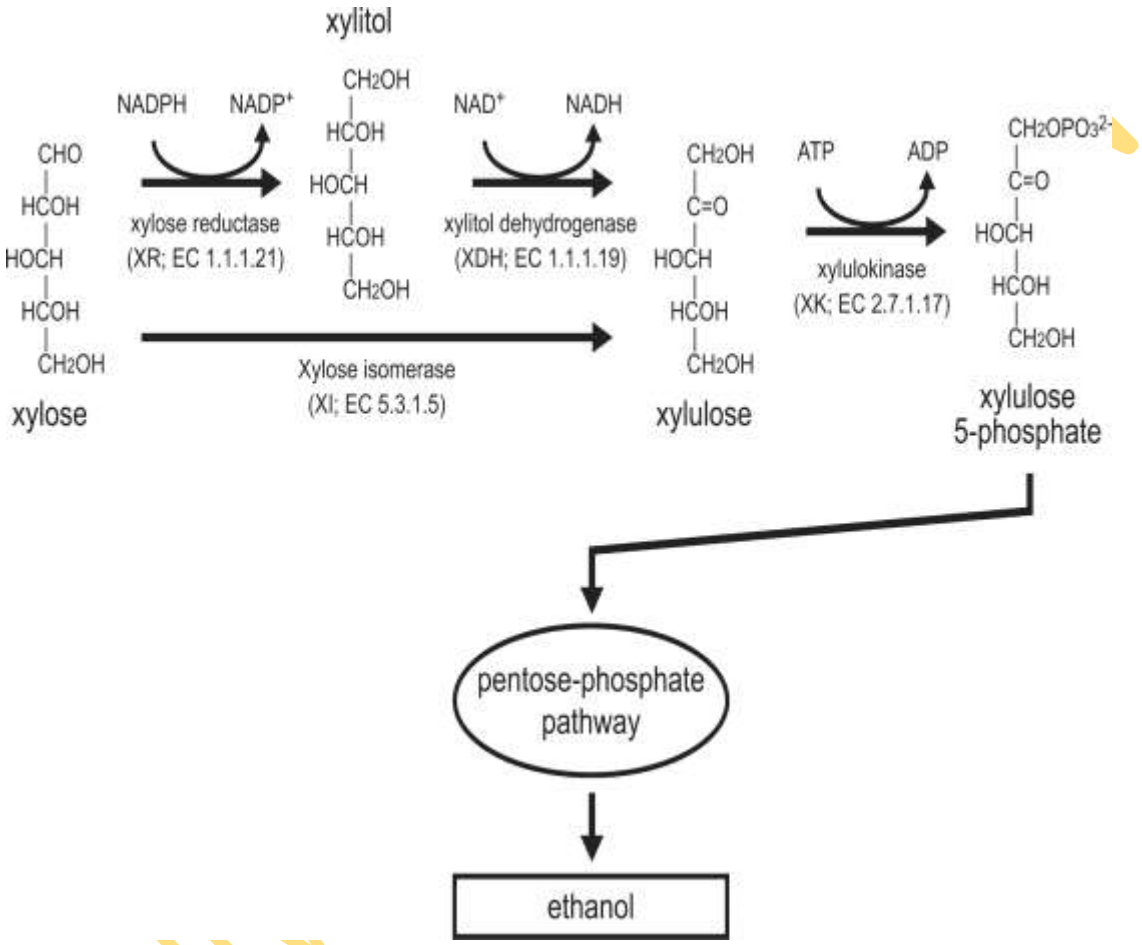


Fig 2.6: *Genes Involved in the metabolism of Xylose (adapted from Watanabe et al., 2005)*
XR, xylose reductase; *XI*, xylose isomerase; *XK*, xylulokinase

position by xylulokinase (XK) to yield xylulose-5-Phosphate, which is further channeled into glycolytic intermediates such as glyceraldehyde-3-phosphate and fructose-6-phosphate via the pentose phosphate pathway (PPP)

S. cerevisiae transformed with these two genes from *P. stipitis* could ferment xylose to ethanol. There is another problem; the excretion of xylitol occurs unless a co-metabolizable carbon source such as glucose is added. This is probably caused by several combined factors. In particular, intercellular redox imbalance due to a different coenzyme specificity of xylose reductase (with NADPH) and XDH (with NAD^+) has been thought to be one of the main factors (http:www.jbc.org/cgi/content/full280/11). The generation of an $NADP^+$-dependent XDH by protein engineering would avoid this problem. Xylulose enters the pentose phosphate (PP) pathway after phosphorylation by xylulokinase (XK), encoded by the *XKS1* gene, which is naturally present in *S. cerevisiae*, and consequently a high capacity of the PP pathway is required.

2.8.3. Saccharomyces cerevisiae

Saccharomyces cerevisiae is the most thoroughly investigated eukaryotic microorganism, which aids our understanding of the biology of the eukaryotic cell and hence, ultimately, human biology (Ostergaad *et al.*, 2000). For several centuries, *S. cerevisiae* has been used in the production of food and alcoholic beverages, and today this organism is also used in a number of different processes within the pharmaceutical industry. *S. cerevisiae* is a very attractive organism to work with since it is nonpathogenic, and due to its long history of application in the production of consumable products such as ethanol and baker's yeast. Also, the well-established fermentation and process technology for large-scale production with *S. cerevisiae* make this organism attractive for several biotechnological purposes. Another important reason for the applicability of *S. cerevisiae* within the field of biotechnology is its susceptibility to genetic modifications by recombinant DNA technology, which has been even further facilitated by the availability of the complete genome sequence of *S. cerevisiae*, published in 1996 (Goffeau *et al.*, 1996). *S. cerevisiae* is relatively tolerant to low pH values and high sugar and ethanol concentrations, i.e., properties which lower the risk of contamination in industrial fermentation. Moreover, this yeast is fairly resistant to inhibitors present in biomass hydrolysates and is able to grow anaerobically. These have been the major reasons for increasing *S. cerevisiae* exploration in industrial ("white") biotechnology, focusing on the fermentative production of industrially relevant biochemicals (Nevoigt, 2008).

The availability of highly efficient transformation methods, Gietz and Woods (2001) has aided *S. cerevisiae* genetic engineering. Furthermore, many specialized expression vectors, including episomal ones (Parent and Bostain, 1995), and numerous other useful tools such as reporter genes, immunotags, and genetically selectable markers (Gueldener *et al.*, 2002; Janke *et al.*, 2004; Sheff and Thorn, 2004) have been available. In addition, the extraordinarily high efficiency of homologous recombination in this species has facilitated targeted manipulations within chromosomes (Klinner and Schafer, 2004). The popularity of *S. cerevisiae* in basic and applied research is undoubtedly influenced by its classification as GRAS (generally regarded as safe) by the U.S. Food and Drug Administration (FDA).

Two tasks are prerequisites for the synthesis of a *S. cerevisiae* strain that efficiently converts xylose to ethanol: (i) expression of heterologous genes should enable *S. cerevisiae* to convert xylose to xylulose; and (ii) consumption of xylulose should be improved since *S. cerevisiae* grows on xylulose with a maximum specific growth rate 10 times lower than that on glucose (Hirosawa *et al*., 2004). Xylulose enters the pentose phosphate (PP) pathway after phosphorylation by xylulokinase (XK), encoded by the *XKS1* gene (Fig 2.7), which is naturally present in *S. cerevisiae*, and consequently a high capacity of the PP pathway is required. A number of steps may contribute to control xylose consumption: the uptake of xylose, the conversion of xylose to xylulose, the phosphorylation of xylulose to xylulose-5-phosphate, and the conversion of xylulose-5-phosphate through the pentose phosphate pathway followed by subsequent conversion to ethanol from pyruvate after glycolysis.

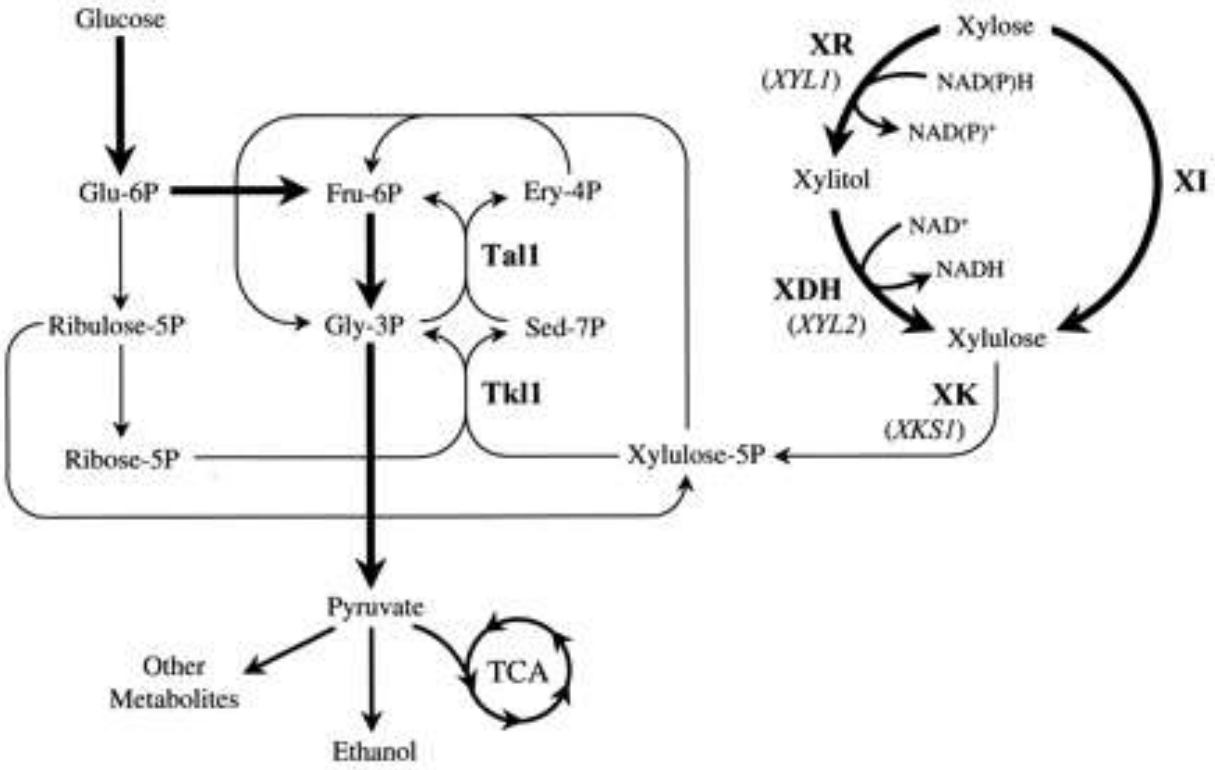


Fig 2.7: *Overview of metabolic pathways for glucose and xylose metabolism. The Embden-Meyerhof-Parnas pathway and the xylose utilization pathway are illustrated by thick lines. The PP pathway is indicated by thin lines. Abbreviations: TCA, tricarboxylic acid cycle; Tkl1, transketolase; Tal1, transaldolase; 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. Genes are depicted in parentheses.(adapted from Ostergaad et al., 2000).*

2.9. Genetic manipulation

Since no naturally-occurring organism can satisfy all the specifications needed for lignocellulosic fermentation, genetic engineering techniques have been utilized with the aim of constructing organisms with most desirable properties for bioprocesses (Ingram *et al.*, 1998; Aristidou and Pentilla, 2000).

Metabolic engineering was introduced by Bailey in 1991 as a subdiscipline of engineering and pertains to "the improvement of cellular activities by manipulation of enzymatic, transport, and regulatory functions of the cell with the use of recombinant DNA technology" (Bailey *et al.*, 1990; Bailey 1991).

Metabolic engineering, i.e., the intentional redirection of metabolic fluxes, has played an exceptional role in improving yeast strains for all industrial applications. In contrast to classical methods of genetic strain improvement such as selection, mutagenesis, mating, and hybridization (Panchal 1990; Attfield and Bell 2003), metabolic engineering has conferred two major advantages:

(1) the directed modification of strains without the accumulation of unfavorable mutations and

(2) the introduction of genes from foreign organisms to equip *S. cerevisiae* with novel traits. The latter is particularly crucial for industrial biotechnology to provide pathways that extend the spectrum of usable industrial media (e.g., lignocellulosic biomass) and/or to produce compounds not naturally formed by *S. cerevisiae*. Since the first introduction of metabolic engineering (Bailey, 1991), there have been tremendous enhancements of its toolbox and have greatly enhanced the potential for using yeast in biotechnological production processes. Ethanol genes have successfully been introduced into bacteria such as *Escherichia coli* and *Klebsiella oxytoca*. Efforts have been undertaken to incorporate pentose-metabolizing pathways into natural ethanol producers. However, the development/use of genetically engineered strains should take account of additional environmental or processing costs of using recombinant organisms.

2.9.1. Pentose fermentation by engineered *S. cerevisiae*

Due to the very attractive properties of *S. cerevisiae* in industrial fermentations, there have been significant efforts made in the past decades to design recombinant xylose and arabinose fermenting strains of this yeast. Xylose fermenting strains of *S. cerevisiae* can in principle be constructed either by introducing genes encoding xylose isomerase (XI) from bacteria and fungi (Walfridsson *et al*., 1997; Karhumaa *et al*., 2005; Kuyper *et al.*, 2005), or genes encoding xylose reductase (XR) and xylitol dehydrogenase (XDH) from fungi (Kòtter *et al*., 1990; Eliasson *et al*., 2000). Transport proteins are needed for uptake of xylose, as well as of other sugars in yeast. In *S. cerevisiae*, xylose has been found to be transported by the hexose transporters, (Meinander and Hahn-Hàgerdal, 1997; Kilian and Uden, 1998), but the affinity for xylose is approximately 200-fold lower than for glucose, (Kòtter and Ciriacy, 1993). Consequently, xylose uptake is competitively inhibited by glucose.

There are 20 different genes encoding sugar transport related proteins, 18 individual systems (Hxt1-17 and Gal2) and two related signal proteins (Snf3p and Rgt2p). The transporters exhibit different affinities for sugars and the expression of their corresponding genes are regulated by the sugar concentrations, *i.e*. the availability of the carbon source, (Boles and Hollenberg, 1997). It has previously been suggested that xylose is taken up by both high- and low-affinity systems of glucose transporters, but the uptake is increased in the presence of low glucose concentrations (Lee *et al*., 2002).

Studies have indicated that the high- and intermediate-affinity hexose transporters; Hxt4, Hxt5 Hxt7 and Gal2 are in fact the most important transporters for xylose (Hamacher *et al*., 2002). Furthermore, it has been shown that a low glucose concentration is needed in the medium for efficient xylose uptake (Meinander *et al*., 1999). This has been explained by a need for glucose for expression of glycolytic enzymes and intermediates (Boles and Zimmermann, 1996), as well as generation of intermediary metabolites for the initial steps of the xylose metabolism and the pentose phosphate pathway (Meinander, 1999). Another possible explanation, inferred was that the glucose is needed for the expression of hexose transporters with favorable xylose transport properties, *e.g*. Hxt4 (Bertilsson, 2008; Pitkanen *et al*., 2003).

2.9.2. Vectors:

Plasmids are circular, double-stranded DNA (dsDNA) molecules that are separate from a cell's chromosomal DNA. These extrachromosomal DNAs, which occur naturally in

bacteria, yeast, and some higher eukaryotic cells ranges in size from a few thousand base pairs to more than 100 kilobases (kb). Like the host-cell chromosomal DNA, plasmid DNA is duplicated before every cell division. During cell division, at least one copy of the plasmid DNA is segregated to each daughter cell, assuring continued propagation of the plasmid through successive generations of the host cell (Brown, 1995).

In order to be useful in DNA cloning, a plasmid vector must contain a *selectable gene;* most commonly a drug-resistance gene encoding an enzyme that inactivates a specific antibiotic like the ampicillin-resistance gene (*amp*^{r}) encodes β-lactamase, which inactivates the antibiotic ampicillin.

An expression vector, otherwise known as an expression construct, is generally a plasmid that is used to introduce and express a specific gene into a target cell (Brown, 1995). Expression vector allows production of large amounts of stable mRNA. Once the expression vector is inside the cell, the protein that is encoded by the gene is produced by the cellular transcription and translation machinery. The plasmid is engineered such that it contains a highly active promoter which causes the production of large amounts of mRNA. After expression of the gene product, the purification of the protein is required; but since the vector is introduced to a host cell, the protein of interest should be purified from the proteins of the host cell. Therefore, to make purification process easy, the cloned gene should have a tag. This tag could be histidine (His) tag or any other marker peptide. (http://www.dualsystems.com/support/vector-information.html)

Expression vectors are used for molecular biology techniques such as site-directed mutagenesis. In general, DNA vectors that are used in many molecular biology gene cloning experiments need not result in the expression of a protein. Expression vectors are often specifically designed to contain regulatory sequences that act as enhancer and promoter regions, and lead to efficient transcription of the gene that is carried on the expression vector. Expression vectors are basic tools for biotechnology and the production of proteins such as insulin that are important for medical treatments of specific diseases like diabetes. Expression vectors must have expression signals like a strong promoter, strong termination codon, and adjustment of distance between promoter and cloned gene, insertion of transcription termination sequence and a portable translation initiation sequence (PTIS). Examples of an expression vectors includes pGAPZαA, pVT100-L, pVT101-U, pVT102-L, pVT103-L, pTEF-MF, pGAL-MF. Others includes p427-TEF, p417-CYC both carrying the aminoglycoside phosphotransferase gene for selection in yeast using G418 (http://www.dualsystems.com/support/vector-information.html)

CHAPTER 3

3.0 MATERIALS AND METHODS

3.1 Sample Collection

3.1.1 Collection of Cocoa Pod Husk Samples

Fresh cocoa pod husks from which cocoa beans have been removed were obtained from the fermentary section of Cocoa Research Institute of Nigeria (CRIN), Ibadan. Suitability was determined to ensure that diseased pods were not selected. The selected cocoa pods were then sundried.

3.1.2 Collection of *Pichia stipitis*

Pichia stipitis Pignal in a glass cryotube (NBRC 10063) CBS 6054 was obtained as a lyophilized powder from the National Institute of Technology and Evaluation (NITE) Biological Resource Centre, Chilba, Japan

3.1.3 Plasmid vectors and other Expression vector

Two yeast expression vectors were used. pGAPZαA vector was obtained from Department of Genetics, Tarbiat Modares University, Iran. The other expression vector used in this study was pVT100-U plasmid vector which was a generous gift from the Yeast Molecular Genetics laboratory of International Centre for Genetic Engineering and Biotechnology (ICGEB), Trieste, Italy.

3.1.4 Restriction Enzymes, DNA Modification Enzymes and other Reagents

Restriction enzymes, DNA modifying enzymes and other molecular reagents were obtained from Yeast Molecular genetics laboratory of ICGEB Italy, Promega and Sigma. Bioinformatics tools used include DNA strider (1.4f7) and Macplasmap programmes of Yeast Molecular Genetics laboratory of ICGEB. The NCBI BLAST search programme (http://www.ncbi.nlm. nih.gov/BLAST) was also employed.

3.2 Primer Designs and Synthesis

Primers for PCR were designed using *Saccharomyces* Genome Database (SGD) (http://www.yeastgenome.org/) ensuring that the primer was within 17 – 18 bases in length and the melting temperature (calculated) of the primer was within 50°C – 60°C. The 3' end of the primer was designed to end in G or C bases inorder to prevent "Breathing" of the

ends, ensure stronger bond and increase the efficiency of priming. The sequences generated were synthesized by Sigma.

3.2.1 *Trichoderma hamatum*

Fungal Isolate: *Trichoderma hamatum* was used for the production of cellulase and xylanase enzymes having established its merit through preliminary investigation. This fungus was obtained from the collection of the Microbial Physiology and Biochemistry laboratory, Department of Microbiology, University of Ibadan. Pure cultures of the fungi were maintained on Potato Dextrose Agar (Oxoid) slants by subculturing every four weeks onto freshly prepared PDA media and preserved at 4°C.

3.3 Isolation and Culture Methods

3.3.1 Isolation of starter cultures from a spontaneously-degrading cocoa pod-derived ecosystem

Dried cocoa pod husk was obtained and fractionated to different particle size forms-coarse, fine particle size and powdered using Electric blender. Spontaneous fermentation of the different samples of cocoa pod husk (coarse, fine and powder) was carried out by steeping 10g substrate in 100ml of sterile distilled water in seven different flasks each and allowed to ferment naturally at room temperature for 7 days. On each day of spontaneous fermentation, 1ml of the samples was put in 9ml of sterile distilled water in a test tube and serially-diluted in 9ml of sterile distilled water in different test tubes (Meynell and Meynell, 1970). One milliliter (1ml) each of appropriate dilutions was inoculated by pour plate method in Yeast Extract Agar (YEA) medium in duplicate containing Streptomycin antibiotics at a concentration of 40.0µg/ml. The plates were incubated at 30^{o}C for 48 hours. Representative colonies were picked randomly and transferred by streaking unto sterile YEA plates until pure cultures were obtained (Harrigan and MacCance, 1966).

3.3.2 Maintenance of Pure Cultures

The purified yeast cultures obtained were kept on YEA slants at 4^{o}C in the refrigerator and subcultured unto fresh slants at intervals of two months inorder to ensure their viability.

3.3.3 Selection of yeast Isolates

For the development of suitable organisms for starter cultures, yeast isolate with the highest frequency of ecological occurrence during isolation were selected.

3.4. Characterization and Identification of Yeast Isolates

Selected yeasts isolates were sub-cultured onto fresh YEA media to obtain pure cultures. Pure cultures of the selected yeasts isolates were identified on the basis of cultural, morphological, biochemical tests. The following morphological, biochemical and physiological tests were used for purpose of identification:

Preliminary identification of yeast isolates was based on their cultural, colony characteristics, microscopic appearance of the cell and other biochemical characteristics. Smear of yeast isolates were air dried, flooded with Lactophenol in cotton blue and then viewed under the microscope using oil immersion lens (Barnett *et al.,* 1990)

3.4.1. Ascopore Formation

The ascoporulation medium (Goroadkawa agar) was used. The components of medium, D-glucose 1g, Sodium chloride 5g, Agar 20g and distilled water 1000ml were measured into a clean conical flask and sterilized at 121°C for 15 minutes. The media was allowed to cool to about 45°C and was poured aseptically into sterile petri dishes. Forty-eight hour-old cultures of the different yeast isolates was streaked on individual plates. The inoculated media was inclubated at 30°C for 3 days. The growth on each of the plates were examined microscopically under the oil immersion objective (x 40) for the presence of ascopores, after staining with lactophenol in cotton blue (lodder and Kreger-van rij, 1952).

3.4.2. Pseudomycelium Formation

The medium used was the commercially prepared Potato Dextrose Agar (PDA). The components were measured accordingly, and sterilized at 121°C for 15 minutes Streptomycin sulphate (0.0lmg/ml) was added aseptically when the media had cooled to about 45°C. Slide cultures were made as follows: Petri dishes were sterilized each dish

containing a piece of filter paper, a U-shaped glass rod support, two clean microscopic slides and clean cover slip. The already prepared potato dextrose Agar was melted and poured aseptically into a boiling tube, wide and deep enough to hold a microscope slide. Each slide was dipped into the agar and replaced on its glass rod support in the petri dish. Yeast cells from an actively growing culture were lightly inoculated with a straight wire along the length of each slide and a cover slip placed over a part of the inoculated agar. The sterile filter paper moistened with sterile water was placed in the petri dishes to prevent drying. The plates were incubated at 30°C. After wiping the agar from the back of each slide, the cultures were examined microscopically every two days for two weeks *(Barnet et al.,* 1990).

3.4.3. Asexual Reproduction

The medium used was Yeast Extract Dextrose Peptone (YEDP) agar. Molten YEDP was poured into sterile petri dishes and allowed to solidify. The medium was inoculated and incubated at 30°C for 24 hours. Lactophenol in cotton blue stain was used and observation was done under oil immersion objective (d40). The yeast isolate can either reproduce by budding or fission (Ethiraj *et al.,* 1980).

The physiological characteristics used for classifying the yeast isolates includes:-

3.4.4. Nitrate Assimilation Test

Filter sterilized 0.07% (w/w) KNO_3 solution was used as source of nitrate in the medium. Modified Bacro Yeasts carbon base (Wickerham, 1946) was used as basal medium. Fifteen mililitres of this was dispensed into McCartney bottles and sterilized at 121°C for 15 minutes in an autoclave. Five mililitre of the filter sterilized 0.07% KNO_3 was added into each McCartney bottle containing the sterile basal medium. Inoculation of the tubes with the test organisms was carried out and tubes were incubated at 30°C for 7 days. The control tube was not inoculated. At the end of 7 days, growth was observed visually in the test tube (Wickerham, 1946*).* The McCartney bottles were shaken to improve aeration (Barnett *et al.,* 1983).

3.4.5. Acid Production Test

The components of the medium were glucose 5g; Calcium Carbonate 0.5g; yeast extract 2g; agar 2g; and distilled water 100ml for acid production were measured out into sterile 250ml conical flask. These were mixed properly and 15ml was then dispensed into sterile McCartney bottles and was sterilized at 121°C for 15 minutes. The bottles were

mixed thoroughly cooled to 50°C and were slanted. Solidification of the medium was done by quickly plunging the test tubes in a slanting position to an ice bath. The yeast isolates were each streaked down the centre of the slant and acid production was observed by dissolution of the constitution of the medium ($CaCO_3$). Incubation was at 30°C and was observed for 5 days (Beech *et al.,* 1968).

3.4.6. Sugar Fermentation Test

This test was performed to determine the ability of certain isolates to ferment particular sugars. Andradeà peptone water was used and 15g of this was weighed and dissolved in 1 litre of distilled water in a litre conical flask. Ten millilitres of phenol red indicator was added. Nine milliliters of the basal medium (Andrade peptone water) was pipetted into test tubes containing inverted Durham tubes. The test tubes were plugged with cotton wool and sterilized at 121°C for 10 minutes. The different sugars, (glucose, lactose, galactose, sucrose, maltose, cellubiose, raffinose, trehalose and xylose) were then added 1% to the basal medium, and the resulting solution was left to cool. Each of the test tubes was then inoculated with the appropriate yeast culture using flame sterilized wine loop. The test tubes were incubated at 30°C for 7 days and observed each day for colour changes and gas production (Seeley and Van Denmark, 1972).

3.4.7. Growth at Different Concentrations of D-glucose

The basal medium used was Yeast Extract Peptone broth. It was prepared in a clean conical flask containing distilled water and homogenized. Two hundred milliliters each of the medium was measured into ten conical flasks and this was followed by the addition of sugar (w/v) 20%, 30%, 40%, 50%, 60% and 70% of glucose. Ten milliliters each of the different sugar concentrations were dispensed into test tubes and autoclaved at 121°C for 10 minutes. The broth was cooled after which the different yeast isolates were inoculated and incubated at 30°C for 3 days. The tubes containing high concentrations of D-glucose 50%, 60% and 70% were kept for up to one week (Barnett *et al,* 1990). Turbidity was used as measure of growth (Gray, 1945).

3.4.8. Growth at Different Temperatures

The test organisms were grown in Yeast Extract Dextrose peptone broth at different temperatures. The components of the medium were measured into a sterile 250ml conical flask containing distilled water. Fifteen milliliters was dispensed into test tubes plugged with a cotton wool and sterilized at 121°C for 15 minutes in an autoclave. After cooling, the test

organisms were inoculated into the medium aseptically and incubated at 25°C, 30°C, 37°C and 40°C for 3 days respectively using turbidity.

3.4.9. Urease Test

The basal medium Christensen's urea agar, Christensen, (1946) was distributed in McCartney bottles, heat sterilized in the autoclave at 121°C for 15 minutes. It was cooled to 45°C. Sufficient 20% urea solution, previously sterilized by filtration was then added to give a final concentration of 2%. The bottles were slanted and allowed to solidify. The slope medium was inoculated and incubated at 30°C for 1 – 7 days. The McCartney bottles were observed each day for change in colour of the medium from yellow to pink (Christensen, 1946).

3.4.10. Ethanol Tolerance

Ethanol tolerance was tested by the method of Ekunsami and Odunfa, (1990). The medium used contained in g/l of distilled water yeast extract 5g; peptone 10g; dextrose 20g. This was homogenized in a hot water bath and was dispensend into 100ml conical flask in proportions corresponding to the amount of ethanol to be added. For 4%, 5%, 6%, 7%, 8%, 9%, 10% and 11% ethanol concentration that were added a corresponding 48ml, 47.5ml, 47ml, 46.5ml, 46ml, 45.5ml, 45.0ml and 44.5ml of the medium was dispensed into the conical flasks. The medium was sterilized at 121°C for 15 minutes and cooled. The corresponding concentration of absolute ethanol was added to the medium aseptically. The medium was swirled to ensure even distribution of the ethanol in the medium.

The yeast isolate for screening was prepared by taking loopful of the culture from an agar slant and suspending in 1ml of sterile distilled water in test tubes. The test tubes were shaken vigorously to ensure dispersion of the yeast isolates in the sterile distilled water. An inoculum concentration of 0.1ml was transferred aseptically from the tubes into the conical flask containing the ethanol. The control was an uninoculated conical flask. The flasks were incubated with shaking at 150 rev./min. and 30°C for 72 hours.

3.5 Preliminary assessment of starters for ethanol tolerance

This was necessary for further selection of two organisms amongst the five selected yeast starters for genetic modification. Isolates were screened for ethanol tolerance on the criterion of Rose (1980) using the medium of Novak *et al*. (1981). The medium contained in g/L, 5g of $MgS0_4.7H_2O$, NH_4SO_4 2g, yeast extract 1.0g and 2.0g of glucose. The medium was dispensed into flasks and autoclaved at 121^OC for 15 minutes. It was allowed to cool to

room temperature before ethanol was added to each flask to make 9%, 10%, 11%, 12% and 13% (w/v) respectively. The samples were then dispensed into pre-sterilized flasks and inoculated with the selected yeast strains. The flasks were then incubated with shaking at 150 rpm in a shaker incubator at 30^{O}C for 48 hours. Initial Optical Density of the flask was taken at 600nm in a spectrophotometer using uninoculated medium as blank. Any increase in the optical density (O.D) reading was taken as evidence of growth.

Ethanol tolerance is defined as the amount of ethanol (% v/v) which completely inhibited the growth under the conditions stated (Rose, 1980).

3.6 Genetic Modifications

3.6.1 Recovery of *P. stipitis* from Cryotube

Lyophilized *Pichia stipitis* Pignal in a glass cryotube (NBRC 10063) CBS 6054 was recovered in the laboratory using a recovery fluid. Rehydration fluid – (YM medium) was prepared in the Yeast Molecular Genetics laboratory, International Centre for Genetic Engineering and Biotechnology (ICGEB) Italy, consisting of 10g glucose, 5g peptone, 3g Yeast extract and 3g of malt extract per litre. The glass tube was first cleaned with ethanol and then scorsed with a metal file to cut the glass opened. The lyophilized *P. stipitis* strain was rehydrated with 200µl of the rehydration medium. 50µl each of the *P. stipitis* rehydration medium was then used to inoculate YMPD agar medium plate which was then incubated at 24^{o}C for 48 hours. Also 50µl was used to inoculate the broth medium (YM)-rehydration fluid in an Erlenmeyer flask with baffles (for oxygenation). The flask was incubated at 24^{o}C in a Rotatory shaker at 120rpm for 2 days.

3.6.2 Microbial strains

The yeast strains MX1 and MX2 selected were identified as *Saccharomyces cerevisiae* with MX2 strain as BY473 with genotype (Mata/α *his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 MET15/met15Δ0 lys2Δ0/LYS2 ura3Δ0/ura3Δ0*). Identification was carried out at the Yeast Molecular Genetics Laboratory of ICGEB. *E.coli* Xl1 Blu MRF' was also obtained from the Yeast Molecular Genetics lab of ICGEB, Italy, preserved and stored in glycerol at -70^{o}C.

3.6.3. Plasmid vectors

Plasmids used in this study were Expression vectors (pGAPZαA and pVT100-U) represented in figure 3.1 and 3.2 respectively. pGAPZαA plasmid uses zeocin as a selectable maker which is bifunctional in both yeast and bacteria while pVT100-U vector uses ampicillin as selectable maker for bacteria and Ura3 for yeast.

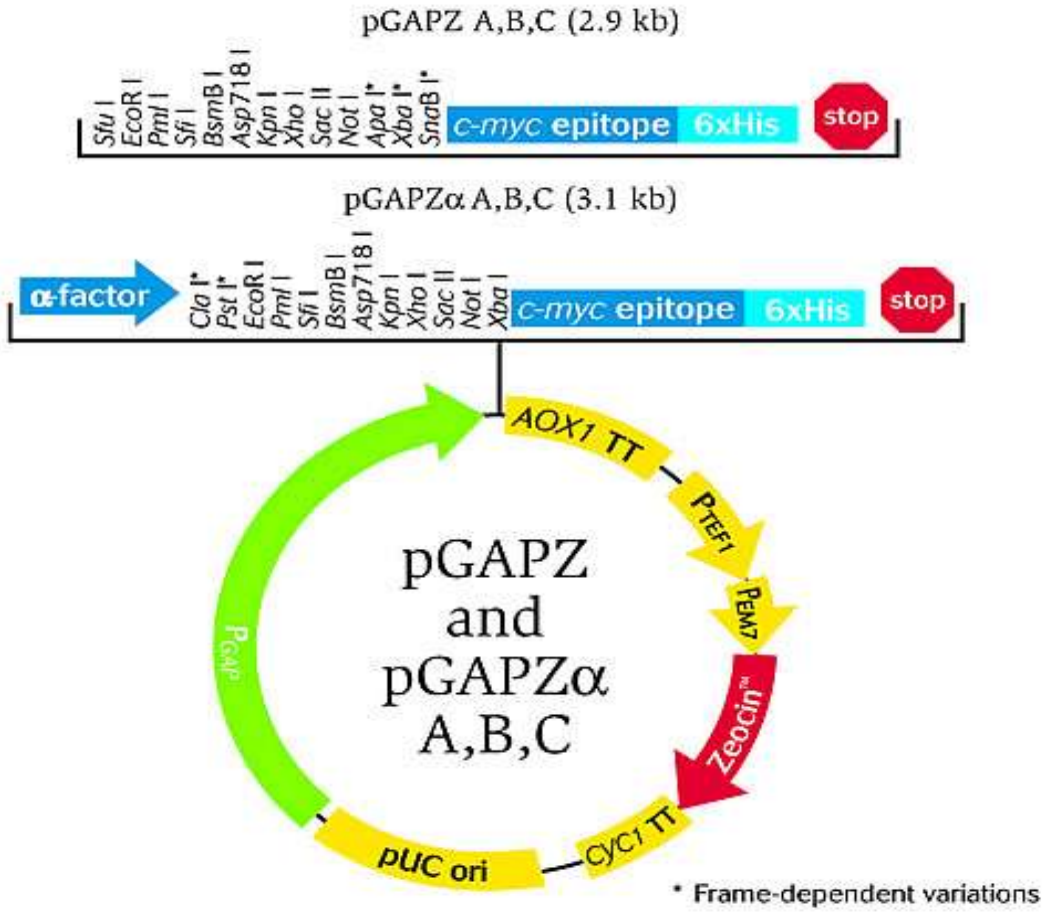


Fig 3.1 pGAPZ Plasmid vector (http:www.invitrogen.com)

Fig 3.2 pVT100-U Plasmid Vector

3.6.4 Media and Culture Conditions

E.coli strain (XL1 BLU, MRF) was grown in standard Luria Bertani (LB) medium for standard bacterial cultivation according to Miller (1992) supplemented with 75µg/ml of Ampicillin. 25µg/ml of Zeocin was added to LB medium used for *E.coli* in transforming pGAPZαA vector and 100µg/ml of Zeocin was added to YPD medium for yeast culture. Synthetic medium (Drop out mix) (see Appendix) was also compounded as described in Rose *et al.* (1990).

3.6.5. Enzymes, Primers and Chemicals

Restriction enzymes, DNA modifying enzymes and other molecular reagents were obtained from Yeast Molecular genetics laboratory of ICGEB Italy, Promega and Sigma. Primers for PCR were designed using SG Database (http://www.yeastgenome.org/) and primers were synthesized by Sigma.

3.6.6. Extraction of Genomic DNA from *Pichia stipitis* CBS 6054

Procedure

Total genomic DNA of *P. stipitis* yeast was extracted using the phenol chloroform method of (Kaiser *et al.,* 1994). A single colony of the yeast cell on YPD plate was used to inoculate 25ml of YPD broth in 250ml Erlemeyer flask. The culture broth was examined microscopically to ensure that there was no contamination. The flasks were incubated overnight at 30^{o}C on a rotatory shaker at 120 rpm, after which 10ml of the broth cultures were dispensed into falcon tubes and centrifuge at 4,000rpm for 5 minutes. The cell pellet was washed by resuspending in 500µl of sterile distilled water and centrifuge for 2 minutes at 5,000rpm. The supernatant was discarded and the washed cell was resuspended in 200ml of breaking buffer (see appendix) with constant pipetting to mix, this was followed by the addition of small quantity of acid-washed glass beads and 200µl of phenol: chloroform (25:25). The content in the tube was vortexed vigorously for 5 minutes under the hood in order to break the chitin cell wall of the yeast. 200µl of sterile water was added and the lysate centrifuged at 12,000rpm for 5 minutes at 4^{o}C to separate the aqueous phase from the organic (phenol:chloroform) phase and the glass beads at the bottom. The aqueous phase was carefully transferred with a sterile suction pipette into a fresh eppendorf tube, after which 1000µl of absolute ethanol (100%) at room temperature was added. The content was placed on dry ice for 5 minutes. Centrifugation followed at 12,000rpm for 10 minutes. The supernatant was discarded; 400µl of sterile water or T.E. buffer and 10µl of RNase (1mg/ml)

was added to the pellet followed by incubation at 37^{o}C for 30 minutes to digest any contaminating RNA. This step was followed by the addition of 10µl of 4M ammonium acetate and 100µl of absolute ethanol. The tube was placed on dry ice for 5 minutes to precipitate the DNA. Thereafter it was centrifuge at 12,000rpm for 10 minutes. The supernatant was discarded and the pellet air dried for 30 minutes. TE buffer (100µl) was added to solubilize the genomic DNA in the tube and stored at -20^{o}C. Small portion was run on 0.8% Agarose gel for verification.

3.6.7 Agarose gel Electrophoresis

The success of DNA extraction or digestion patterns of fragment generated by restriction enzymes was investigated by running a DNA agarose gel electrophoresis. 0.8% agarose gel was prepared by dissolving 2.0g of agarose powder in 250ml of 0.5X TBE buffer in a 500ml conical flask. The flask was placed in a microwave for 15 minutes for the agarose powder to melt and gel. Thereafter the molten agarose gel was allowed to cool to about 50^{o}C, followed by the addition of 30µl of 2.5mg/ml of ethidium bromide solution. The molten gel was poured into electrophoretic tank ensuring the comb was put in place. The gel was allowed to solidify at room temperature after which 0.5X TBE buffer was poured on the solidified gel. The comb was removed to create wells for loading the DNA samples. Aliquot of 2.0µl of 6X loading buffer was added to 3.0µl of sterile water and 2.0µl of DNA samples and mix by pipetting. This was followed by loading the mixture into the wells of the agarose gel. DNA marker (1kb+) of known size was also loaded along side the samples. The electrodes were then connected to the electric power source to run the gel at 100volt for 1 hour. The gel was observed under the U.V light.

3.6.8 Preparation of Competent cells from *E. coli (* XL1 BLU MRF')

Competent cells of *E. coli* strain (XL1 BLU MRF') were prepared using the method of Miller (1992).

A single colony of *E. coli* XL1 BLU MRF' was used to inoculate 10ml LB medium and allowed to grow overnight on a rotatory shaker at 200rpm at 37^{o}C. An aliquot of 300µl of the overnight culture was then used to inoculate 10ml of LB medium incubated on a rotatory shaker at 37^{o}C until the cell density was between 5x10^{7} and 1x10^{8}cells/ml. This corresponds to an Optical density (O. D_{600}) of between 0.2 - 0.25, usually obtained in two hours. This was followed by transferring the subculture to a round bottom tube and centrifugation at

3,000rpm in a refrigerated centrifuge at (4^{o}C) for 10 minutes. The supernatant was discarded and the pellet was re-suspended in 100mM $CaCl_2$ solution and place on ice for 30 minutes. This was then centrifuge again at 5000rpm for 5 minutes in a refrigerated centrifuge (4^{o}C). The supernatant was discarded and the pellet was resuspended in 3ml of 100mM $CaCl_2$ solution and used directly. It was also stored by dispensing on aliquot at 0.5ml in a microfuge tubes after the addition of 20% glycerol and freeze at -70^{o}C for subsequent use.

3.6.9 Transformation of competent *E. coli* cell with pGAPZA Plasmid

Transformation of *E. coli* was performed according to the method of Sambrook *et al.,* (1989). An aliquot of 100µl of *E. coli* competent cell was pipette in an Eppendorf tube and placed on ice for 30 minutes.

In another eppendorf tube, 3µl of DNA plasmid (pGAPZA) was placed along with 10µl of 500mM $MgCl_2$, 100mM $CaCl_2$, 8µl of 30% PEG and the volume made up to 100µl by the addition of deionized distilled water. 100µl of the competent cells was added to this tube and the mixture vortexed. Also placed in another tube was 100µl of competent cells without the DNA plasmid (control). Both tubes were incubated on ice for 1 hour.

Heat shock Treatment

The cells were heat-shocked by placing the tubes in a water-bath at 42°C for 90 seconds. The tubes were immediately transferred back to ice for 3 minutes. This was followed by the addition of 1.0ml of LB broth to both tubes and incubated at 37°C in a rotating shaker for 1 hour to allow the expression of the antibiotic resistance gene. 200µl of the transformation mix was then placed directly on dried LB plated supplemented with 25µg/ml of zeocin using sterile glass spreader. Furthermore, 200µl of the control competent cells without DNA plasmid was plated in another plate. Both plates were incubated at 30°C for 24 hours wrapped with foil paper and kept in the dark to prevent photo-oxidation of zeocin. The cells that received the plasmid grow on the plate, but there was no growth on the control (without the plasmid).

3.6.10 Transformation of competent *E. coli* cell with pVT100-U Plasmid

An aliquot of 100µl of *E. coli* competent cell was pipetted in an Eppendorf tube and placed on ice for 30 minutes. In another Eppendorf tube, 2µl of DNA plasmid pVT100-U was placed along with 10µl of 500mM $MgCl_2$, 100mM $CaCl_2$, 8µl of 30% PEG and the volume made up to 100µl by the addition of deionized distilled water. 100µl of the

competent cells was added to this tubes and the mixture vortexed. Also placed in another tube was 100µl of competent cells without the DNA plasmid (control). Both tubes were incubated on ice for 1 hour.

Heat shock Treatment: The cells were heat-shocked by placing the tubes in water-bath at 42°C for 90 seconds. Immediately the tubes were transferred back to ice for 3 minutes. This was followed by the addition of 1.0ml of LB broth to both tubes and incubated at 37°C in a rotating shaker for 1 hour to allow the expression of the antibiotic resistance gene. 200µl of the transformation mix was then placed directly on dried LB plated supplemented with 75µg/ml of ampicillin using sterile glass spreader. Also 200µl of the control competent cells without DNA plasmid was plated in another plate. And both plates were incubated at 37°C for 24 hour and examined for the emergence of colonies. The cells that received the plasmids grew on the ampicillin plate, but there was no growth on the control (without the plasmid)

3.6.11. Measurement of DNA Concentration

DNA concentration was accurately measured using ultraviolet absorbance spectrophotometry method in which the amount of ultraviolet radiation absorbed by a solution of DNA is directly proportional to the amount of DNA in the sample. The absorbance was measured at 260nm; at this wavelength an absorbance (A_{260}) of 1.0 corresponds to 50ηg of double stranded DNA per ml. This was also used to check the purity of the DNA preparation. With a pure sample of DNA the ratio of absorbance at $^{260}/_{280}$ is 1.8. A ratio of less than 1.8 indicates that the preparation was contaminated either with protein or with phenols and need to be re-purified. 6µl of the DNA was dissolve in 300µl water (polar solvent) or TE buffer to give the dilution factor (d_f) of 50.

DNA concentration (µg/ml) = $O.D_{260}$ x 50 x dilution factor (df)

3.6.12 Preparation of Plasmid DNA (pGAPZA) using Chemical Method

Transformed *E. coli* cells with plasmid vector pGAPZA were subcultured into 30ml LB broth supplemented with 25µg/ml of zeocin. This was incubated in a rotatory shaker at 200rpm overnight at 37^{o}C. Solution I, II and III were prepared (see appendix). 10ml of the saturated overnight culture was pipetted into a falcon tube and centrifuge at 5,000rmp for 5 minutes. The supernatant was discarded and the pellet re-suspended in 100µl of ice cold

solution I and vortex. It was placed on ice for 10minutes. This was followed by the addition of 200µl of freshly prepared solution II which was mix gently by inverting the tube until a slightly milky solution was observed and then placed on ice for 10 minutes. Thereafter 150µl of ice cold solution III was added to the tube; it was mixed gently and placed on ice for another 10 minutes. This was followed by centrifugation in a refrigerated centrifuge at (4^{o}C) for 5 minutes. With suction micropipette, the supernatant was transferred to a new eppendorf tube and an equal volume (400µl) of phenol:chloroform:isoamyl alcohol (25:24:1) was added to the supernatant and vortexed, followed by centrifugation for 5 minutes at 5,000 rpm. The aqueous top layer of the supernatant was transferred into a new tube and 400µl of chloroform: isoamyl alcohol (24:1) was added and centrifuge for 5 minutes.

The top layer of the supernatant was transferred to a new tube followed by precipitation by adding 40µl of 3M sodium acetate (pH 5.2) and 1000µl of absolute cold ethanol (100% at -20°C) to the supernatant, placed on dry ice (-70°C) and allowed to precipitate for 15 minutes. This was followed by centrifugation for 5 minutes at 4°C. Thereafter the supernatant was discarded and the pellet was rinsed gently with 70% ethanol by pipetting 100µl over each pellet and immediately removing the ethanol without vortexing. The pellet was air dried and re-suspended in 50µl of TE buffer in which 3µl of RNase was added, incubated at 37°C for 1 hour to digest the RNA and then stored at -20°C.

3.6.13 Preparation of Plasmid DNA (pVT100-U) Using Kits (Talent Plasmix Miniprep)

Transformed *E. coli* cells with plasmid vector pVT100-U were subcultured into 30ml LB broth supplemented with 75µg/ml of ampicillin. This was incubated in a rotatory shaker at 200rpm overnight at 37^{o}C. 10ml of the saturated overnight culture was pipette into a falcon tube and centrifuged at 5,000rpm for 5 minutes. The supernatant was discarded and the pellet was re-suspended in 70µl of resuspension solution, this was followed by the addition of 70µl of lysing solution. Mixing was done by inverting the tubes until the suspension was clear. 70µl of neutralizing solution was added and the tubes were inverted several times until a white precipitate was formed. The tubes were then spun in a microcentrifuge at 14,000rpm for 2 minutes. The cleared supernatant was transferred with a micropipette into a new eppendorf tube. 350µl of Plasmix-High capacity purification resin (strongly shaken before use) was added to the supernatant and mix by inverting the tubes

several times. Plasmix filters were put into 2.0ml eppendorf tube and the mixture were transferred into the filter and centrifuged at 14,000 rpm for 30 minutes. The resin with the bound DNA was retained on the filter and the liquid in the bottom tube was discarded. 400µl of washing solution containing added ethanol to final concentration of 50% was added to the filter and centrifuged at 14,000rpm for 30 minutes. To elute the plasmid DNA, 50µl of preheated TE buffer at 65^{o}C was added to the filter and allowed to resusupend the DNA for 1 minute, this was followed by centrifugation of the mini column in the eppendorf tube for 20 minutes. After removal of the filter the resuspended Plasmid DNA was ready for use and left over in TE buffer was stored at -20^{o}C.

3.6.14. Generation of Restriction enzyme sites for Plasmids (pGAPZA and pVT100-U) and insert genes (XL1 and XL2)

The Bioinformatic tools (Macplasmap and DNA Strider 1.4f7) of Yeast Molecular Genetics lab of ICGEB, Italy was used to sequence and generate restriction enzyme digest sites on the multiple cloning sites (MCS) of the plasmids for the flexibility of inserting gene into the vectors as well as the entire plasmid vectors genome. The genes (XL1 and XL2) to be inserted into the vectors were also sequenced in order to generate restriction enzyme sites to avoid using enzymes that will destroy the insert. Neoschizomers and enzymes with two or multiple restriction sites were not chosen.

3.6.15 Designing of Primers with Attached Restriction Enzyme Sites (RES)

Based on the published gene sequences of *P. stipitis*, the genes of interest (Xylose reductase (XL1) and xylitol dehydrogenase (XL2) were identified using the BLAST search programme of NCBI (www.ncbi.ntm.nitigov/blast) XL1 gene, accession number X59465 and XL2 with accession number X55392 were used in the BLAST search programme and the genes were located on chromosome 1 and 5 of *P. stipitis* genome respectively. The nucleotide sequences of the open reading frame (ORFs) were downloaded from the Gene Bank and aligned to find the coding sequence of the genes.

XL1 gene on chromosome 5 has an ORF of 2025bp and a coding sequence of 957bp starting at ATG codon nucleotide 356 and extending to TAA codon at nucleotide position 1312.

XL2 gene on chromosome 1 has an ORF of 1963bp with a coding sequence of 1092bp starting at ATG codon nucleotide 319 and extending to TAA codon at nucleotide position 1410. The primers for the coding sequence of the XL1 and XL2 genes were designed using the Saccharomyces Genome Data base (SGD) (http://www.yeastgenome.org/) of yeast

molecular genetic laboratory of the International Centre for Genetic Engineering and Biotechnology Trieste, Italy (Table 3.1).

After the generation of the forward and reverse sequences of the primers, chosen restriction enzymes sites were attached to the sequences. As a general rule, 6 to 4 extra bases were added to the end of the primers based on the chosen enzyme when engineering restriction enzyme sites into it.

For XL1 gene, *BstB1* restriction enzyme site was attached to the forward primer and *SacII* restriction enzyme site attached to the reverse primer.

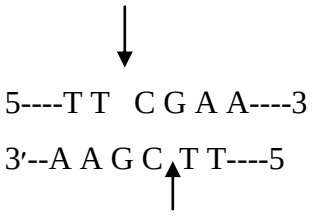
XL2 gene has *Pst1* restriction site attached to the forward primer and *BamH1* restriction site attached to the reverse primer. This was followed by synthesis of the primers by SIGMA.

Table 3.1: Oligonucleotide primers used in this study

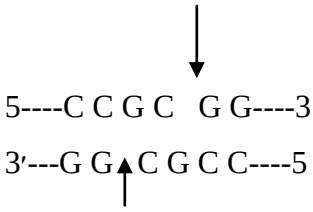
Target gene	Primer	Sequence (5' – 3')	Amplicon size
XL1 (XR)	Fwd Rev	ATGCCTTCTATTAAGTTGAAC TTAGACGAAGATAGGAATC	957bp
XL2 (XDH)	Fwd Rev	ATGACTGCTAACCCTTCCTT TTACTCAGGGCCGTCAATGA	1092bp

Xylose Reductase gene (XL1)

The sequences of restriction enzyme sites for *BstBI* and *SacII* were introduced into the Xylose reductase gene at the 5 and 3 ends respectively.



Restriction site for ***BstBI*** (*Bacillus stearothermophilus B22*5) – Fwd primer



Restriction site for ***SacII*** (*Streptomyces achromogenes*) – Rev primer

Attachment of RE sites to XR gene Primers

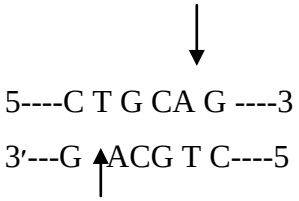
Forward primer, ----gtcgagtt cgaaATGCCTTCTATTAAGTTGAAC (33mers) (restricted with *BstB1*)

Reverse primer,----actggtccgc ggTTAGACGAAGATAGGAATC (31mers) (restricted with SacII)

As a general rule, these two restriction enzymes used require 6 base pairs on either side of their recognition site to cleave effectively.

Xylitol Dehydrogenase gene (XL2) Primer

The sequences of restriction enzyme sites for **PstI** and **BamHI** were introduced into the Xylitol dehydrogenase gene at the 5 and 3 ends respectively.



Restriction site for ***PstI*** (*Providencia stuartii* 164) - Fwd primer

Restriction site for ***BamHI*** (*Bacillus amyloliquefaciens H*) - Rev primer

Attachment of RE sites to XDH gene Primers

Forward primer,----atcgctgca gATGACTGCTAACCCTTCCTT (30mers) (attached with *Pst1*)

Reverse primer,-acgtg gatccTTACTCAGGGCCGTCAATGA (30mers) attached with *BamHI*)

3.6.16 Preparation of Oligonucleotide Primers Solutions

Solutions of oligonucleotide primers used in PCR amplification of target genes (Table 3.1) from *P. stipitis* (CBS 6054) were prepared as follow: The genes were located using Bioinformatics tools of NCBI search blast programme. The coding sequences of the genes were removed from the Open Reading Frame (ORF) of the genes and subjected to SGD data base of the Yeast Molecular Genetics laboratory of International Centre for Genetics Engineering and Biotechnology (ICGEB), Italy for primer design in which the chosen restriction site were attached. The sequences generated were sent to SIGMA for synthesis of the primers.

Sigma oligonucleotides were provided deprotected and desalted. Prior to opening and resuspension, the oligonucleotides tubes were centrifuged at 12,000rpm for 30 minutes in order to concentrate the salt and prevent loss of pelleted oligonucleotide. The quantity of sterile water or TE buffer needed to make the primer stock solutions of 100µM concentration was estimated by SIGMA. The synthesized dried oligonucleotide salt was resuspended in 1xTE buffer (10mM Tris, pH 7.5 – 8-0, 1mM EDTA) aseptically followed by mixing on a vortex mixer for about 3 minutes and incubation on ice for 45 minutes. This help to keep the oligos buffered at pH above neutral to avoid degradation due to acidic conditions. Once suspended, the stock was stored at -20^{o}C for several weeks. To minimize degradation of the primers due to freeze/thaw, aliquots of 10µM working solutions were prepared by dilution of the 100µM stock solution with TE buffer and kept at 4^{o}C for short term use.

3.6.17 Calculation of annealing temperature of Primers

The melting temperature (Tm) of primers in which hybridization of the DNA will take place was calculated using the formular below:

$Tm = 4(G + C) + 2 (A + T)\ ^{o}C$ (Sambrook *et al.*, 1989)

As a general rule, the chosen temperature of the primer should be 2 – 5^{o}C less than the calculated value.

3.6.18 PCR Amplification of Target Genes (XL1 and XL2)

To obtain more copy number of the desired genes (XL1 and XL2) from (*Pichia stipitis)* for cloning into *Saccharomyces cerevisiae*. Two primers each with forward and reverse regions were designed with SGD data base to span the full coding region of the genes with attached restriction enzyme sites. PCR reaction was carried out on 120ηg of isolated genomic DNA of *P. stipitis* using CELBIO thermal cycler. The PCR mixture of 50µl volume contained deionized water 34.2µl, 5x Gotaq DNA polymerase buffer 10µl, dNTPs 1.0µl, forward primer 2.0µl, Reverse primer 2.0µl, Gotaq DNA polymerase enzyme 0.3µl and DNA template of 0.5µl respectively. The PCR was carried out for 30 cycles at initial denaturation of 94^{o}C for 3 minutes, annealing temperature of 56^{o}C which is usually calculated from the melting temperature for 30 seconds and extension at 72^{o}C for 4 minutes. It was then put on hold at 16^{o}C.

The amplified products were analysed by electrophoresis in 0.8% Agarose gel. A small portion of the gel was stained in (0.5µg/ml) ethidium bromide (EtBr) solution for 10-30 minutes and then photographed on trans UV illuminator (Biorad) using Quantity one gel imaging software (Biorad) The stained EtBr gel was used to mark and cut out the amplified gene from the unstained Agarose gel which was purified and used for gene cloning. This portion was not stained with EtBr to prevent DNA intercalation.

3.6.19 Double digestion of pGAPZA plasmid and insert gene (XL1) with Restriction Enzymes

The restriction enzyme sites fragment generation of the plasmid vectors and the inserts gene (XL1) were generated with Bioinformatics tools of Yeast Molecular Genetics laboratory of ICGEB, Italy using DNA strider 1.4f7 and Macplasmap programmes respectively (see appendix 3). Two enzyme restriction sites were carefully selected from the multiple cloning sites (MCS) of the plasmid such that the enzymes do not have a restriction sites on the gene (XL1) to be inserted to avoid digestion and destruction of the gene. The same R.Es (*SacII and BstB1*) used in the digestion of the plasmid was also used to restrict the insert gene so as to create complementarity during ligation.

The 120µl reaction volume of the plasmid digest in an Eppendorf tube consists of 76µl deionized water, 30µl plasmid DNA, 12µl of Nebuffer 4 and 1.0µl of 1000µg/ml *SacII* enzyme, while that of the insert (XL1) gene in a separate reaction consists of 96µl deionized water, 10µl XL1 gene, 10µl of Nebuffer 4 and 1.0µl of 1000µg/ml *SacII* enzyme. The tubes

were incubated at 37^{o}C for 2 hours, after the restriction, the reaction of *SacII* enzyme was inactivated by heating at 65^{o}C in a heating block for 20 minutes. This was followed by the addition of 1.0µl of *BstB1* RE to the tube and incubated at 65^{o}C for another 2 hours. The inactivation temperature of *SacII* R.E was the incubation temperature for *BstB1* R.E and both uses same Nebuffer 4. After the digestion, it was subjected to agarose gel electrophoresis and the fragments of interest were gel purified.

3.6.20 Double digestion of pVT100-U plasmid and insert gene (XL2) with Restriction Enzymes

The restriction enzyme sites fragment generation of the pVT100-U plasmid and the inserts gene (XL2) were generated with Bioinformatics tools of Yeast Molecular Genetics laboratory of ICGEB, Italy using DNA strider 1.4f7 and Macplasmap programmes respectively (see appendix). Two enzyme restriction sites (*Pst1* and *BamH1*) were carefully selected from the multiple cloning sites (MCS) of the plasmid such that the enzymes do not have a restriction sites on the gene (XL2). The same R.Es used in the digestion of the plasmid was also used to restrict the insert gene so as to create complementarity during ligation. The 30µl reaction volume of the plasmid digest in an Eppendorf tube consists of 18.0µl deionized water, 5.0µl plasmid DNA, 3.0µl of Nebuffer3 and 0.5µl of 1000µg/ml *BamHI* enzyme, while that of the insert (XL2) gene in a separate reaction consists of 17.0µl deionized water, 3.0µl XL2 gene, 3.0µl of Nebuffer 3 and 0.5µl of 1000µg/ml *BamHI* enzyme. The tubes were incubated at 37^{o}C for 2 hours, after the restriction, the reaction of *BamHI* enzyme was inactivated by heating at 65^{o}C in a heating block for 20 minutes. This was followed by the addition of 1.0µl of *Pst1* RE and 3.0µl of BSA (Bovine serum albumin) to the tubes and incubated at 65^{o}C for another 2 hours. The inactivation temperature of *BamHI* R.E was the incubation temperature for *Pst1* R.E and both uses same Nebuffer 3. After the digestion, it was subjected to agarose gel electrophoresis and the fragments of interest were incised and gel purified.

3.6.21 Recovering DNA from Agarose gel using kit (Talent Plasmix Miniprep)

The fragment of DNA in the gel containing the DNA of interest was cut out with a sharp knife after aligning it with a stained Etbr portion to visualize the portion. The excise gel was cut into smaller pieces and weighed. This was followed by placing the sliced gel

into a tube and 1000µl of Solubilization buffer (SB) was added. The tubes were heated at 65^{O}C in a heating block for 15 minutes while vortexing every 2 minutes until the gel in the solubilization buffer melted completely and homogenized. This was followed by pipetting 750µl of the homogenized gel into a separating column and centrifuged at 13,000rpm for 1 minute, discarding the eluent from each centrifugation.

After the last elution, 750µl of buffer PE (with added ethanol) was pipetted into the separating column and allowed to stayed for 1 minute to washed the DNA trapped in the separating column and centrifuged at 13,000rpm for 1 minute discarding the eluent. This step was repeated with 750µl of PE solution followed by centrifugation of the empty columns to remove residual PE solution. The bottom collection tube of the separating column was discarded and replaced with a new Eppendorf tube. The tubes were allowed to dry at room temperature for 5 minutes before the DNA entrapped in the column by electrostatic attraction were eluted with 30µl of Elution buffer (EB) added directly to the separating column for 1 minute before centrifugation at 13,000rpm for 1 minute. The purified eluted DNA was then collected and stored at -20^{o}C.

3.6.22 Ligation Reaction (Construct)

The digested products (plasmid and insert gene) were gel purified and used in a ligation reaction. A control mixture that lacks the insert DNA gene was also included to investigate the background of uncut or re-ligated vector DNA. The ligation reaction of 20µl volume consists of 5.0µl deionized water, 5.0µl of insert gene, 7.0µl of plasmid DNA, 2.0µl of 10X ligase buffer (with 10mM ATP) and 1.0µl of T4 DNA ligase enzyme (400,000U/ml). The ligation mixture was incubated at 16°C in a water-bath overnight and then used to transform competent *E.coli* host cells for multiplication before transformation of the host yeast cell.

3.6.23 Transformation of competent *E. coli* cells with ligation mixture of (pGAPZA and XLl gene)

Ten microlitre of ligation mixture (pGAPZA and XR gene) were added separately to 200µl of competent *E. coli* cells in a sterile Eppendorf tubes and gently pipette to mix. The mixture were incubated on ice for 1 hour and then heat-shocked in a water bath at 42°C for 90 seconds, the heat-shocked cells were immediately transferred back to ice for another 3 minutes before 1000µl of LB broth was added to each tube of the transformed cells. The

tubes were then incubated at 37°C on a rotatory shaker at 150rpm for 1 hour to allow the cells to express the antibiotic gene (zeocin) on the plasmid.

After 1 hour, the tubes were centrifuge at 5,000rpm for 60 seconds, the supernatant was removed by pipetting with a suction pipette and the cells resuspended in 150µl of LB broth, which was then used to inoculate dried plates of LB agar containing 25µg/ml of Zeocin using sterile glass spreader. The plates were wrapped with foil paper, incubated at 37°C overnight and examined for the presence of colonies.

3.6.24 Transformation of competent *E. coli* cells with ligation mixture of (pVT100-U and XL2 gene)

Ten microlitre (10µl) of ligation mixture (pVT100-U and XDH gene) were added separately to 200µl of competent *E. coli* cells in a sterile eppendorf tubes and gently pipette to mix. The mixture were incubated on ice for 1 hour and then heat-shocked in a water bath at 42°C for 90 seconds, the heat-shocked cells were immediately transferred back to ice for another 3 minutes before 1000µl of LB broth was added to each tube of the transferred cells. The tubes were then incubated at 37°C on a rotatory shaker at 150rpm for 1 hour to allow the cells to express the antibiotic gene (Ampicillin) on the plasmid.

After 1 hour, the tubes were centrifuged at 5,000rpm for 60 seconds. The supernatant was removed by pipetting with a suction pipette and the cells resuspended in 150µl of LB broth, which was then used to inoculate dried plates of LB agar containing 75µg/ml of ampicilin using sterile glass spreader. The plates were incubated at 37°C overnight and examined for the presence of colonies.

3.7 GENETIC TRANSFORMATION OF MX1 AND MX2 YEAST WITH CONSTRUCTS

Yeast transformation was carried out using the modified Lithium acetate method as described in Rose *et al.*, (1990).

3.7.1 Preparation of Yeast competent cells and Transformation

Competent cells of MX1 and MX2 yeast strains of *Saccharomyces cerevisiae* were prepared according to the published protocol of Gietz and Woods (1994).

Inoculum from a yeast cell colony was used to inoculate 5ml YPD broth in a 250ml Erlemeyer flask with baffles (to allow for oxygenation) and allowed to grow overnight at

30^{o}C in a rotatory shaker of 120rpm. The overnight cultures were examined microscopically to ensure that there was no contamination. 2.0ml of the overnight culture was used to inoculate 50ml YPD broth, incubated at 30^{o}C in a rotatory shaker for 2 hours. The cells was harvested at mid-log phase with cell density of 1.7x10^{7}cells/ml. 50ml of the yeast broth was centrifuged at 13,000rpm for 5 minutes and the supernatant was discarded.

The cell pellet obtained was washed by resuspending in 20ml of sterile water and centrifuged at 8,000rpm for 5 minutes. Water was removed and the cell transferred to a new Eppendorf tube and resuspended in 1.0ml of sterile water, centrifuged at 3,000rpm for 2 minutes. This was followed by resuspending the cell in 250µl of 0.1M (100mM) Lithium Acetate solution, vortexed and incubated at 30^{o}C for 20minutes to make the cells competent. 100µl of the competent cell was placed in a new tube with 10µl of DNA (plasmid DNA with insert gene) (construct) and 10µl of Henry sponge DNA (heated at 94^{o}C to make it single stranded), the tube was incubated at 30^{o}C in a rotatory shaker for 20 minutes.

Also prepared in another eppendorf tube was 50% Poly ethylene glycol (PEG) of Molecular weight (3350) solution consisting of 200µl sterile water, 200µl of 1.0M Lithium Acetate solution and 1,600µl of 50% PEG solution. This was followed by the addition of 300µl of the PEG solution to 300µl of the yeast competent cell prepared in duplicate. The tubes were vortexed and incubated at 42^{o}C in a water bath for 20 minutes.

Heat shocked: The cells were heat-shocked by incubating the tubes in a water-bath at 42^{o}C for 20 minutes and thereafter placed on ice for 90 seconds.

The tubes were centrifuge at 3,000rpm for 5 minutes and the PEG solution was removed with micro pipette. The cell pellet was resuspended in 1.0ml of YPD broth and incubated at 30^{O}C in a rotatory shaker for 1 hour. After the incubation, the mix was vortexed and centrifuged to remove the YPD medium. The pellet cell was resuspended in 200µl of sterile water.

For pGAPZA and XL1 construct: The resuspended cells in 200µl of water was plated out on dry YPD medium containing 100µg/ml of zeocin antibiotics, incubated at 30^{o}C for 2 – 3 days to observe cell colony of transformed cells.

For pVT100-U and XL2 construct: The resuspended cells in 200µl of water was plated out on dry ura3^{-} synthetic drop-out mix medium (appendix for composition and method of preparation). The plates were incubated at 30^{o}C for 2 -3 days to obtained colony of transformed cells.

3.7.2 Colony PCR

This protocol was designed to quickly screen transformants for insert genes from colonies of transformed cells. The colonies obtained from the transformation were streaked on dried plate of the appropriate medium to obtained "patch plates". To each PCR tubes containing 50µl of sterile water were numbered and a small amount of the colony from the numbered patch plate was added with micropipette tips, care was taken to avoid the addition of air into the tubes. This was followed by the addition of zymolase enzyme (10µg/ml) to each tube and incubated at 37°C for 30 minutes. The tubes were centrifuged at 500rpm for 1 minute. The enzyme and the water layer were pipetted and thrown away leaving the cell pellete which was then resuspended in sterile distilled water and used in a PCR reaction.

The PCR mixture of 50µl volume contained deionized water 34.5µl, 5x Gotaq DNA polymerase buffer 10µl, dNTPs 1.0µl, forward primer 2.0µl, Reverse primer 2.0µl and Gotaq DNA polymerase enzyme 0.5µl respectively. The PCR was carried out for 30 cycles at initial denaturation of 94^{o}C for 4 minutes, annealing temperature of 54^{o}C for 30 seconds and extension at 72^{o}C for 4 minutes. It was then put on hold at 16^{o}C. The amplified products were analysed by electrophoresis in 0.8% agarose gels.

3.8 PHYSIOLOGICAL STUDIES OF MODIFIED AND WILD YEAST ISOLATES

3.8.1 Effect of different carbon sources on the growth of yeasts Isolates

The carbon sources tested included glucose, xylose, arabinose, lactose, mannitol, raffinose, fructose and maltose. The basal medium used was Yeast Extract Peptone broth, prepared by dissolving 5g of yeast extract, 10g of peptone in 1000ml of sterile distilled water, 30ml each was dispensed into 100ml conical flasks and sterilized at 121°C for 15 minutes. The different carbon sources were filter-sterilized and added appropriately. The different yeast isolates were grown in YPD broth for 24 hours. The medium was centrifuged to obtain the cell pellet, which was thereafter suspended in 5ml of sterile distilled water. 200µl aliquot of the washed cell suspension was used to inoculate the flasks in duplicate. The flasks were incubated at 30°C in a rotatory shaker for 24 hours. Growth was determined by measuring the absorbance at 600nm using Perkin Spectrophotometer.

3.8.2 Effect of different temperatures on the growth of yeasts

The test organisms were grown in Yeast Extract Dextrose peptone broth at different temperatures. The YPD medium was prepared and dispensed into different tubes and sterilized at 121°C for 15 minutes in an autoclave. The tubes were left to cool and 200µl of the test organisms were inoculated into the medium aseptically. These test tubes were incubated at 25°C, 30°C, 40°C, 45°C and 50°C for 24 hours. Results were observed by measuring the turbidity at 600nm of the Spectrophotometer.

3.8.3 Effect of different pH on the growth of yeasts

Yeast Extract Dextrose Peptone broth was the basal medium used, it was prepared by dissolving 5g of yeast extract, 10g of peptone and 20g of glucose in 1000ml of sterile distilled water, 30ml each was dispensed into 100ml conical flasks. The pH of the different flasks were varied from 4.0 to 7.0 using 0.5M of NaOH and HCL before sterilization at 121°C for 15 minutes. The flasks were left to cool and 200µl of the test organisms were inoculated into the medium aseptically. The flasks were incubated at 30°C in a rotatory shaker for 24 hours. Growth was determined by measuring the absorbance at 600nm using Perkin Spectrophotometer.

3.8.4 Effect of different cations and anions on the growth of yeasts

Yeast Extract Dextrose peptone broth was prepared and dispensed into different tubes prepared in duplicate. Varying concentrations of anions (NO_3^-) ranging from 0.01, 0.05, 0.1, 0.15 and 0.2M were added to the tubes and sterilized at 121°C for 15 minutes in an autoclave. For the cations (Cu^{2+}) in the same concentration was added to another separate tubes containing the same YPD medium and steririlized in an autoclave at 121°C for 15 minutes. The tubes were left to cool and 200µl of the test organisms was used to inoculate the medium aseptically. The test tubes were incubated at 30°C for 24 hours. Growth obtained was determined by measuring the absorbance at 600nm of the Spectrophotometer.

3.8.5 Effect of different nitrogen sources on the growth of yeasts Isolates

The nitrogen sources tested included ammonium sulphate [$(NH_4)_2SO_4$], ammonium hydrogen phosphate [NH_4HPO_4], sodium nitrate ($NaNO_3$), ammonium citrate and urea. A modification of the basal medium of (Tani *et al*., 1988) was used containing carbon source (sugar) 30g; ammonium chloride [(NH_4Cl] 4g; Potassium dihydrogen phosphate [KH_2PO_4] 1.0g; magnesium sulphate [$MgSO_4.7H_2O$] 0.5g; yeast extract 2g in 1000ml of distilled water. The nitrogen sources present in the basal medium were replaced by the nitrogen

source (1% w/v) being tested. 30ml of the basal medium containing each nitrogen source was distributed into flasks in duplicates and sterilized at 121°C for 15 minutes. On cooling, the medium was inoculated with 200µl aliquot of the washed cell suspension of the organisms. The flasks were incubated at 30°C in a rotatory shaker for 24 hours. Growth was determined by measuring the absorbance at 600nm using Perkin Spectrophotometer.

3.8.6 Effect of different concentration of Ethanol on the growth of yeasts

Yeast Extract Dextrose peptone broth (YPD medium) was prepared and dispensed into different tubes prepared in duplicate and sterilized at 121°C for 15 minutes in an autoclave. The medium was allowed to cool followed by the addition of varying concentration ot ethanol (4%, 5%, 6%, 7%, 8%, 9% and 10%) to the tubes. The tubes were inoculated with 200µl of the test organisms and incubated at 30°C for 24 hours. Results were observed by measuring the turbidity at 600nm of the Spectrophotometer.

3.9 Hydrolysis of Cocoa Pod Husk (CPH)

3.9.1 Cellulase Induction from *Trichoderma hamatum*

For enzyme synthesis, the modified method of Mandel and Reese (1956) was used. The basal medium used for enzyme production in g/l was NH_4SO_4 1.4g, Urea 0.3g, KH_2PO_4 2.0g, $MgSO_4.7H_2O$ 0.3g, $CaCl_2$ 0.3g, yeast extract 0.1g. 1ml of trace elements solution was added and the pH adjusted to 5.3 with NaOH. 5g of carboxymethylcellulose (CMC) sterilized separately was added to the medium to serve as the carbon source. 50ml of the medium was dispensed into different Erlemeyer flasks and autoclaved for 20mins at 121°C. After cooling, the flasks were inoculated with 1ml of spore suspension containing $2.0x10^6$spores ml^{-1}. The flasks were incubated in a rotary shaker (100rpm) at 30°C for 7 days. After incubation, the medium was filtered through Watman No. 1 filter paper and the filterate was centrifuged to obtain the clear supernantant used for enzyme assay.

3.9.2 Cellulase Assay

This was done according to the modified method of Ghose (1987). Cellulase activity was determined by mixing 0.9ml of 1% (w/v) CMC (prepared in 50mM Na-citrate buffer, (pH 5.3) with 0.1ml of the fungus filtrate. The mixture was incubated at 50°C for 15 minutes. The reaction was stopped by addition of 1.5ml of 3,5-dinitrosalicyclic acid (DNSA) and the content was boiled for 5 minutes. The absorbance of the colour developed was read at 540nm using Jenway 640 Spectrophotometer. Transmittance was set at 100% with the

filterate CMC reaction mixture of the uninoculated control. DNSA reagent was prepared by combining 1.0g DNSA with 20ml 2N NaOH and 20g potassium sodium tartrate in 100ml distilled water. The transmittance of standard aqueous solution of D-glucose of various concentrations (0 – 10mg per ml) was determined and used to construct a graph of % transmittance as related to mg of glucose per ml. One unit of cellulose is defined as the amount of enzyme that liberates 1µ mole of glucose equivalents per unit under the specified assay conditions.

3.9.3 Xylanase production from *Trichoderma hamatum*

The fungi were cultured in Erlenmeyer flasks (250 ml) in submerged fermentation moistened with 10ml of mineral salts solution. The composition of the mineral salts solution was ($g.L^{-1}$): KCl, 0.5; $MgSO_4$, $7H_2O$, 0.5; $(NH_4)_2HPO_4$, 2.5; NaH_2PO_4, 0.5; $CaCl_2$-$2H_2O$, 0.01; $FeSO_4$, $7H_2O$, 0.01; $ZnSO_4$, $7H_2O$, 0.002 and 1% of birch wood xylan (Nair *et al*., 2008). The pH was adjusted to 5. The medium was then autoclaved for 20min at 121°C (15 lbs). After cooling, the flasks were inoculated with 1 ml of spore suspension containing $1x10^6$ spores ml^{-1}. The spore suspension was obtained from 7 day-old pure cultures. After mixing, flasks were incubated at 30°C under static conditions for 7 days.

After incubation, the enzyme was harvested in sodium citrate buffer (50 mM, pH 5.3). The fermented slurry was filtered and centrifuged at 10,000 x g for 20 min at 4°C. The clear supernatant was used for enzyme assays.

3.9.4 Xylanase Assay

Xylanase activity was determined by mixing 0.9ml of 1% (w/v) birch wood xylan (prepared in 50mM Na – citrate buffer, pH 5.3) with 0.1ml of the filtrate enzyme and the mixture was incubated in a water bath at 50°C for 5 minutes (Bailey *et al.*, 1992). The reaction was stopped by the addition of 1.5ml of 3,5 Dinitrosalicyclic acid (DNSA) and the content was boiled for 5 minutes (Miller, 1959). After cooling the colour developed was read at 540nm using Jenway 640 Spectrophotometer. The amount of reducing sugar liberated was quantified using xylose as standard. One unit of xylanase is defined as the amount of enzyme that liberates 1 µmole of xylose equivalent per minute under the specified assay conditions.

3.9.5 Pretreatment of Cocoa pod husk and enzymatic hydrolysis

Pretreatment of CPH was carried out by particle size reduction of the pod using sterilized grinder. 10g of the CPH was added to 300ml of sterile distilled water in different flasks and subjected to High pressure liquid hot water at 130°C for 1 hour in an autoclave. After cooling, the pH was adjusted to 5.0 using 0.5M HCl and enzyme prepared was added in the ratio of 10:1 (30ml of the crude enzyme to 300ml of the cocoa pod hydrolysate). Tween-80 was also added at 2.5g/L. Hydrolysis was carried out at 45°C for 72 hours in a stirred hydrolysis vessel. After the enzymatic hydrolysis, the content was filtered using sterilized filter to obtain the hydrolysate.

3.9.6 Acid hydrolysis of Cocoa Pod Husk

Acid hydrolysis of CPH was carried out using the modified method of Takahashi *et al*. (2000) this was done by adding 30g of fine grinded CPH to 300ml of water containing 1% hydrochloric acid (v/v) for 24 hours. It was then subjected to moist heat treatment in an autoclave at 121°C for 40 minutes. The liquid phase of the acid hydrolysis was recovered.

3.9.7 Detoxification of CPH acid hydrolysate

Due to the presence of inhibitors like acetic acids, phenolic compounds, furfural and 5-hydroxy-methyl furfural that are usually generated during acid hydrolysis of lignocellulose, the hydrolysate was subjected to "overliming" by adding CaO powder sequentially until the pH reaches 10.5 and then supplemented with sodium sulphite (1.0g/L). After cooling at room temperature, the resulting precipitate was removed by centrifugation and filtration. The pH of the hydrolysate was readjusted to pH 5.1 using concentrated Hydrochloric acid and the liquid was filtered again to remove the precipitate.

3.10 HPLC Sugar analysis of Acid and Enzymatically Hydrolysed CPH

Procedure:

1.0g of grinded dried cocoa pod husk was dissolved in 10ml of deionised water (1 in 10 dilutions) and allowed to stay overnight. The content was filtered and centrifuged to remove the sediment. The supernantant was hydrolysed by adding equal amount of conc. HCl to the supernantant to give a conc. of 6N HCl. The content was neutralized by adding $NaHCO_3$ in equal amount to bring the pH to 6.0. The flask was centrifuged and 1.0ml of the

clear solution (which contains the hydrolysed sugar) was pippeted into a test tube and allowed to evaporate in a water bath. The residue was dissolved in 1.0ml of acetonitrile.

Instrumentation: A High Performance Liquid Chromatograpy system (HPLC), AKTA model was used for the monomeric sugars analysis.

20ul of the sample (the residue dissolve in acetronitrile) was injected into HPLC with,

Mobile phase (M/P) of acetronitrile: water (1:1).

The Column was C18 (pack columnS5 ODS2 – octadecylsilane) (25.0cm length x 3.00mm bredth of column), Flow rate (F/R) = 1.0ml/min and Detector = UV 205nm.

Calculation

$$\text{Conc. of sugar} = \frac{peak\ area\ of\ sugar}{peak\ area\ of\ std\ sugar} \times conc.\ of\ std\ sugar \times dilution\ \ factor$$

3.11 Fortification and Fermentation of the Hydrolysate in a Chemically - Defined Medium

Fermentation of the hydrolysates was carried out according to the method of Martin-Carlos *et al.,* (2002). The CPH-hydrolysates were supplemented with nutrients to a final concentrations of per litre: 1.0g yeast extract, 0.5g $(NH_4)_2$ HPO_4, 0.025g $MgSO_4$. $7H_2O.7H_2O$ and 1.38g NaH_2PO_4 (0.1M), the pH was adjusted to 5.5 with 3M NaOH. The fermentation medium was then inoculated with a ($3x10^7$ cells/ml) of yeasts isolates and fermented at room temperature for 120 hours.

Fermentation of sugar solutions consisting of mixtures of glucose and xylose in similar concentrations as in the hydrolysates and supplemented with the same nutrient as stated above was performed as references. The fermentation was carried out in small fermentation tanks, sealed with rubber stopper and equipped with cannulas for sampling and CO_2 removal.

3.12 Calculation of fermentation parameters

The maximum yield of ethanol was calculated as g/l. The maximum amount theoretical yield of ethanol from glucose is 0.51g ethanol/g sugar (Takahashi *et al.,* 2000). This value was used as a basis for the calculation of conversion efficiency (%), the volumetric productivity (Qp) was calculated by dividing the maximum ethanol concentration by the time required to achieve such concentration (g/l h). The ethanol yield (Yp/s) was calculated as the maximum concentration of ethanol produced divided by the concentration of sugar initially present in the medium (g ethanol/g sugar).

CHAPTER 4

4.0 RESULTS

4.1 Yeast isolation from CPH and selection

Thirty yeasts isolates were obtained from a sun-dried submerged cocoa pod husk subjected to spontaneous fermentation for seven days. The colonial morphologies, physiological and biochemical characteristics of some of the isolates were studied as shown in Table 4.1. The colour varied from cream to white, some smells of palmwine while some have fruity odours.

Five of the isolates were selected based on their frequency of occurrence as shown in Table 4.2. Majority of the isolates (80%) coded as MX1, MX2, MX4 and MX5 were identified as *S. cerevisiae* and (20%) coded MX3 was identified as *S. uvarum*.

Ethanol tolerance of the selected five yeast isolates is shown in Table 4.3. This provided a preliminary screening of all the isolates. It was observed that all the yeast isolates were able to grow at 4% ethanol concentration tested. Appreciable growth of the yeast were observed up to 8% of ethanol concentration, above this concentration, the tolerance decreases slightly for the *S. cerevisiae* isolate MX4 and MX5. However, *S. cerevisiae* isolates MX1 and MX2 were still able to tolerate ethanol to an appreciable extent above 9% to 10% concentration of ethanol. The tolerance exhibited by these two strains of yeast necessitated their selection for further genetic modification. Isolate MX3 was less tolerant to ethanol at higher concentration but it was selected based on the frequency of occurrence during preliminary isolation from the CPH medium.

Table 4.1: Physiological and Biochemical Characteristics of yeast Isolates

Yeast isolates	Sugar Fermentation										Sugar Tolerance												Probable Identity
	Glucose	Galactose	Maltose	Sucrose	Trehalose	Lactose	Cellobiose	Melliobiose	Raffinose	Xylose	20% glucose	30% glucose	30% glucose	40% glucose	50% glucose	60% glucose	Urease Test	Nitrate Assimilation	Acid Production	Asexual Reproduction	Pseudomycelium Formation	Ascospore Formation	
MX1	+	+	+	+	+	-	-	+	+	-	+	+	+	+	+	+	-	-	-	B	+	+	*Saccharomyces cerevisiae*
MX3	+	+	+	+	+	-	-	+	+	-	+	+	+	+	+	+	W+	+	+	B	+	+	*Saccharomyces uvarum*
MX1	+	+	+	+	+	-	-	+	+	-	+	+	+	+	+	+	-	-	-	B	+	+	*Saccharomyces cerevisiae*
MX2	+	+	+	+	+	-	-	+	+	-	+	+	+	+	+	+	-	-	-	B	+	+	*Saccharomyces cerevisiae*
MX4	+	+	+	+	+	-	-	+	+	-	+	+	+	+	+	+	-	-	-	B	+	+	*Saccharomyces cerevisiae*
MX2	+	+	+	+	+	-	-	+	+	-	+	+	+	+	+	+	-	-	-	B	+	+	*Saccharomyces cerevisiae*
MX5	+	+	+	+	+	-	-	+	+	-	+	+	+	+	+	+	-	-	-	B	+	+	*Saccharomyces cerevisiae*
MX4	+	+	+	+	+	-	-	+	+	-	+	+	+	+	+	+	-	-	-	B	+	+	*Saccharomyces cerevisiae*
MX1	+	+	+	+	+	-	-	+	+	-	+	+	+	+	+	+	-	-	-	B	+	+	*Saccharomyces cerevisiae*
MX3	+	+	+	+	+	-	-	+	+	-	+	+	+	+	+	+	W+	+	+	B	+	+	*Saccharomyces uvarum*
MX1	+	+	+	+	+	-	-	+	+	-	+	+	+	+	+	+	-	-	-	B	+	+	*Saccharomyces cerevisiae*
MX5	+	+	+	+	+	-	-	+	+	-	+	+	+	+	+	+	-	-	-	B	+	+	*Saccharomyces cerevisiae*
MX3	+	+	+	+	+	-	-	+	+	-	+	+	+	+	+	+	W+	+	+	B	+	+	*Saccharomyces uvarum*
MX4	+	+	+	+	+	-	-	+	+	-	+	+	+	+	+	+	-	-	-	B	+	+	*Saccharomyces cerevisiae*
MX1	+	+	+	+	+	-	-	+	+	-	+	+	+	+	+	+	-	-	-	B	+	+	*Saccharomyces cerevisiae*
MX1	+	+	+	+	+	-	-	+	+	-	+	+	+	+	+	+	-	-	-	B	+	+	*Saccharomyces cerevisiae*
MX2	+	+	+	+	+	-	-	+	+	-	+	+	+	+	+	+	-	-	-	B	+	+	*Saccharomyces cerevisiae*
MX	+	-	+	+	D	-	+	-	+	W+	+	+	+	+	-	-	-	+	-	B	+	+	*Pichia sp.*
MX3	+	+	+	+	+	-	-	+	+	-	+	+	+	+	+	+	-	-	-	B	+	+	*Saccharomyces cerevisiae*
MX5	+	+	+	+	+	-	-	+	+	-	+	+	+	+	+	+	-	-	-	B	+	+	*Saccharomyces cerevisiae*
MX2	+	+	+	+	+	-	-	+	+	-	+	+	+	+	+	+	-	-	-	B	+	+	*Saccharomyces cerevisiae*
MX4	+	+	+	+	+	-	-	+	+	-	+	+	+	+	+	+	-	-	-	B	+	+	*Saccharomyces cerevisiae*

Table 4.2: Frequency of occurrence of the different yeast isolates obtained from spontaneously fermenting Cocoa Pod Husk (CPH) submerged medium

Yeast Isolates	Frequency of occurrence (%)
MX1	28.5
MX2	27.3
MX3	15.2
MX4	14.6
MX5	14.4

Keys:

Saccharomyces cerevisiae MX1

Saccharomyces cerevisiae MX2

Saccharomyces uvarum MX3

Saccharomyces cerevisiae MX4

Saccharomyces cerevisiae MX5

Table 4.3: Ethanol Tolerance of the Selected Yeast Isolates

Yeast Isolates	Concentration of Ethanol						
	4%	5%	6%	7%	8%	9%	10%
MX1	2.60± 0.0079^{a}	2.45 ± 0.0020^{a}	2.38 ± 0.00430^{a}	2.34 ± 0.03700^{a}	2.19± 0.00295^{b}	2.15 ± 0.00110^{a}	1.65 ± 0.00600^{b}
MX2	2.54± 0.0041^{a}	2.47 ± 0.00100^{a}	2.41 ± 0.01190^{a}	2.18± 0.13850^{ab}	2.28 ± 0.01765^{a}	2.10 ± 0.00505^{a}	1.79 ± 0.05550^{a}
MX3	1.95± 0.0233^{b}	1.26 ± 0.00195^{d}	0.95 ± 0.00480^{d}	0.90 ± 0.00855^{c}	0.81 ± 0.01140^{d}	0.78 ± 0.00980^{d}	0.58 ± 0.00045^{d}
MX4	2.44± 0.09585^{a}	2.34 ± 0.01775^{b}	2.13 ± 0.04465^{c}	2.07 ± 0.04075^{b}	1.99 ± 0.03500^{c}	1.92 ± 0.04590^{b}	1.49 ± 0.03240^{c}
MX5	2.41 ± 0.05770^{a}	2.25 ± 0.01205^{c}	2.24 ± 0.02425^{b}	2.05 ± 0.02510^{b}	2.02 ± 0.01460^{c}	1.61 ± 0.00290^{c}	1.47 ± 0.01125^{c}

Values with same superscript are not significantly different

SD = 0.05

Keys

Saccharomyces cerevisiae MX1

Saccharomyces cerevisiae MX2

Saccharomyces uvarum MX3

Saccharomyces cerevisiae MX4

Saccharomyces cerevisiae MX5

4.2 Preparation and Isolation of Genomic DNA from *P. stipitis*

The extraction and separation of genomic DNA of *P. stipitis* (CBS 6054) was carried out using 0.8% agarose gel in 0.5x TBE buffer incoporated with ethidium bromide and viewed with trans illuminator. The gel bands revealed a total size of 15.4Mbp (15,400kb) of the extracted genomic DNA. Also a DNA ladder (1kb+ DNA marker) was placed alongside with the extracted *P. stipitis* DNA sample in the gel electrophoresis, as shown in figure 4.1. The bioinformatic study of *P. stipitis* DNA genome sequence (figure 4.2) revealed that it has 8 chromosomes; the length of chromosome 1 is 3.5 containing 1,276 genes occupy two motifs (1.1 and 1.2) while chromosome 5 has a length of 1.7 containing 679genes.

The BLAST search programme of NCBI used located Xylose reductase gene (XR) sequence on chromosome 5 of *P. stipitis* (CBS 6054) with an Open Reading Frame (ORF) of 2052bp nucleotide sequence (figure 4.3). The gene has a poly A site located on nucleotide 1335 - 1339. DNA Stider 1.4f7 of analysis revealed that of this 2052bp nucleotide sequence, 957bp represents the coding sequence of the gene (XR) that drives the expression of the gene, this occurs in the prim transcript from nucleotide 356 – 1312 figure 4.4. Further bioinformatic analysis of the xylose reductase gene confirms that the gene (XR) has a protein sequence of 319 with deduced 318 amino acids polypeptide encoded by a coding sequence of 954bp with a predicted molecular weigth of 35.8kDa, (figure 4.5).

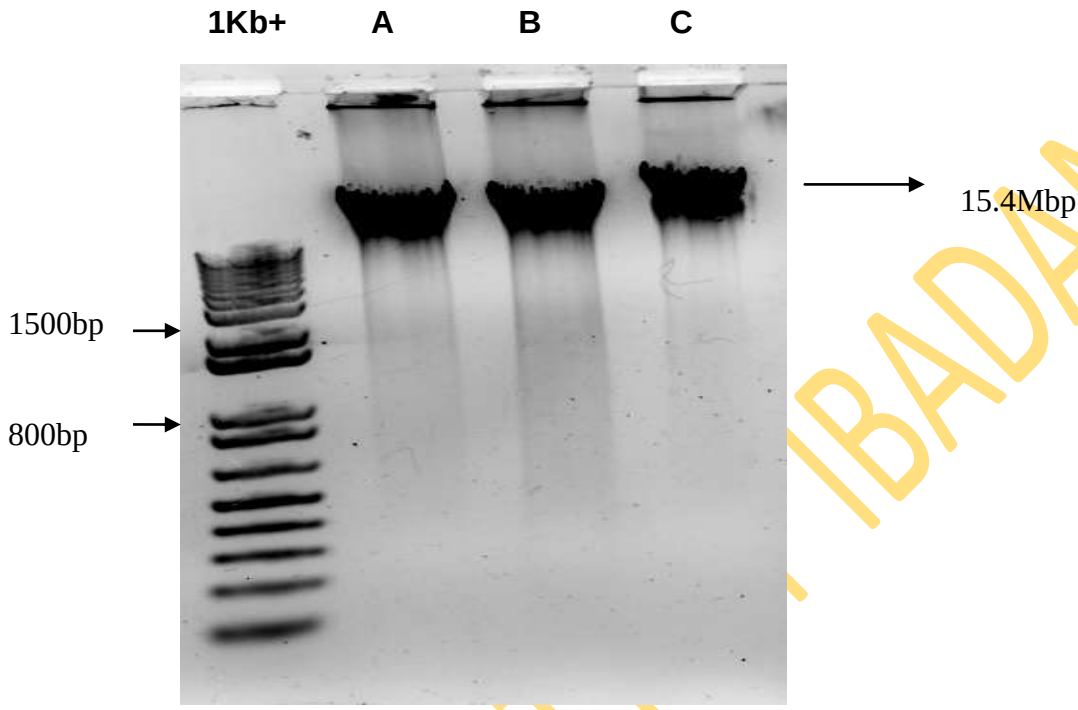


Fig. 4.1 Genomic DNA of *P. stipitis* (15.4Mbp) – lane A, B and C in 0.8% agarose gel with 1kb+ DNA marker.

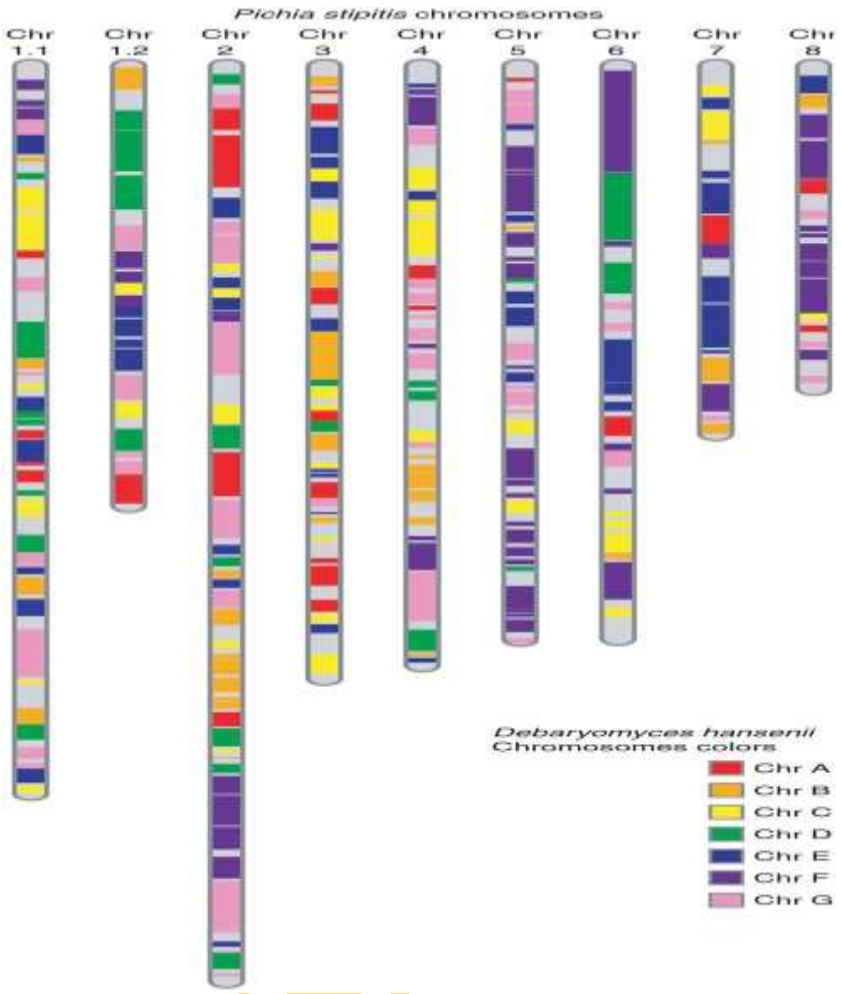


Fig. 4.2 *Pichia stipitis* CBS 6054 Showing 8 chromosomes

DNA Sequence 2052 base pairs gatccacagaca ...tttcgttttcgg Linear

```
   1 gatccacaga cactaattgg ttctacatta ttcgtgttca gacacaaaccccagcgttgg
  61 cggtttctgt ctgcgttcct ccagcacctt cttgctcaac cccagaaggtgcacactgca
 121 gacacacata catacgagaa cctggaacaa atatcggtgt cggtgaccgaaatgtgcaaa
 181 cccagacacg actaataaac ctggcagctc caataccgcc gacaacaggtgaggtgaccg
 241 atggggtgcc aattaatgtc tgaaaattgg ggtatataaa tatggcgattctccggagaa
 301 tttttcagtt ttcttttcat ttctccagta ttcttttcta tacaactatactacaatgcc
 361 ttctattaag ttgaactctg gttacgacat gccagccgtc ggtttcggctgttggaaagt
 421 cgacgtcgac acctgttctg aacagatcta ccgtgctatc aagaccggttacagattgtt
 481 cgacggtgcc gaagattacg ccaacgaaaa gttagttggt gccggtgtcaagaaggccat
 541 tgacgaaggt atcgtcaagc gtgaagactt gttccttacc tccaagttgtggaacaacta
 601 ccaccaccca gacaacgtcg aaaaggcctt gaacagaacc ctttctgacttgcaagttga
 661 ctacgttgac ttgttcttga tccacttccc agtcaccttc aagttcgttccattagaaga
 721 aaagtaccca ccaggattct actgtggtaa gggtgacaac ttcgactacgaagatgttcc
 781 aattttagag acctggaagg ctcttgaaaa gttggtcaag gccggtaagatcagatctat
 841 cggtgtttct aacttcccag gtgctttgct cttggacttg ttgagaggtgctaccatcaa
 901 gccatctgtc ttgcaagttg aacaccaccc atacttgcaa caaccaagattgatcgaatt
 961 cgctcaatcc cgtggtattg ctgtcaccgc ttactcttcg ttcggtcctcaatctttcgt
1021 tgaattgaac caaggtagag ctttgaacac ttctccattg ttcgagaacgaaactatcaa
1081 ggctatcgct gctaagcacg gtaagtctcc agctcaagtc ttgttgagatggtcttccca
1141 aagaggcatt gccatcattc caaagtccaa cactgtccca agattgttggaaaacaagga
1201 cgtcaacagc ttcgacttgg acgaacaaga tttcgctgac attgccaagttggacatcaa
1261 cttgagattc aacgacccat gggactggga caagattcct atcttcgtctaagaaggttg
1321 ctttatagag aggaaataaa acctaatata cattgattgt acatttaaaattgaatattg
1381 tagctagcag attcggaaat ttaaaatggg aaggtgattc tatccgtacgaatgatctct
1441 atgtacatac acgttgaaga tagcagtaca gtagacatca agtctacagatcattaaaca
1501 tatcttaaat tgtagaaaac tataaacttt tcaattcaaa ccatgtctgccaaggaatca
1561 aatgagattt ttttcgcagc caaacttgaa tccaaaaata aaaaacgtcattgtctgaaa
1621 caactctatc ttatctttca cctcatcaat tcattgcata tcataaaagcctccgatagc
1681 atacaaaact acttctgcat catatctaaa tcatagtgcc atattcagtaacaataccgg
1741 taagaaactt ctatttttt agtctgcctt aacgagatgc agatcgatgcaacgtaagat
1801 caaacccctc cagttgtaca gtcagtcata tagtgaacac cgtacaatatggtatctacg
1861 ttcaaaataga ctccaataca gctggtctgc ccaagttgag caactttaatttagagacaa
1921 agtcgtctct gttgatgtag gcaccacaca ttcttctctt gcccgtgaactctgttctgg
1981 agtggaaaca tctccagttg tcaaatatca aacactgacc aggcttcaactggtagaaga
2041 tttcgttttc gg
```

Fig. 4.3 Open reading frame (OFR) of *Pichia stipitis* CBS 6054 showing 2052bp, arrow indicates the coding sequence (CD) of xylose reductase (XR) gene on chromosome 5.

DNA sequence 957 base pairs atgccttctatt ……atcttcgtctaa Linear

```
   1 ATGCCTTCTA TTAAGTTGAA CTCTGGTTAC GACATGCCAG CCGTCGGTTTCGGCTGTTGG
  61 AAAGTCGACG TCGACACCTG TTCTGAACAG ATCTACCGTG CTATCAAGACCGGTTACAGA
 121 TTGTTCGACG GTGCCGAAGA TTACGCCAAC GAAAAGTTAG TTGGTGCCGGTGTCAAGAAG
 181 GCCATTGACG AAGGTATCGT CAAGCGTGAA GACTTGTTCC TTACCTCCAAGTTGTGGAAC
 241 AACTACCACC ACCCAGACAA CGTCGAAAAG GCCTTGAACA GAACCCTTTCTGACTTGCAA
 301 GTTGACTACG TTGACTTGTT CTTGATCCAC TTCCCAGTCA CCTTCAAGTTCGTTCCATTA
 361 GAAGAAAAGT ACCCACCAGG ATTCTACTGT GGTAAGGGTG ACAACTTCGACTACGAAGAT
 421 GTTCCAATTT TAGAGACCTG GAAGGCTCTT GAAAAGTTGG TCAAGGCCGGTAAGATCAGA
 481 TCTATCGGTG TTTCTAACTT CCCAGGTGCT TTGCTCTTGG ACTTGTTGAGAGGTGCTACC
 541 ATCAAGCCAT CTGTCTTGCA AGTTGAACAC CACCCATACT TGCAACAACCAAGATTGATC
 601 GAATTCGCTC AATCCCGTGG TATTGCTGTC ACCGCTTACT CTTCGTTCGGTCCTCAATCT
 661 TTCGTTGAAT TGAACCAAGG TAGAGCTTTG AACACTTCTC CATTGTTCGAGAACGAAACT
 721 ATCAAGGCTA TCGCTGCTAA GCACGGTAAG TCTCCAGCTC AAGTCTTGTTGAGATGGTCT
 781 TCCCAAAGAG GCATTGCCAT CATTCCAAAG TCCAACACTG TCCCAAGATTGTTGGAAAAC
 841 AAGGACGTCA ACAGCTTCGA CTTGGACGAA CAAGATTTCG CTGACATTGCCAAGTTGGAC
 901 ATCAACTTGA GATTCAACGA CCCATGGGAC TGGGACAAGA TTCCTATCTT CGTCTAA
```

Fig. 4.4: 957bp coding sequence of xylose reductase gene on chromosome 5 of *P. stipitis*

"MPSIKLNSGYDMPAVGFGCWKVDVDTCSEQIYRAIKTGYRLFDG

AEDYANEKLVGAGVKKAIDEGIVKREDLFLTSKLWNNYHHPDNVEKALNRTLSDLQVD

YVDLFLIHFPVTFKFVPLEEKYPPGFYCGKGDNFDYEDVPILETWKALEKLVKAGKIR

SIGVSNFPGALLLDLLRGATIKPSVLQVEHHPYLQQPRLIEFAQSRGIAVTAYSSFGP

QSFVELNQGRALNTSPLFENETIKAIAAKHGKSPAQVLLRWSSQRGIAIIPKSNTVPR

LLENKDVNSFDLDEQDFADIAKLDINLRFNDPWDWDKIPIFV"

Fig. 4.5: Protein Translation of XR gene (318 Amino acids)

4.3 Primer Design for Xylose Reductase (XL1) Gene and PCR

The primer designed for the coding sequence (957 bp) of the xylose reductase (XL1) gene (Genebank 59465) using Saccharomyces Genome Database (SGD) is shown in Table 4.4. The primers were designed within a base length of 17 – 28 bases, with sequence ends of C nucleotide. The forward primer consisted of 21 bases in length, with 33% GC content and a melting temperature of 45°C while the reverse primer has a base length of 19, with 36% GC content and a melting temperature (Tm) of 40°C. This primer set specific for the component gene (XR) was used in a normal PCR to amplify the component gene of this operon from the genomic DNA of *Pichia stipitis*.

Analysis of the PCR product of XR gene in 0.8% agarose gel electrophoresis shows that DNA fragments with expected size (957bp) were successfully amplified from the genomic DNA of *Pichia stipitis* (figure 4.6).The correct amplification of this primer necessitated the attachement of two restriction enzyme sites into the sequence.

Table 4.4: Designed primer sequence of xylose reductase gene for PCR reaction using SGD

Forward-Primer	**ATGCCTTCTATTAAGTTGAAC**	**Reverse-Primer**	**TTAGACGAAGATAGGAATC**
Length:	21	Length:	19
Percent GC:	33	Percent GC:	36
Tm:	45	Tm:	40
Self-Anneal:	16	Self-Anneal:	10
End-Anneal:	8	End-Anneal:	8
Offset:	1	Offset:	957

Pair-Anneal:12

Pair-End-Anneal:6

Total valid primer pairs: 6

Primer sequence: The primer sequence derivable was sent to SIGMA for synthesis.

Fwd 5'----- ATGCCTTCTATTAAGTTGAAC……3'

Rev 5'…..TTAGACGAAGATAGGAATC ……..3'

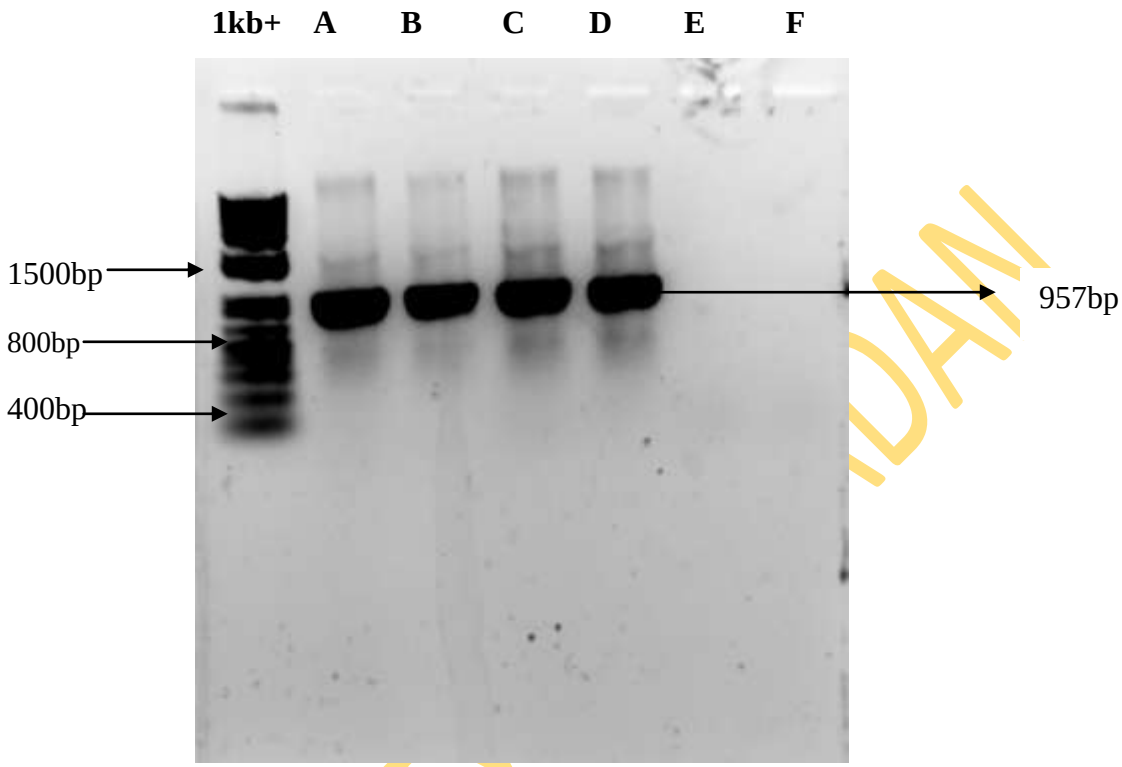
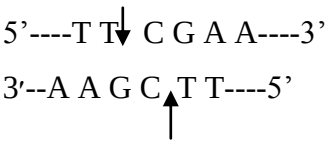


Fig. 4.6: **PCR reaction Lane A to D showing 957bp of XR gene product amplified from the genomic DNA of *P. stipitis* in 0.8% agarose gel electrophoresis, Lane E and F (control)**

4.4 Attachment of Enzyme Restriction sites into designed XR Primer sequence

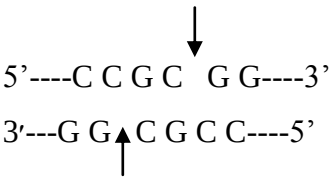
Based on the successful amplification of the XR gene primer, the insert gene (XR) DNA coding sequence 957bp linear was subjected to DNA Strider 1.4f7 analysis generated whole restriction enzyme sites usage and sites with no enzyme restriction respectively (Fig. 4.7) (see whole sequence restriction report on appendix 5) two different enzymes restriction sites *BstBI* and *SacII* were chosen after confirming with DNA strider 1.4f7 that these RE has no restriction sites on the insert gene (XL1) sequence, but has one restriction enzyme site each on the plasmid vector. The restriction enzymes sites had six bases each attached into the Xylose reductase gene (957bp) at the 5' and 3' ends respectively along with six bases each at both 3' and 5' position increases the efficiency of the digest by the restriction enzymes (Fig. 4.8), Thus 24 bases added to the sequence brought the total size of the gene to 981bp (Fig. 4.9). After the digest by the two enzymes, 18 bases were removed giving a total size of the gene to be 963bp as shown in the 0.8% agarose gel purified PCR product Fig. 4.10. The two different restriction enzymes were used in order to prevent the generation of pallidromic sequences and self ligation of the sequence.

Bst B1 **tt/cga**



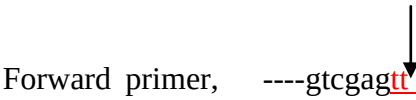
Restriction site for ***BstBI* restriction enzyme** (*Bacillus stearothermophilus* B225)

SacII **ccgc/gg**



Restriction site for ***SacII* restriction enzyme** (*Streptomyces achromogenes*)

Fig. 4.7: Restriction enzyme sites usage of XR gene generated by 1.4f7 DNA strider



Forward primer, ----gtcgagtt cgaaATGCCTTCTATTAAGTTGAAC (33mers) (attached with ***BstB1***)

Reverse primer,----actggtccgc ggTTAGACGAAGATAGGAATC (31mers) (attached with ***SacII***)

Fig. 4.8 Attachment of *BstB1* and *SacII* restriction enzyme sites into XR primer sequence

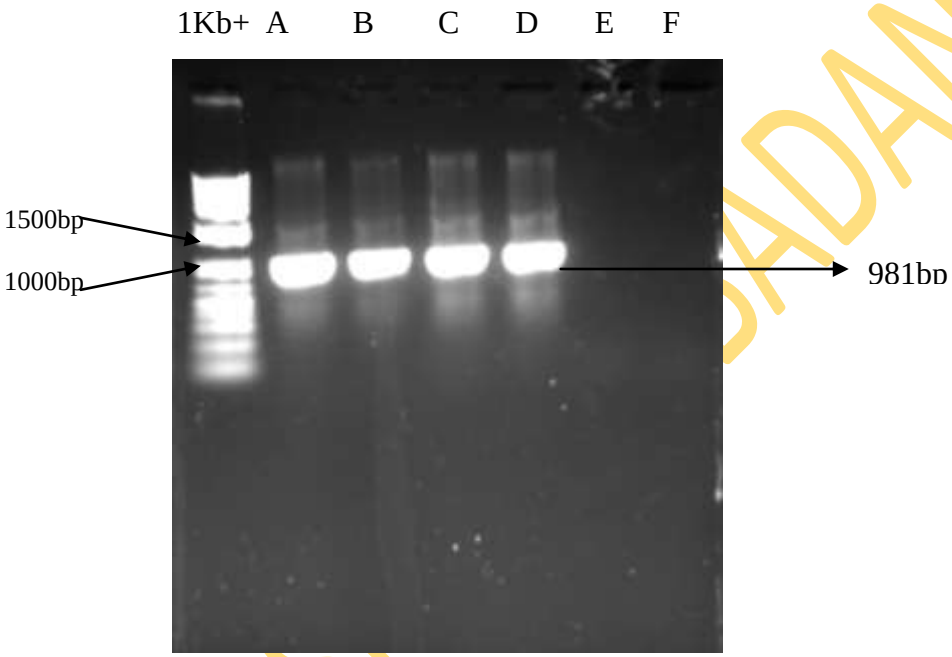


Fig. 4.9: The PCR amplified product of XR gene with attached *BstB1 and Sac11* restriction enzymes sites sequences in 0.8% agarose gel (lanes A to D), lane E and F (control) and 1Kb+ DNA marker

Fig. 4.10: **Gel Purification of XL1 gene (963bp) digested with *BstB1 and Sac11* Enzymes lane A and B in 0.8% agarose gel electrophoresis with 1Kb+ DNA marker.**

4.5 Extraction and Restriction Digest of pGAPZA Plasmid with Restriction Enzymes

The extraction and purification of the plasmid vector (pGAPZA) in 0.8% agarose gel electrophoresis gave a size of 3.147kb of the plasmid genomic DNA (Fig.4.11). The restriction enzyme site map of the plasmid vector gave a total of 174 different restriction enzymes with the specified number of cut on the plasmid genome and the positions (see Appendix 5). The choice of the two enzymes was based on the fact that both enzymes used same buffer system (Nebuffer 4) and do not require the purification of the DNA sample after each enzyme digestion. The *SacII* enzyme cut at 37°C for 2 hours and inactivated at 65°C which is the activation temperature for BstB1 restriction enzyme. The linearization of the plasmid with the same two restriction enzymes used for the insert gene (XR) is shown in Fig 4.12, the gel purification of the double digested plasmid reveal a size of 2.825kb Fig. 4.13. The multiple cloning sites of the plasmid shows that *BstB1* enzyme restricted the plasmid once at position 486 nucleotide sequence while *SacII* enzymes also cut once at position 808 of the plasmid (fig.4.14); both restrictions removed 322bp containing the alpha factor segment giving a plasmid size of 2.825kb (Fig. 4.15).

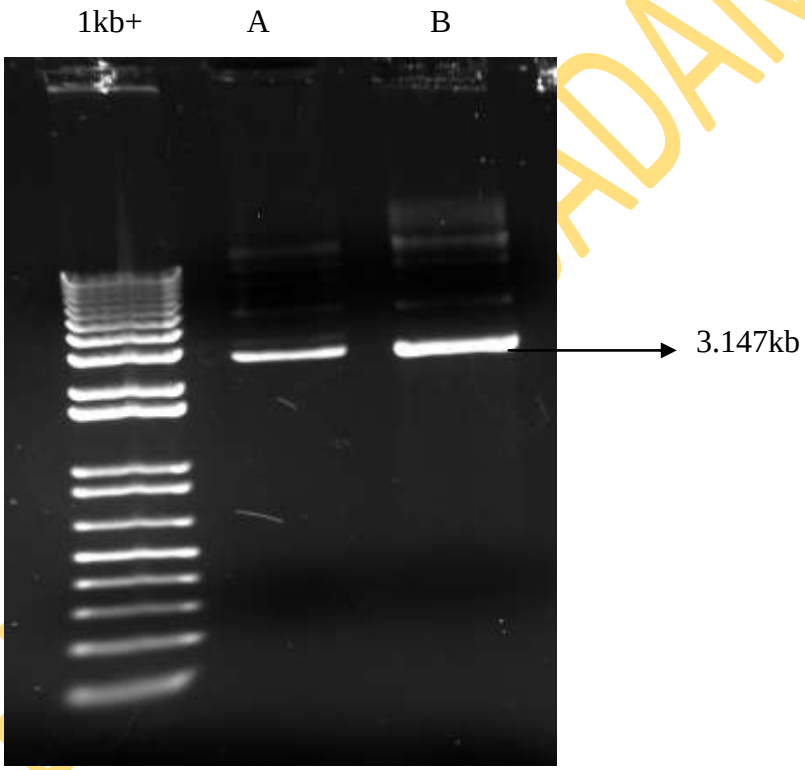


Fig. 4.11: Electrophoresis of the genomic DNA of pGAPZA plasmid vector (3.147kb) lane A and B and 1kb+ DNA marker in 0.8% agarose gel

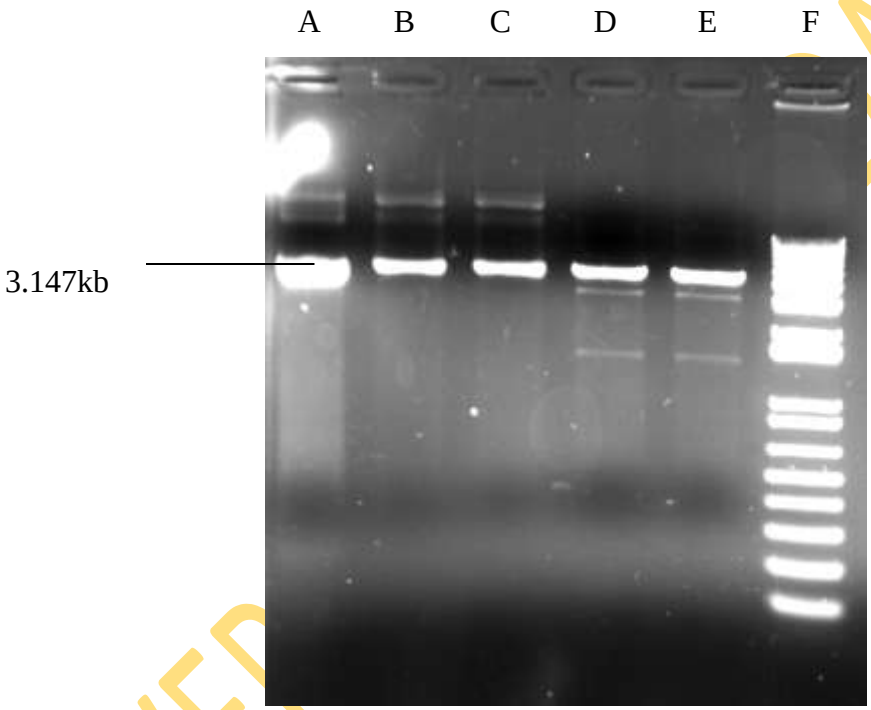


Fig. 4.12: pGAPZA plasmid (3.147kb) lane A; lane B and C is digested plasmid with *SacII* enzyme (linearization) (3.147kb); lane D and E is plasmid vector (2.825kb) digested with *SacII* and *BstB1* restriction enzymes

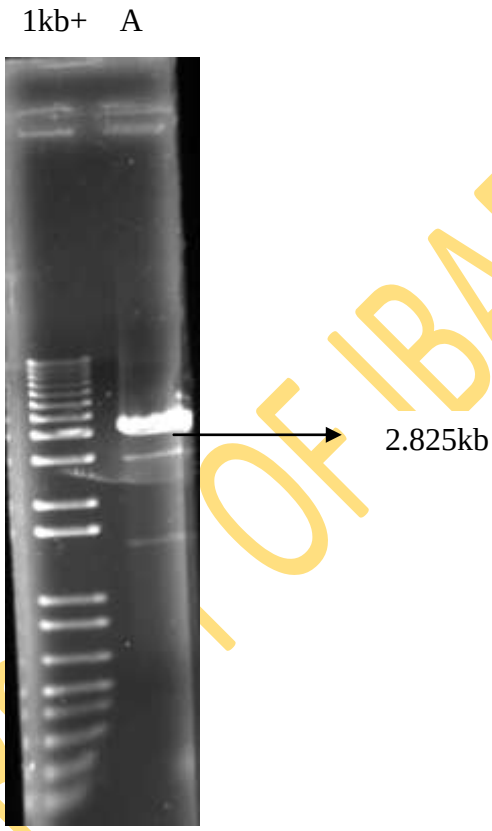


Fig. 4.13. Lane A is Gel Purified product of pGAPZA plasmid (2.825kb) digested with *SacII and BstB1* restriction enzymes with DNA ladder

Fig. 4.14: Open reading frame of pGAPZαA plasmid vector (3.147kb) showing restriction sequence sites of *BstB1* enzymes at position 486 and *SacII* enzyme at position 808.

AGATCTTTTTTGTAGAAATGTCTTGGTGTCCTCGTCCAATCAGGTAGCCATCTCTGAAATATCTGGCTCCGT
TGCAACTCCGAACGACCTGCTGGCAACGTAAAATTCTCCGGGGTAAAACTTAAATGTGGAGTAATGGAACCA
GAAACGTCTCTTCCCTTCTCTCTCCTTCCACCGCCCGTTACCGTCCCTAGGAAATTTTACTCTGCTGGAGAG
CTTCTTCTACGGCCCCCTTGCAGCAATGCTCTTCCCAGCATTACGTTGCGGGTAAAACGGAGGTCGTGTACC
CGACCTAGCAGCCCAGGGATGGAAAAGTCCCGGCCGTCGCTGGCAATAATAGCGGGCGGACGCATGTCATGA
GATTATTGGAAACCACCAGAATCGAATATAAAAGGCGAACACCTTTCCCAATTTTGGTTTCTCCTGACCCAA
AGACTTTAAATTTAATTTATTTGTCCCTATTTCAATCAATTGAACAACTATTTCGAAACGAGGAATTCACGT
GGCCCAGCCGGCCGTCTCGGATCGGTACCTCGAGCCGCGGCGGCCGCCAGCTTGGGCCCGAACAAAAACTCA
TCTCAGAAGAGGATCTGAATAGCGCCGTCGACCATCATCATCATCATTGAGTTTTAGCCTTAGACATGA
CTGTTCCTCAGTTCAAGTTGGGCACTTACGAGAAGACCGGTCTTGCTAGATTCTAATCAAGAGGATGTCAGA
ATGCCATTTGCCTGAGAGATGCAGGCTTCATTTTTGATACTTTTTTATTTGTAACCTATATAGTATAGGATT
TTTTTTGTCATTTTGTTTCTTCTCGTACGAGCTTGCTCCTGATCAGCCTATCTCGCAGCTGATGAATATCTT
GTGGTAGGGGTTTGGGAAAATCATTCGAGTTTGATGTTTTTCTTGGTATTTCCCACTCCTCTTCAGAGTACA
GAAGATTAAGTGAGACCTTCGTTTGTGCGGATCCCCCACACACCATAGCTTCAAAATGTTTCTACTCCTTTT
TTACTCTTCCAGATTTTCTCGGACTCCGCGCATCGCCGTACCACTTCAAAACACCCAAGCACAGCATACTAA
ATTTTCCCTCTTTCTTCCTCTAGGGTGTCGTTAATTACCCGTACTAAAGGTTTGGAAAAGAAAAAAGAGACC
GCCTCGTTTCTTTTTCTTCGTCGAAAAAGGCAATAAAAATTTTTATCACGTTTCTTTTTCTTGAAATTTTTT
TTTTTAGTTTTTTTCTCTTTCAGTGACCTCCATTGATATTTAAGTTAATAAACGGTCTTCAATTTCTCAAGT
TTCAGTTTCATTTTTCTTGTTCTATTACAACTTTTTTTACTTCTTGTTCATTAGAAAGAAAGCATAGCAATC
TAATCTAAGGGCGGTGTTGACAATTAATCATCGGCATAGTATATCGGCATAGTATAATACGACAAGGTGAGG
AACTAAACCATGGCCAAGTTGACCAGTGCCGTTCCGGTGCTCACCGCGCGCGACGTCGCCGGAGCGGTCGAG
TTCTGGACCGACCGGCTCGGGTTCTCCCGGGACTTCGTGGAGGACGACTTCGCCGGTGTGGTCCGGGACGAC
GTGACCCTGTTCATCAGCGCGGTCCAGGACCAGGTGGTGCCGGACAACACCCTGGCCTGGGTGTGGGTGCGC
GGCCTGGACGAGCTGTACGCCGAGTGGTCGGAGGTCGTGTCCACGAACTTCCGGGACGCCTCCGGGCCGGCC
ATGACCGAGATCGGCGAGCAGCCGTGGGGGCGGGAGTTCGCCCTGCGCGACCCGGCCGGCAACTGCGTGCAC
TTCGTGGCCGAGGAGCAGGACTGACACGTCCGACGGCGGCCCACGGGTCCCAGGCCTCGGAGATCCGTCCCC
CTTTTCCTTTGTCGATATCATGTAATTAGTTATGTCACGCTTACATTCACGCCCTCCCCCCACATCCGCTCT
AACCGAAAAGGAAGGAGTTAGACAACCTGAAGTCTAGGTCCCTATTTATTTTTTTATAGTTATGTTAGTATT
AAGAACGTTATTTATATTTCAAATTTTTCTTTTTTTTCTGTACAGACGCGTGTACGCATGTAACATTATACT
GAAAACCTTGCTTGAGAAGGTTTTGGGACGCTCGAAGGCTTTAATTTGCAAGCTGGAGACCAACATGTGAGC
AAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGCTGGCGTTTTTCCATAGGCTCCGCCCCCC
TGACGAGCATCACAAAAATCGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGATACCAGGC
GTTTCCCCCTGGAAGCTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCCTT
TCTCCCTTCGGGAAGCGTGGCGCTTTCTCAATGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGTTCG
CTCCAAGCTGGGCTGTGTGCACGAACCCCCCGTTCAGCCCGACCGCTGCGCCTTATCCGGTAACTATCGTCT
TGAGTCCAACCCGGTAAGACACGACTTATCGCCACTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAG
GTATGTAGGCGGTGCTACAGAGTTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGGACAGTATTTGG
TATCTGCGCTCTGCTGAAGCCAGTTACCTTCGGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAACAAACCAC
CGCTGGTAGCGGTGGTTTTTTTGTTTGCAAGCAGCAGATTACGCGCAGAAAAAAAGGATCTCAAGAAGATCC
TTTGATCTTTTCTACGGGGTCTGACGCTCAGTGGAACGAAAACTCACGTTAAGGGATTTTGGTCATGCATGA
GATC

Fig. 4.15: 2.825kb of pGAPZA restricted with BstB1 and SacII enzymes removing 322bp

4.6 Multiple cloning site of pGAPZ*α*A Plasmid and cloning of XL1 gene into yeast

The GAP promoter region encoding GAPDH protein is located between bases 1 – 483, with a multiple cloning site located between bases 760 – 828. The C – terminal polyhistidine tag encodes 6 histidine residues that form a metal binding site for purification of recombinant protein was found between bases 872 - 889. The 3' AOX1 form the transcription termination (TT) region is located between bases 893 – 1233 this region permits the efficient 3' mRNA processing, including polyadenylation for increased mRNA stability (Fig. 4.16)

Ligation of the insert gene (XL1) and the plasmid (Fig.4.17) gave series of bands showing that ligation mixture may contain in addition to the desired recombinant molecule, unligated vector molecules, unligated DNA (insert) fragments, self ligated vectors and recombinants with wrong insertion of DNA fragment. Some transformant colonies were picked, numbered A-E and analysed for true transformants as revealed in gel eletrophoresis, Fig. 4.18. The cells were transformed into competent *E.coli* cell for multiplication and purified. The multiplied, purified construct were used in the transformation of *Saccharomyces cerevisiae* starter yeast (Isolate MX1). The gel electrophoresis of the transformed yeast DNA gave a band size of 3.788kb (2.825kb of plasmid and 963bp of XR gene) shown in Fig. 4.19.

Fig. 4.16: Multiple cloning site of pGAPZA confirmed by sequencing and functional testing

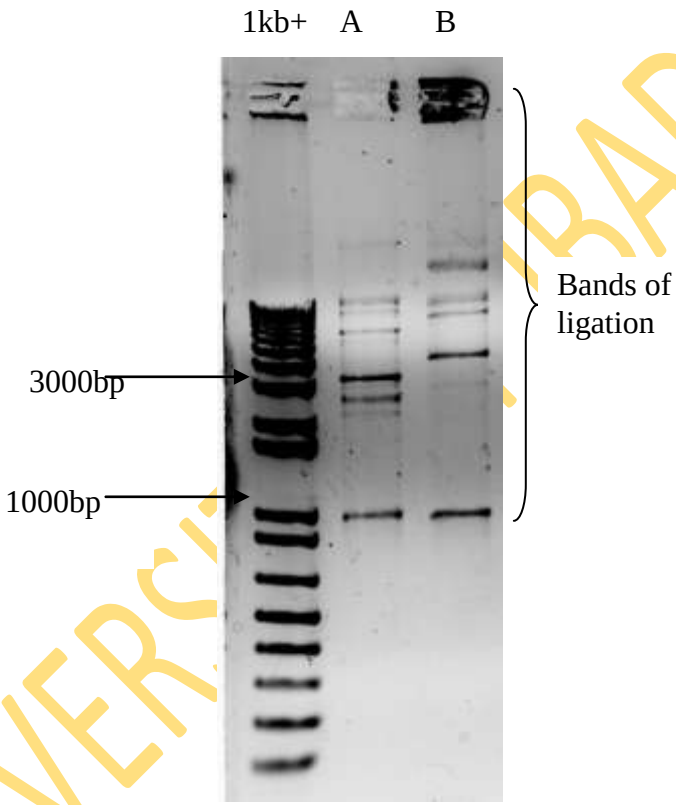


Fig. 4.17 Bands of ligation reaction of pGAPZA plasmid vector (2825bp) with XL1 (963bp) gene (lane A and B) and 1kb+ DNA marker in 0.8% agarose gel

1Kb+ A B C D E F G H 1kb+

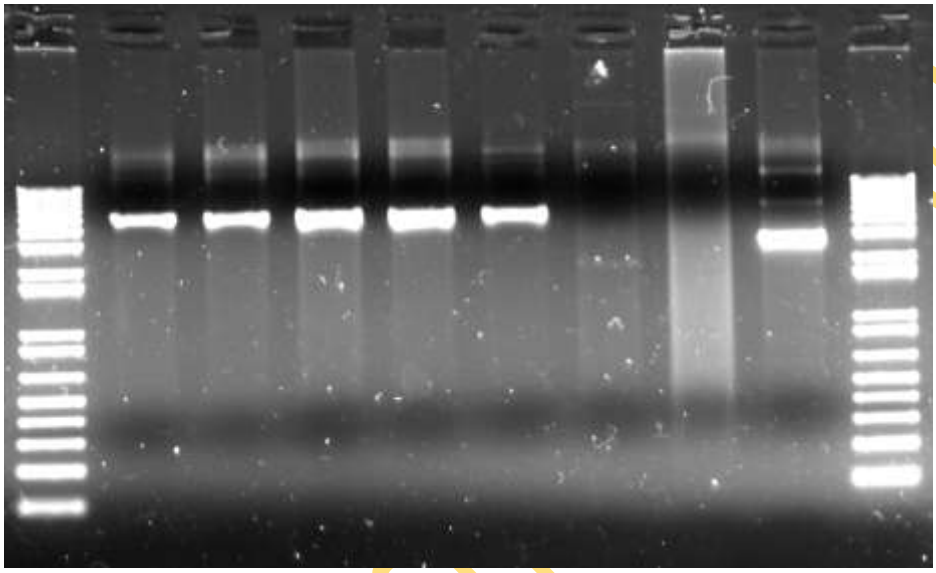


Fig. 4.18 Transformation of *E. coli* competent cells with ligation mixture, Lane A to E (pGAPZA and XL1 gene 3788bp), lane H is 3.147kb of pGAPZA plasmid and 1kb+ DNA markers on both sides in 0.8% agarose gel. Lane F and G is control

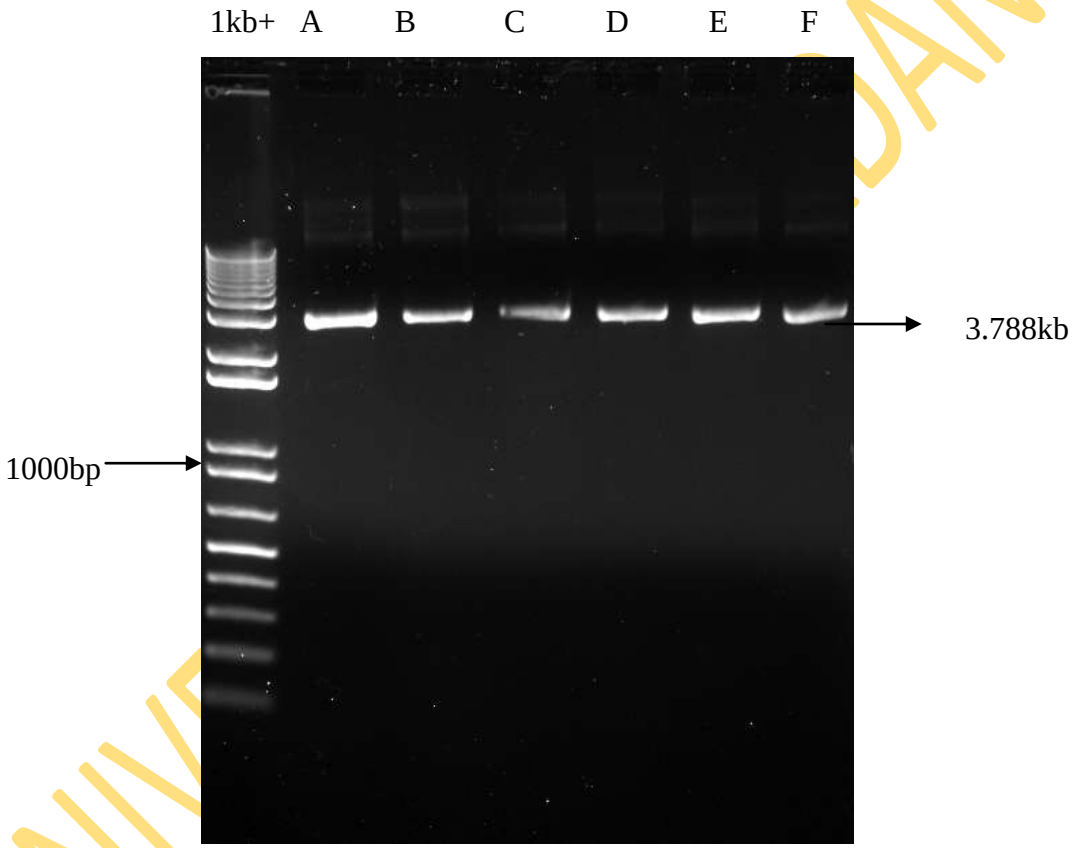


Fig. 4.19: Transformation of competent MX1 yeast cell (lane A to F) showing 3.788kb of gel purified transformant (pGAPZA and XL1) in 0.8% agarose gel

4.7 Transformation of MX1 yeast and verification of transformants

Confirmation of the transformants – MX1 yeast isolate (ligation of pGAPZA and XL1 gene) was verified using restriction enzyme analysis. The digestion of the DNA with *Stu1* restriction enzyme shows that the gene integrated into the yeast genome. From the restriction enzyme analysis of both insert gene and plasmid shows that the *Stu1* RE cuts once on the plasmid vector at position 2118 and the XL1 gene at position 268 giving two bands in 0.8% agarose gel (Fig. 4.20). Some of the transformants restricted with the enzyme fail to cut as shown on the gel electrophoresis, this probably show that some of the transformant colonies contains self ligated plasmid. The true transformants were carefully picked, multiplied and purified by streaking. It was observed that the transformants colonies obtained grew less than the parental strains.

5’----AG G C C T----3’

3′---T C C G G A----5’

Restriction site for **Stu1 restriction enzyme** (*Streptomyces tubercidius*)

The position of the XR gene on *S. cerevisiae* yeast (MX1) is depicted in Fig.4.21 showing that the XR gene is on chromosome 8 at position 104 and at the right arm of the Watson strand.

A B

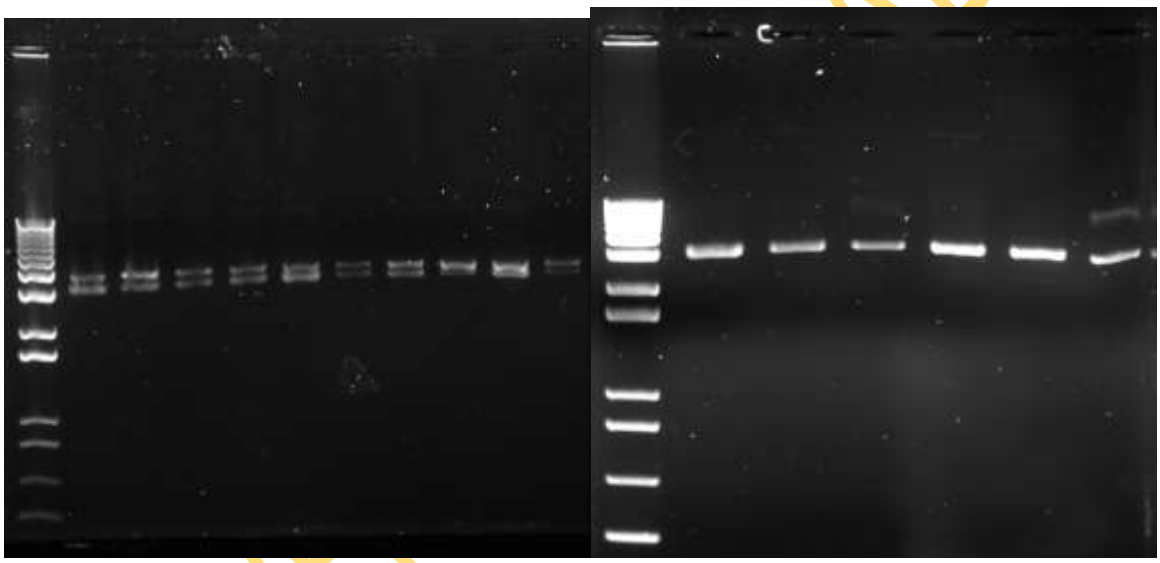


Fig. 4.20: (Gel A) Verification of MX1 yeast transformants with *Stu1* R.E showing double band formation of the DNA restricted. (Gel B) Restriction of untransformed MX1 yeast DNA with *Stu1* restriction enzyme

Fig. 4.21: Chromosome 8 of *Saccharomyces cerevisiae* showing the position of xylose reductase gene (YHR104w)

4.8 Xylitol dehydrogenase (XDH) gene

The BLAST search programme of NCBI used located Xylitol dehydrogenase gene (XDH) sequence on chromosome 1 of *P. stipitis* (CBS 6054) with an Open Reading Frame (ORF) of 1963bp nucleotide sequence (Fig. 4.22). The gene has a poly A site located on nucleotide 1335 - 1339. DNA Stider 1.4f7 analysis revealed that of this 1963bp nucleotide sequence, 1092bp nucleotide sequence represents the coding sequence of the gene (XDH), this occurs in the prim transcript from nucleotide 319 – 1410 shown in Fig. 2.23. Further bioinformatic analysis of the gene confirms that the XDH gene has a protein sequence of 364, representing 363 amino acids (Fig. 4.24) encoded by a coding sequence of 1092bp.

```
   1 tctagaccac cctaagtcgt ccctatgtcg tatgtttgcc tctactacaa agttactagc
  61 aaatatccgc agcaacaaca gctgccctct tccagcttct tagtgtgttg gccgaaaagg
 121 cgctttcggg ctccagcttc tgtcctctgc ggctgctgca cataacgcgg ggacaatgac
 181 ttctccagct tttattataa aaggagccat ctcctccagg tgaaaaatta cgatcaactt
 241 ttactctttt ccattgtctc ttgtgtatac tcactttagt ttgtttcaat cacccctaat
 301 actcttcaca caattaaaat gactgctaac ccttccttgg tgttgaacaa gatcgacgac
 361 atttcgttcg aaacttacga tgccccagaa atctctgaac ctaccgatgt cctcgtccag
 421 gtcaagaaaa ccggtatctg tggttccgac atccacttct acgcccatgg tagaatcggt
 481 aacttcgttt tgaccaagcc aatggtcttg ggtcacgaat ccgccggtac tgttgtccag
 541 gttggtaagg gtgtcacctc tcttaaggtt ggtgacaacg tcgctatcga accaggtatt
 601 ccatccagat tctccgacga atacaagagc ggtcactaca acttgtgtcc tcacatggcc
 661 ttcgccgcta ctcctaactc caaggaaggc gaaccaaacc caccaggtac cttatgtaag
 721 tacttcaagt cgccagaaga cttcttggtc aagttgccag accacgtcag cttggaactc
 781 ggtgctcttg ttgagccatt gtctgttggt gtccacgcct ccaagttggg ttccgttgct
 841 ttcggcgact acgttgccgt ctttggtgct ggtcctgttg gtcttttggc tgctgctgtc
 901 gccaagacct tcggtgctaa gggtgtcatc gtcgttgaca ttttcgacaa caagttgaag
 961 atggccaagg acattggtgc tgctactcac accttcaact ccaagaccgg tggttctgaa
1021 gaattgatca aggctttcgg tggtaacgtg ccaaacgtcg ttttggaatg tactggtgct
1081 gaaccttgta tcaagttggg tgttgacgcc attgccccag gtggtcgttt cgttcaagtt
1141 ggtaacgctg ctggtccagt cagcttccca atcaccgttt tcgccatgaa ggaattgact
1201 ttgttcggtt ctttcagata cggattcaac gactacaaga ctgctgttgg aatctttgac
1261 actaactacc aaaacggtag agaaaatgct ccaattgact ttgaacaatt gatcacccac
1321 agatacaagt tcaaggacgc tattgaagcc tacgacttgg tcagagccgg taagggtgct
1381 gtcaagtgtc tcattgacgg ccctgagtaa gtcaaccgct tggctggccc aaagtgaacc
1441 agaaacgaaa atgattatca aatagcttta tagaccttta tcgaaattta tgtaaactaa
1501 tagaaaagac agtgtagaag ttatatggtt gcatcacgtg agtttcttga attcttgaaa
1561 gtgaagtctt ggtcggaaca aacaaacaaa aaaatatttt cagcaagagt tgatttcttt
1621 tctggagatt ttggtaattg acagagaacc cctttctgct attgccatct aaacatcctt
1681 gaatagaact ttactggatg gccgcctagt gttgagtata tattatcaac caaaatcctg
1741 tatatagtct ctgaaaaatt tgactatcct aacttaacaa aagagcacca taatgcaagc
1801 tcatagttct tagagacacc aactatactt agccaaacaa aatgtccttg gcctctaaag
1861 aagcattcag caagcttccc cagaagttgc acaacttctt catcaagttt acccccagac
1921 cgtttgccga atattcggaa aagccttcga ctatagtgga tcc
```

Fig. 4.22: Open Reading Frame (ORF) 1963bp of Xylitol dehydrogenase gene (whole sequence), arrows indicating the coding sequence of the gene on Chromosome 1 of *Pichia stipitis* CBS 6054

```
1    atgactgcta acccttcctt ggtgttgaac aagatcgacg acatttcgtt cgaaacttac
61   gatgccccag aaatctctga acctaccgat gtcctcgtcc aggtcaagaa aaccggtatc
121  tgtggttccg acatccactt ctacgcccat ggtagaatcg gtaacttcgt tttgaccaag
181  ccaatggtct tgggtcacga atccgccggt actgttgtcc aggttggtaa gggtgtcacc
241  tctcttaagg ttggtgacaa cgtcgctatc gaaccaggta ttccatccag attctccgac
301  gaatacaaga gcggtcacta caacttgtgt cctcacatgg ccttcgccgc tactcctaac
361  tccaaggaag gcgaaccaaa cccaccaggt accttatgta agtacttcaa gtcgccagaa
421  gacttcttgg tcaagttgcc agaccacgtc agcttggaac tcggtgctct tgttgagcca
481  ttgtctgttg gtgtccacgc ctctaagttg ggttccgttg ctttcggcga ctacgttgcc
541  gtctttggtg ctggtcctgt tggtcttttg gctgctgctg tcgccaagac cttcggtgct
601  aagggtgtca tcgtcgttga cattttcgac aacaagttga agatggccaa ggacattggt
661  gctgctactc acaccttcaa ctccaagacc ggtggttctg aagaattgat caaggctttc
721  ggtggtaacg tgccaaacgt cgttttggaa tgtactggtg ctgaaccttg tatcaagttg
781  ggtgttgacg ccattgcccc aggtggtcgt ttcgttcaag tcggtaacgc tgctggtcca
841  gtcagcttcc caatcaccgt ttcgccatg aaggaattga ctttgttcgg ttctttcaga
901  tacggattca acgactacaa gactgctgtt ggaatctttg acactaacta ccaaaacggt
961  agagaaaatg ctccaattga ctttgaacaa ttgatcaccc acagatacaa gttcaaggac
1021 gctattgaag cctacgactt ggtcagagcc ggtaagggtg ctgtcaagtg tctcattgac
1081 ggccctgagt aa
```

Fig. 4.23: Coding sequence (CDS) of XDH gene 1092bp

Translation="MTANPSLVLNKIDDISFETYDAPEISEPTDVLVQVKKTGICGSD

IHFYAHGRIGNFVLTKPMVLGHESAGTVVQVGKGVTSLKVGDNVAIEPGIPSRFSDEY

KSGHYNLCPHMAFAATPNSKEGEPNPPGTLCKYFKSPEDFLVKLPDHVSLELGALVEP

LSVGVHASKLGSVAFGDYVAVFGAGPVGLLAAAVAKTFGAKGVIVVDIFDNKLKMAKD

IGAATHTFNSKTGGSEELIKAFGGNVPNVVLECTGAEPCIKLGVDAIAPGGRFVQVGN

AAGPVSFPITVFAMKELTLFGSFRYGFNDYKTAVGIFDTNYQNGRENAPIDFEQLITH

RYKFKDAIEAYDLVRAGKGAVKCLIDGPE"

Fig. 4.24: Protein Translation of XDH gene (363 amino acids)

4.9 Primer Design for Xylitol dehydrogenase (XL2) Gene and PCR

The primer designed for the coding sequence (1092 bp) of the xylitol dehydrogenase gene (XL2) [GeneBank: X55392] using Saccharomyces Genome Database (SGD) is shown in Table 4.5. The forward primer consists of 20 bases in length, with 45% GC content and a melting temperature (TM) of 49°C while the reverse primer has a base length of 20, with 50% GC content and a melting temperature (Tm) of 55°C. This primer set specific for the component gene (XDH) in PCR reaction was able to amplify the component gene of this operon from the genomic DNA of *Pichia stipitis*.

Analysis of the PCR product of XDH gene in 0.8% agarose gel shows that DNA fragments with expected size (1092bp) was successfully amplified from the genomic DNA of *Pichia stipitis* (Fig.4.25). The correct amplification of this primer necessitated the attachement of two restriction enzyme sites into the sequence.

Table 4.5: Primers Design for XL2 gene with 1092 base pairs

Forward-Primer	**ATGACTGCTAACCCTTCCTT**	**Reverse-Primer**	**TTACTCAGGGCCGTCAATGA**
Length:	20	Length:	20
Percent GC:	45	Percent GC:	50
Tm:	49	Tm:	55
Self-Anneal:	10	Self-Anneal:	18
End-Anneal:	4	End-Anneal:	8
Offset:	1	Offset:	1092

Pair-Anneal: 14

Pair-End-Anneal: 6

Total valid primer pairs: 16

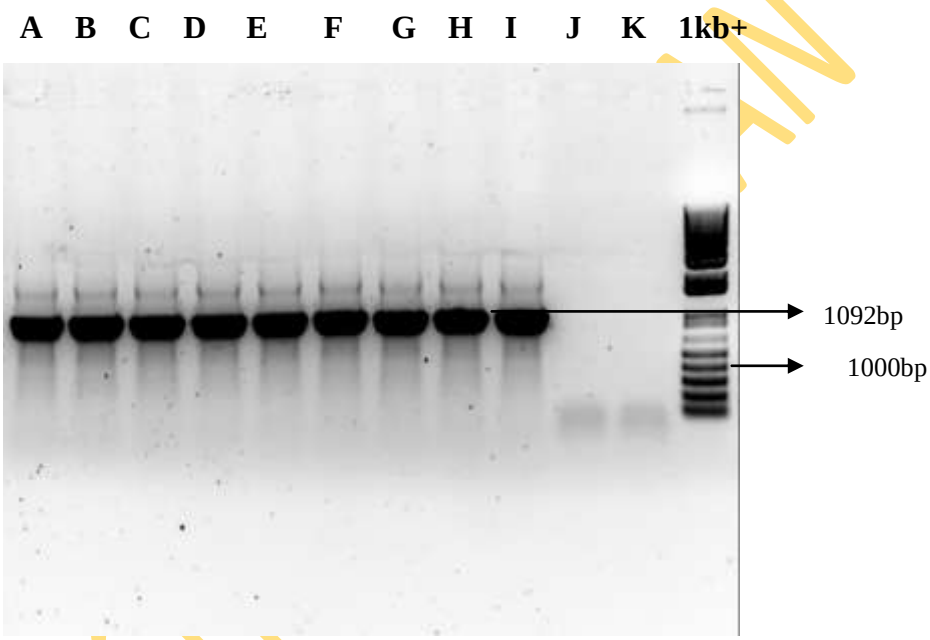


Fig. 4.25: PCR showing 1092bp of Xylitol dehydrogenase gene product (lane A to I) amplified from genomic DNA of *P. stipitis* in 0.8% agarose gel electrophoresis, lane J and K (control) with 1kb+ DNA ladder

4.10 Attachment of Enzyme Restriction sites into Primer

Coding sequence of Xylitol dehydrogenase gene (1092bp) subjected to DNA Strider 1.4f7 generated whole restriction enzyme sites and sites with no restriction enzymes respectively (Fig. 4.26, also see appendix 5) two different enzymes restriction sites sequences *PstI* and *BamHI* were chosen after confirming with DNA strider 1.4f7 that both has no restriction sites on the insert gene (XL2) but has a restriction site on the pVT100U-plasmid vector. The restriction enzymes sites with (six bases each) attached into the XL2 gene (1092bp) at the 5' and 3' ends respectively providing the cleavage site for the enzyme along with four bases each at both 3' and 5' position (to increase the efficiency of the cut by the restriction enzymes) brought the total size of the gene to 1112bp (Fig. 4.27). The 0.8% agarose gel purified PCR product of the gene with attached restriction enzyme sites digested with *Pst1 and BamHI*, gave a total size of 1098bp of the gene (Fig.4.28). The different restriction enzymes used prevented the generation of pallidromic sequences that can bring about self ligation.

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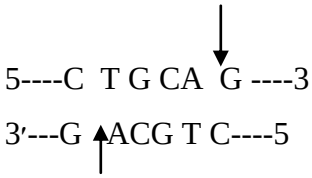
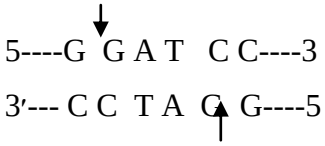
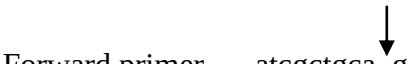


Restriction site for ***PstI*** (Forward primer)



Restriction site for ***BamHI*** (Reverse primer)

Fig. 4.26 Chosen restriction enzymes of XL2 gene generated by 1.4f7 DNA Strider.



Forward primer,----atcgctgca gATGACTGCTAACCCTTCCTT (30mers)

(attached with ***Pst1***)

Reverse primer,-acgtg gatccTTACTCAGGGCCGTCAATGA (30mers)

(attached with ***BamHI***)

Fig. 4.27 Attachment of *Pst1* and *BamH1* RE sites to XDH primers sequence

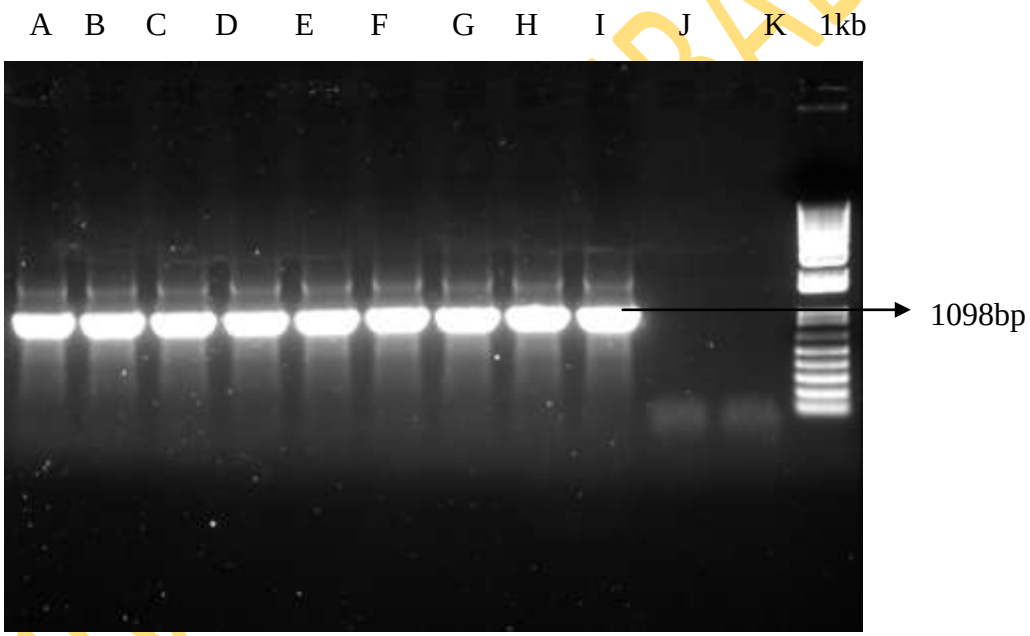


Fig. 4.28 Eletrophoresis of PCR amplified XL2 gene with attached RE site sequences (1098bp) in lane A to I; lane J and K , no amplification (control) with 1kb+ DNA ladder

4.11 Extraction and Restriction Digest of pVT100-U Plasmid with Restriction Enzymes

The extraction and purification of the yeast expression vector pVT100-U DNA in 0.8% agarose gel gave a size of 6.9 kb (Fig.4.29). The vector habours the promoter ADC1 gene encoding alcohol dehydrogenase gene from *S. cerevisiae*. Immediately downstream from the promoter, at position – 12 upstream of the translation – initiation codon ATG, lies a polylinker sequence comprising unique restriction enzyme sites of 161bp. The plasmid has Ura3 selection markers for *S. cerevisiae* and ampicillin for *E.coli.*

The restriction map of the multiple cloning sites (MCS) of the plasmid subjected to DNA strider 1.4f7 yielded series of different restriction enzymes sites with the number of cut and the positions (see Appendix 5). The linearization of the plasmid with the same restriction enzymes used for the insert gene (XL2) at the multiple cloning sites of the plasmid shows that *BamHI* enzyme cut the plasmid once at position 71 and *Pst1I* enzymes also cut once at position 101 of the plasmid multiple cloning site respectively, the gel electrophoresis of the double digested plasmid (6.87kb) is shown in (Fig.4.30).

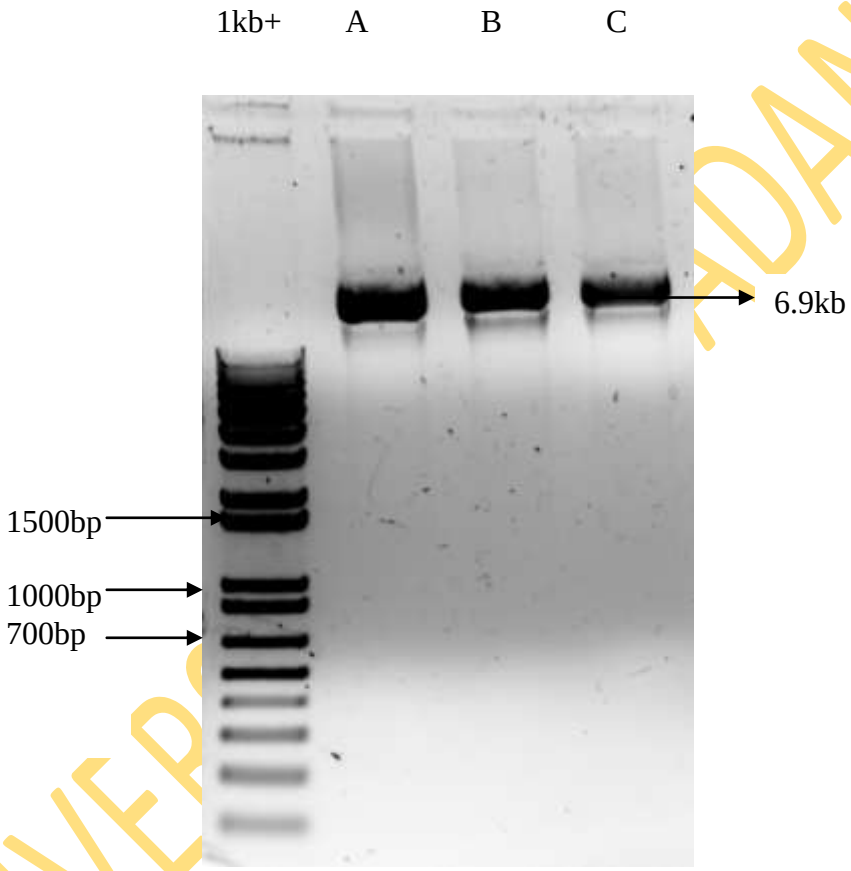


Fig. 4.29 Gel purified Plasmid DNA vector - pVT100-U (6.9kb) in lane A to C with 1kb+ DNA marker

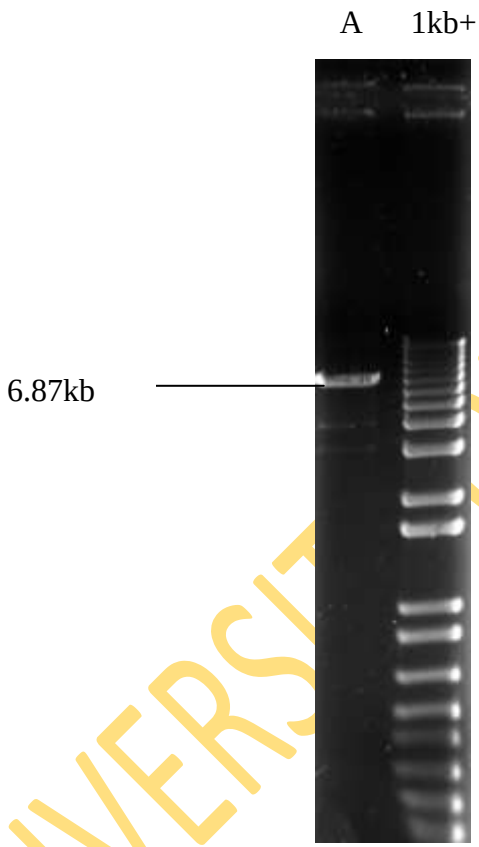


Fig. 4.30 Gel purification of pVT100-U plasmid digested with *Pst1* and *BamH1* Res (6.87kb) in lane A with 1kb+ DNA ladder

4.12 Ligation of digested - purified XL2 gene to pVT100-U expression vector and yeast transformation

Ligation reaction carried out on gel purified XL2 gene with the plasmid vector pVT100-U in a reaction mixture containing the ligase buffer and the T4 ligase enzyme gave series of bands showing that ligation mixture may contain in addition to the desired recombinant molecule, unligated vector molecules, unligated DNA (insert) fragments, self ligated vectors and recombinants with wrong insertion of DNA fragment (Fig. 4.31).

Some colonies of competent cells of *E. coli* strain XL1 BLU MRF transformed with the ligation mixture grew on ampicillin antibiotic plate showing that the insert gene was ligated to the plasmid vector. This was multiplied in competent *E.coli* cell, extracted, gel purified and used in transforming competent cells of MX2 yeast. Few colonies of yeast cells were also observed when MX2 yeast was transformed with this construct using Ura3 drop out synthetic complete medium as the selectable marker for pVT100-U plasmid with XL2 gene. The transformant colonies that grew were picked from the synthetic drop-out plate medium and multiplied. It was observed that the transformant colonies grew less on plate than the parent wild yeast strain. The gel elctrophoresis gave a band of 7.96kb (Fig. 4.32).

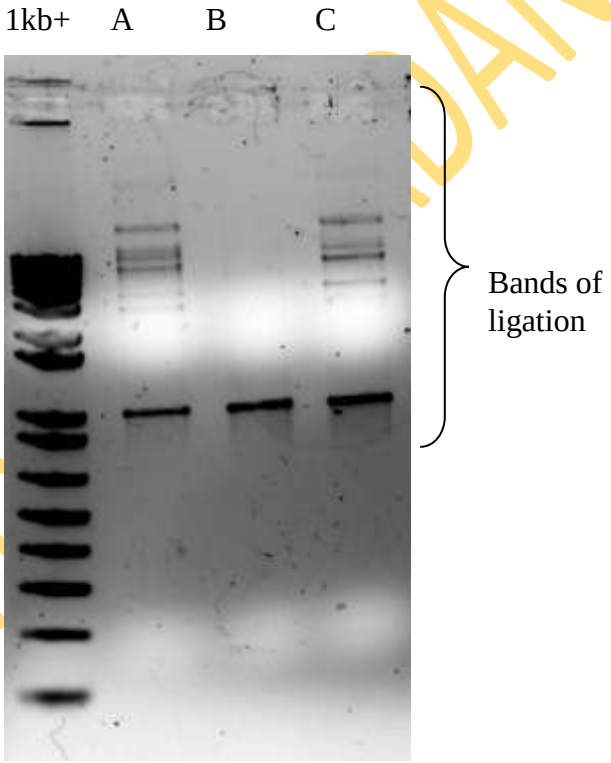


Fig. 4.31: Bands of ligation reaction of digested XL2 gene with digested pVT100-U Plasmid vector, lane A to C with 1kb+ DNA ladder

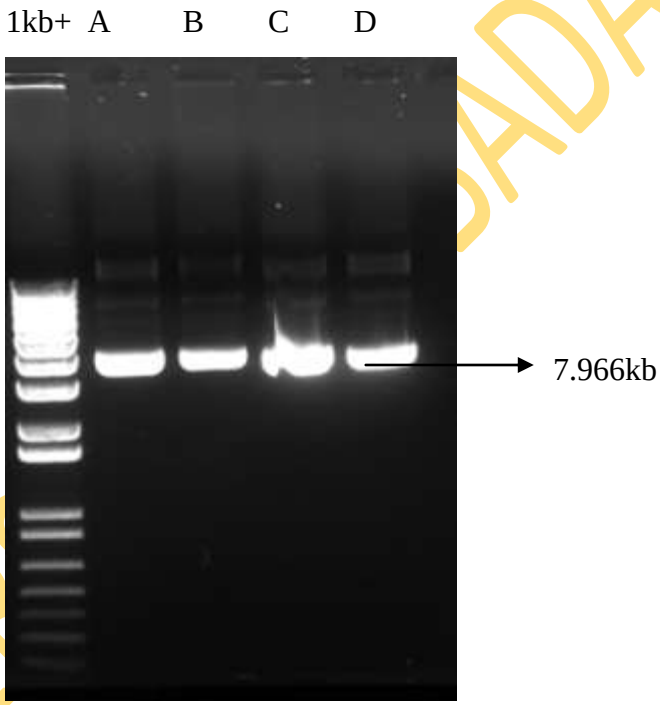


Fig. 4.32 Gel electrophoresis of DNA transformed MX2 yeast (XL2 gene 1096bp and pVT100-U plasmid vector 6870bp) lane A to D with 1kb+ DNA marker

4.13 Verification of MX2 Yeast Transformants – Colony PCR and RE analysis

Each *Saccharomyces cerevisiae* (MX2) transformant colony was verified by the Restriction enzyme analysis test. *Bacillus stearothermophilus* (*Bstx1*) restriction enzyme digest of the extracted transformed yeast DNA gave two bands on gel electrophoresis (Fig.4.33), this restriction enzyme from the bioinformatic analysis done has one restriction site only on the plasmid vector at position 42 and on the insert gene (XL2) at position 176 only, showing that the gene was inserted to the plasmid. Other test was done using colony PCR test reaction to further ascertain the presence of XDH gene in the cell. The yeast transformant cell having been hydrolysed by zymolase enzyme was used in a PCR reaction with the gene design primer gave amplification of the gene as shown in Fig. 4.34. The position of XDH gene in the genome of *S. cerevisiae* MX2 is shown in Fig. 4.35. The XDH gene is located on chromosome 12 at position 70 and to the right arm of the Crick strand.

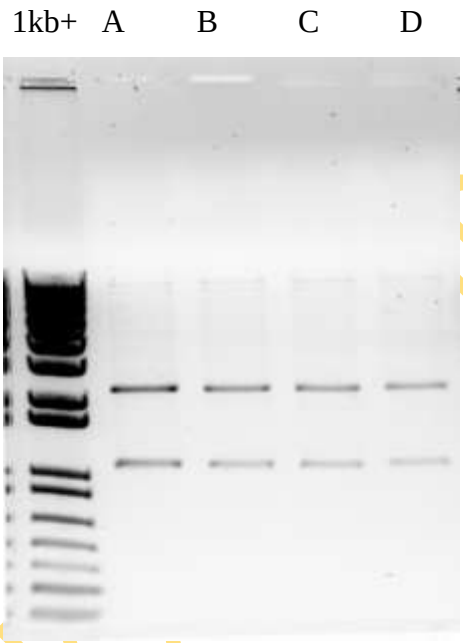


Fig.4.33 Restriction Enzyme analysis of transformants (MX2 yeast) with *BstX1* showing 2 bands of restrictions, lane A to D with 1kb+ DNA marker

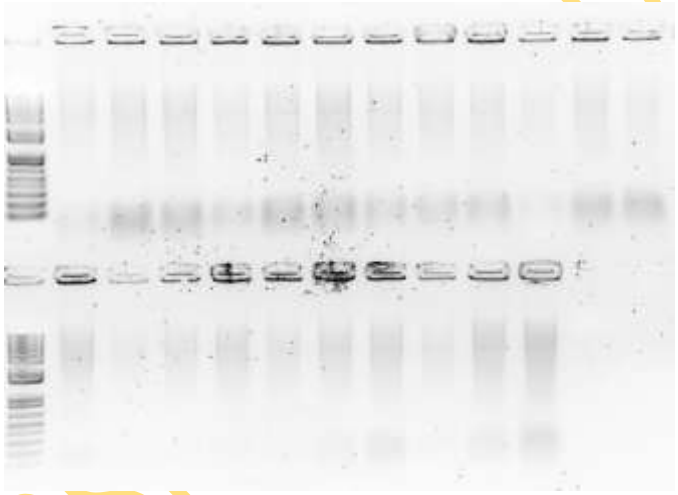


Fig. 4.34: Colony PCR of transformant MX2 yeast colonies

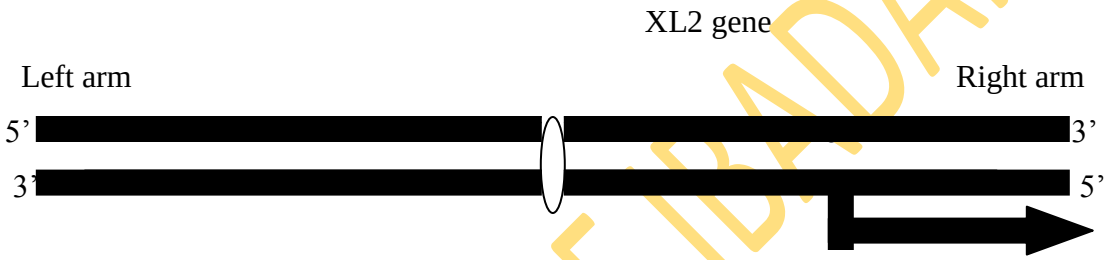


Fig. 4.35 Chromosome 12 of *Saccharomyces cerevisiae* showing the position of Xylitol Dehydrogenase gene (YLR070c)

4.14 Physiological studies of genetically-modified and other selected yeasts

The physiological studies conducted on the selected *Saccharomyces* yeast isolates showed that *Saccharomyces cerevisiae* MX1 and MX2 being genetically modified with XR and XDH gene and the 3 other selected unmodified yeasts revealed an optimum growth temperature of between 30^{o} – 35^{o}C as presented in Figure 4.36. The growth performances of the yeasts were determined at different temperature ranges of 25, 30, 35, 40 and 45^{o}C respectively. Four of the isolates (MX1, MX2, MX4 and MX5) maintained an increase in growth up to 40^{o}C beyond which their growth declined. However, isolate MX3 exhibited an optimum temperature at 30^{o}C beyond which the growth density declined sharply.

Figure 4.37 shows the comparative effects of different carbon sources on the growth of the yeast isolates. The growth density was determined by measuring their spectrophotometer absorbance at 650nm. Glucose, mannitol, raffinose and maltose appeared to be the best carbon sources for the optimum growth of all the yeast isolates. However maximum growth was achieved by MX5 in fructose medium. Xylose and Arabinose sugars supported poor growth in all the unmodified yeast. Comparatively, the genetically modified yeasts (MX1 and MX2) exhibited good growth in these carbon sources with growth density of $1.78x10^{9} \pm 0.089$cells/ml in xylose medium and $2.04\ x10^{9} \pm 0.078$cells/ml in arabinose medium for MX1 and $1.57\ x10^{9} \pm 0.020$cells/ml and $1.91\ x10^{9} \pm 0.024$cells/ml for MX2 respectively at 650nm.

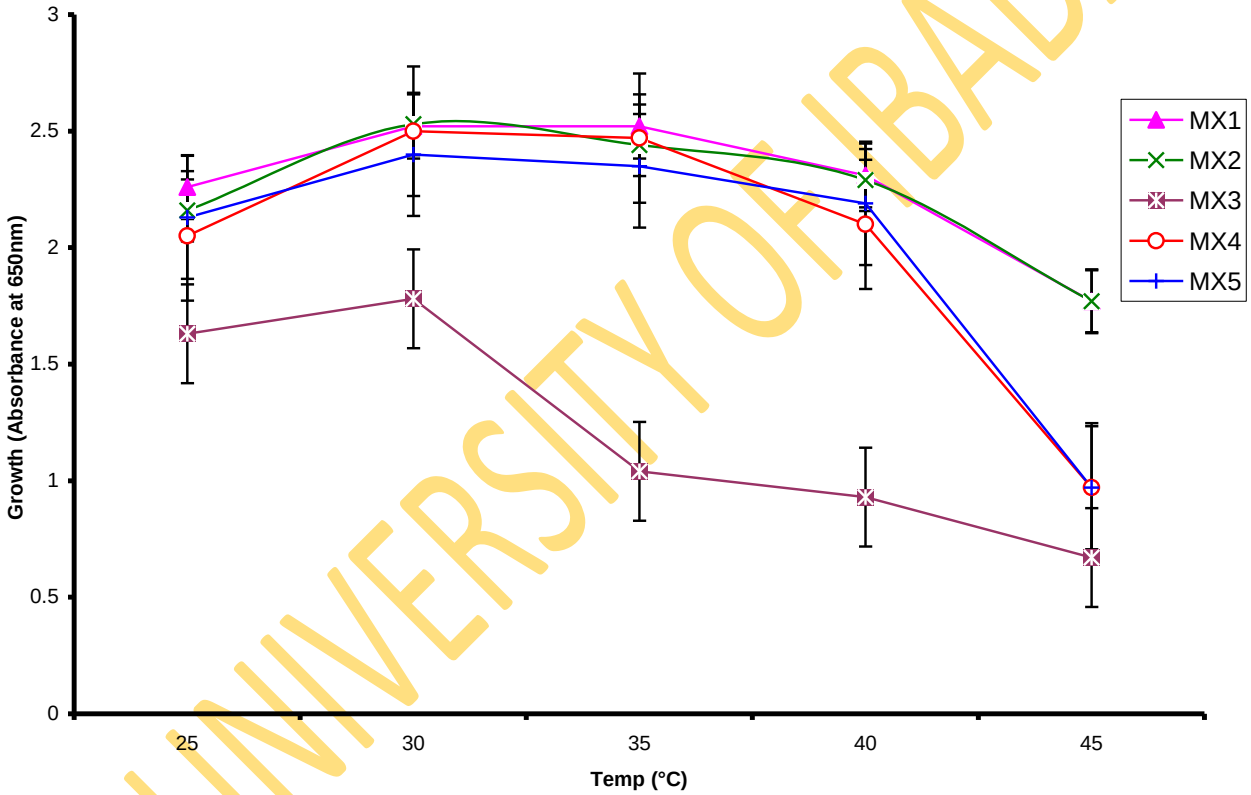


Fig. 4.36 Effect of different temperatures on the growth of selected yeast isolates Bar indicates standard error

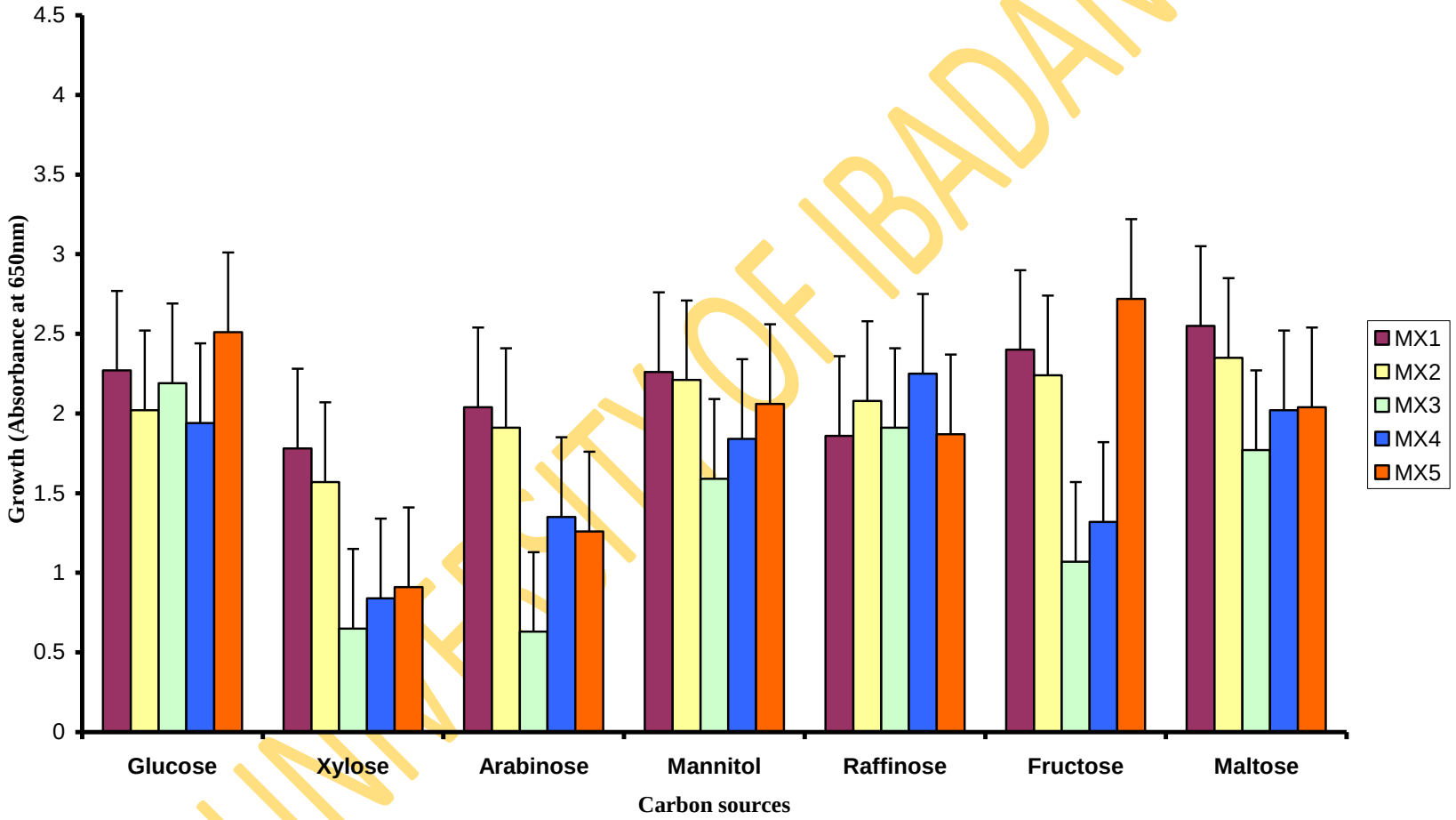


Fig.4.37 Effect of different carbon sources on the growth of selected yeast isolates Bar indicates standard error

The effect of different pH ranges on the growth of the yeast isolates is depicted in Fig.4.38. Optimum growth of all the isolates was observed at pH of 4 – 5.0, only MX3 got to 5.5. However, the MX5 had an optimum pH within 4 – 5, beyond which there was a decline in growth with increase in pH. There was an appreciable growth observed with the genetically modified yeasts, isolate MX1 up to pH 5.5 before slight decline was observed.

The ethanol tolerance, measured as survival and growth showed that the test medium except isolate MX3 as presented in Fig. 4.39. The modified isolates MX1 and MX2 were very tolerant to the ethanol environment and maintained fairly constant growth up to 9% of ethanol concentration before their growth declined slightly. Isolate MX4 displayed similar property and the modified strain isolate MX5 also exhibited tolerance to ethanol up to 8% concentration of ethanol beyond which a decrease in cell density was observed. It was observed generally that the ethanol tolerance of the MX3 yeast was low. The sensitivity of isolate MX3 to ethanol concentration was observed to start at 4% concentration with a sharp decrease in growth up to 6% concentration. It subsisted at this level of growth up to 9% and finally declined at 10% of concentration.

The effect of different concentrations of acetic acid on the growth of the selected yeast isolates is depicted in Fig. 4.40. The result indicates that all the yeast cells were able to withstand the effect of acetic acid at a lower concentration of 0.2%, with the genetically modified strains having the highest growth density. Above this concentration, the yeast cells showed growth inhibition by acetic acid in a dose-dependent manner. However, the XR and XDH – based strains (MX1 and MX2) displayed more tolerance to acetic acid growth medium than the three other test strains.

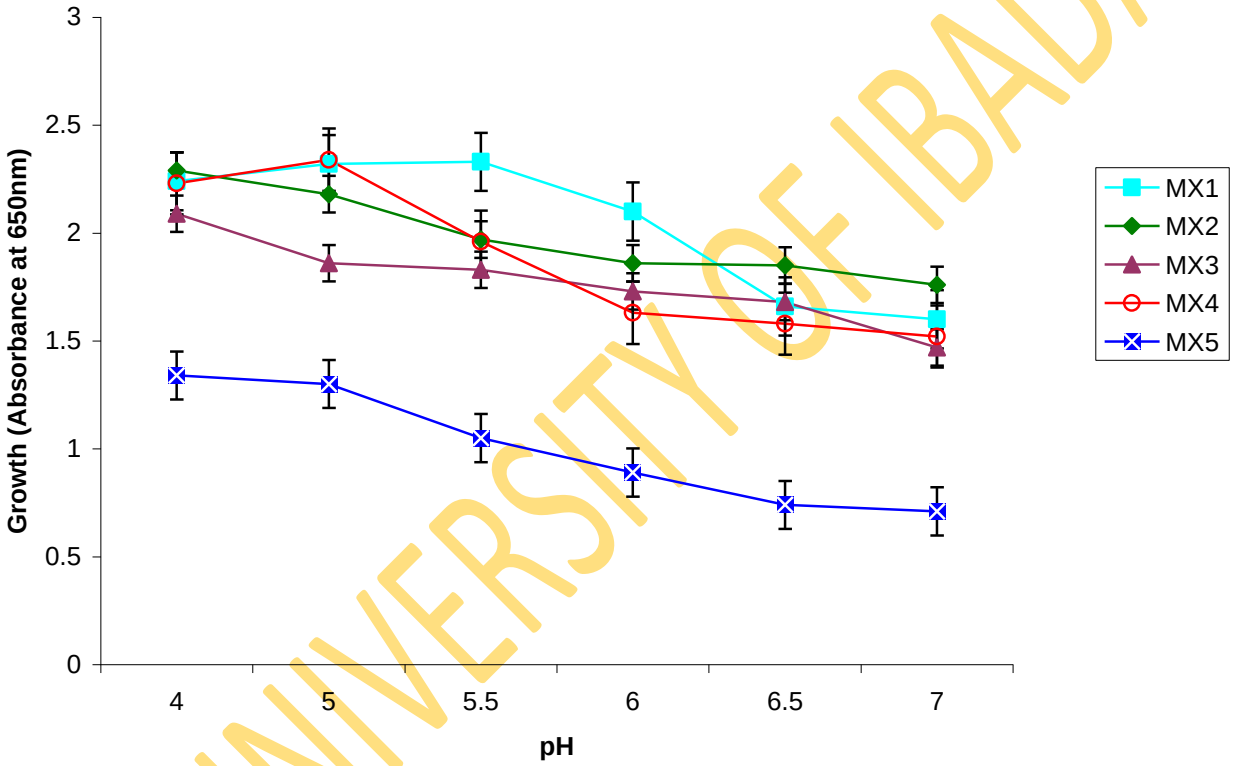


Fig. 4.38 Effect of different pH on the growth of selected yeast isolates Bar indicates standard error

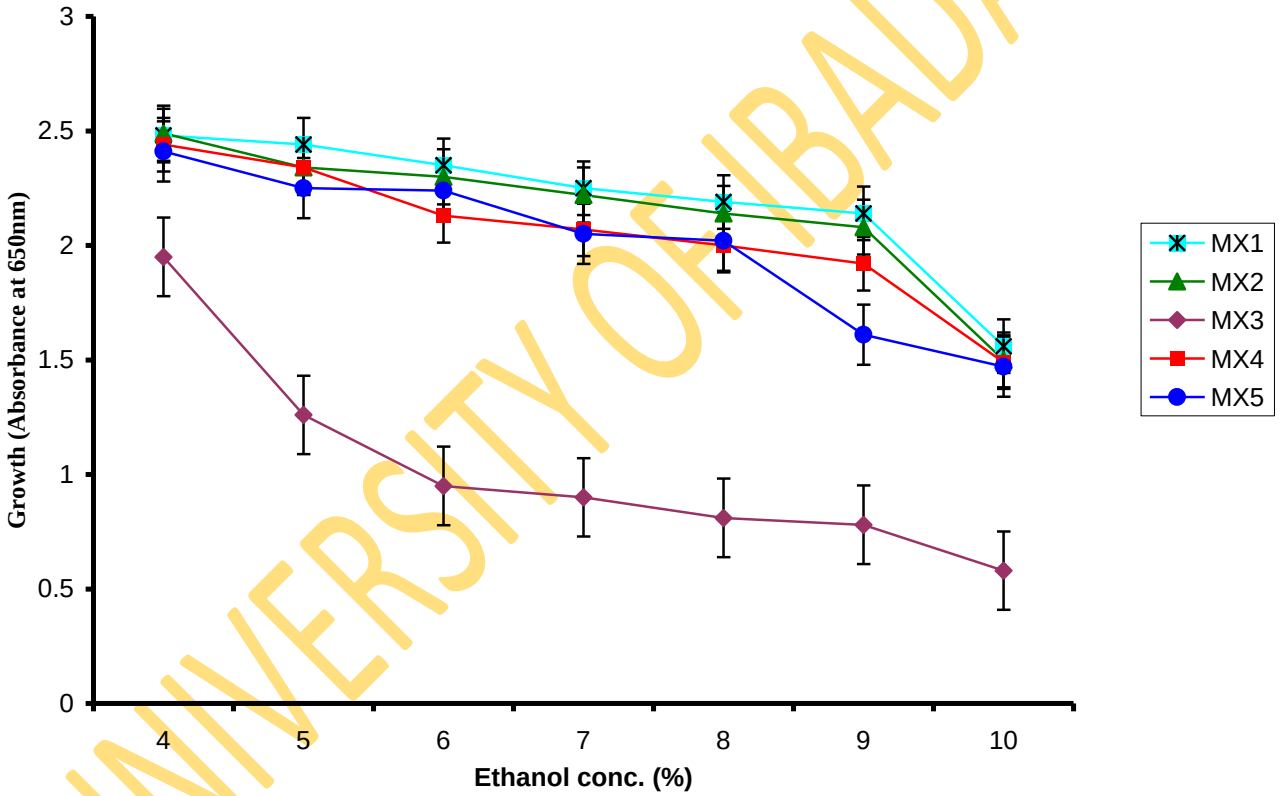


Fig. 4.39 Effect of different ethanol concentration on the growth of selected yeast isolates Bar indicates standard error

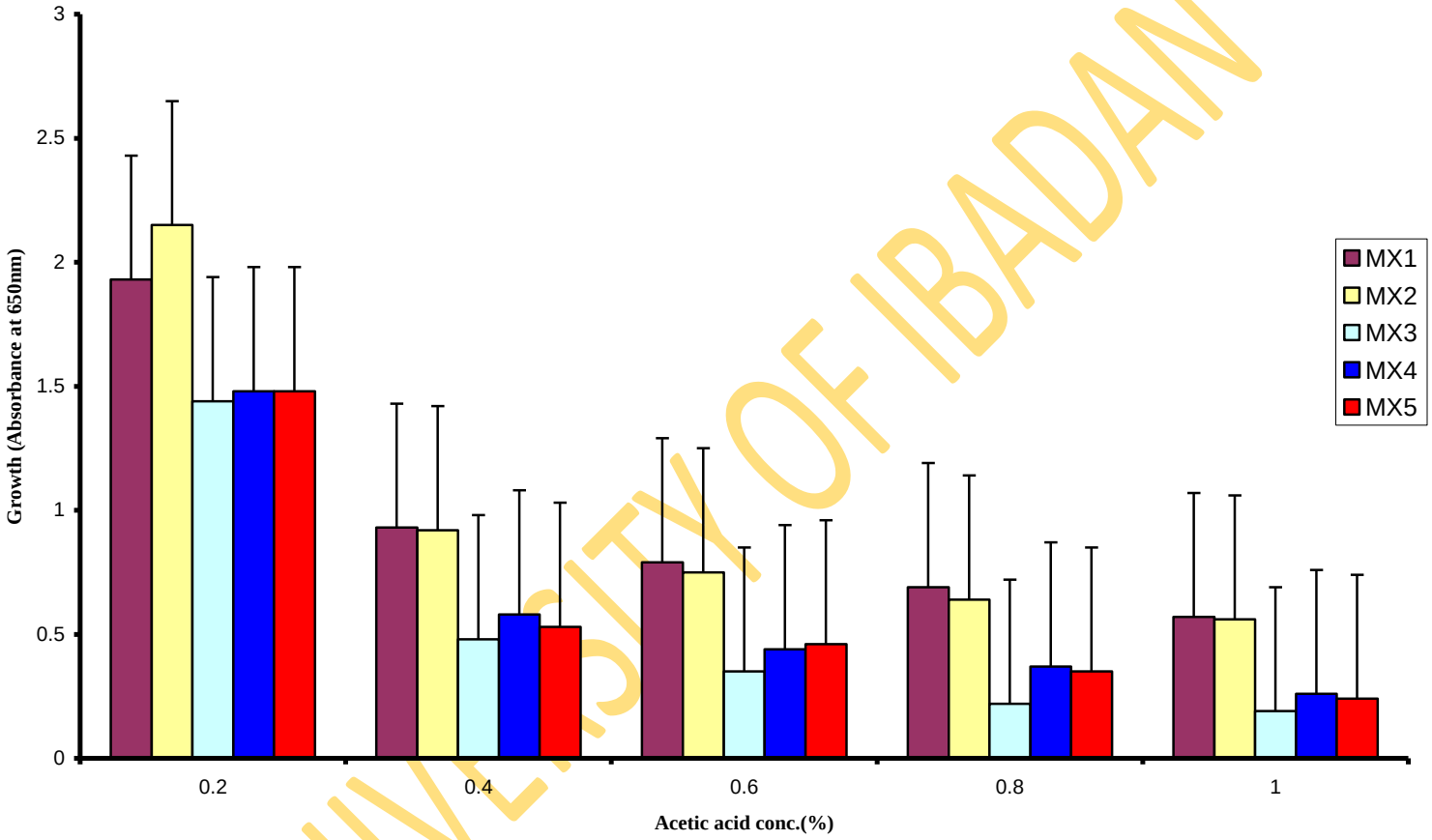


Fig.4.40 Effect of different concentration of Acetic acid on the growth of selected yeast isolates Bar indicates standard error

At 1% concentration of acetic acid the growth densities of the three wild strains (MX3, MX4 and MX5) was observed to 0.1080 ± 0.0112, 0.1546 ± 0.0045 and 0.1446 ± 0.00149 x10^9 cells/ml respectively. The addition of acetic acid caused a marked reduction in the growth densities of the yeast cells as observed, causing the formation of petite mutants as shown by strain MX4 and MX5.

The effect of some Nitrogen sources on the growth of the selected yeasts is presented in Fig.4.41. Ammonium sulphate supported the best growth in all the yeasts tested. Isolate MX3 grew better in this nitrogen source compared to others. Also other nitrogen sources including ammonium hydrogen phosphate, sodium nitrate and ammonium citrate equally supported good growth of the yeasts. However, urea appeared not to be a good nitrogen source to the yeasts as the lowest growth density was observed with this nitrogen source medium.

Figure 4.42 shows the effects of different concentrations of cations (Fe^{2+}) on the growth of the selected yeast isolates. The growth of all the yeasts was enhanced from 0.01 – 0.15 molar concentration, with a very slight decrease in growth densities at a concentration of 0.2M. The modified yeast strains (MX1 and MX2) had the best growth in all the molar concentrations tested.

The comparative effect of different concentrations of anions (NO_3^-) on the growth densities of the yeast strains is shown in Figure 4.43. Predominant growth of yeast strain MX5 was observed in all the concentrations tested. All other yeasts equally exhibited good growth in all the concentrations tested, except isolate MX3 which showed a poor growth density at 0.1M concentration. The growth increase dramatically at 0.05M concentration, and stabilized at 0.1M concentration. It then decreased at 0.15M and later increases at 0.2 M concentration.

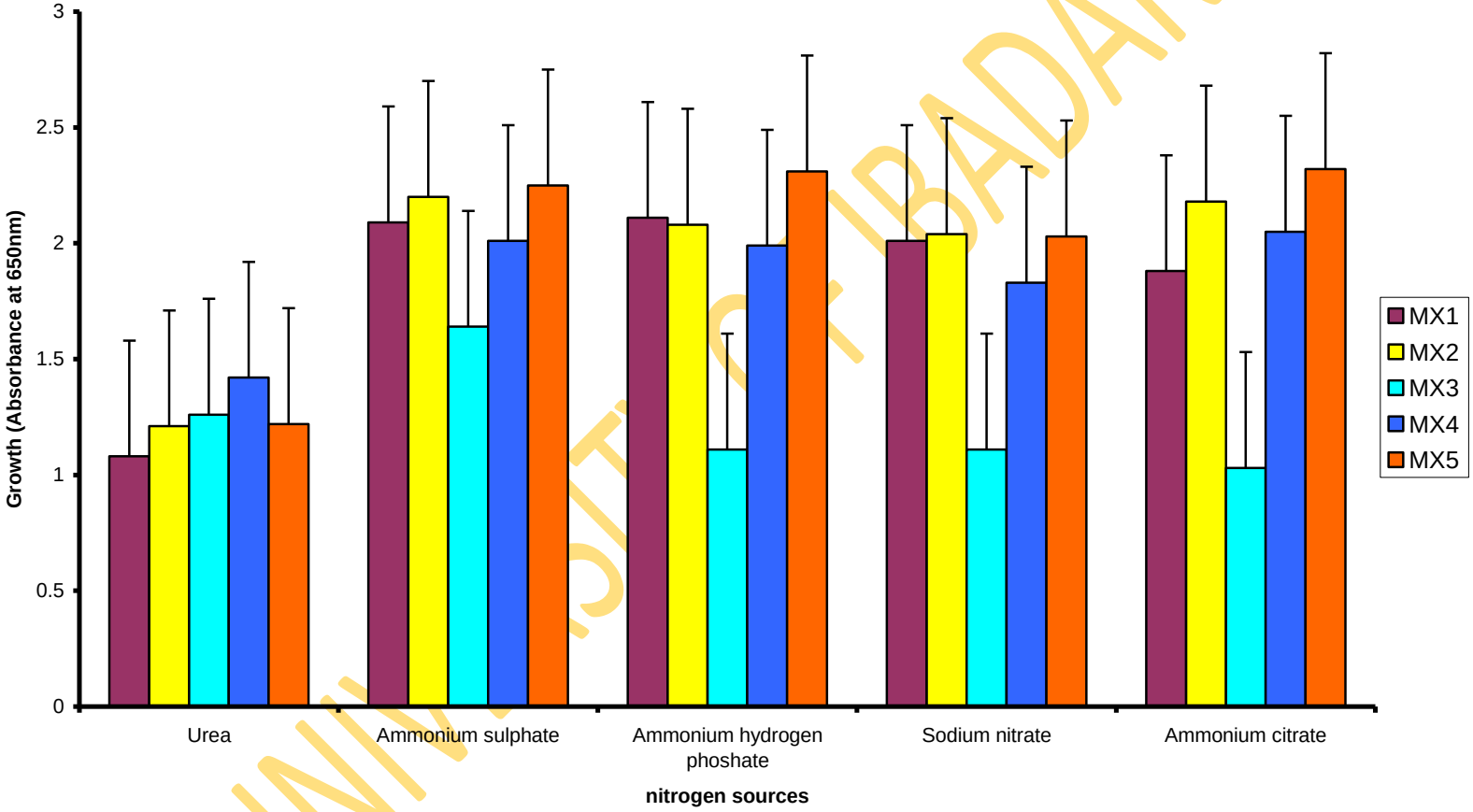


Fig.4.41 Effect of different nitrogen sources on the growth of selected yeast isolates

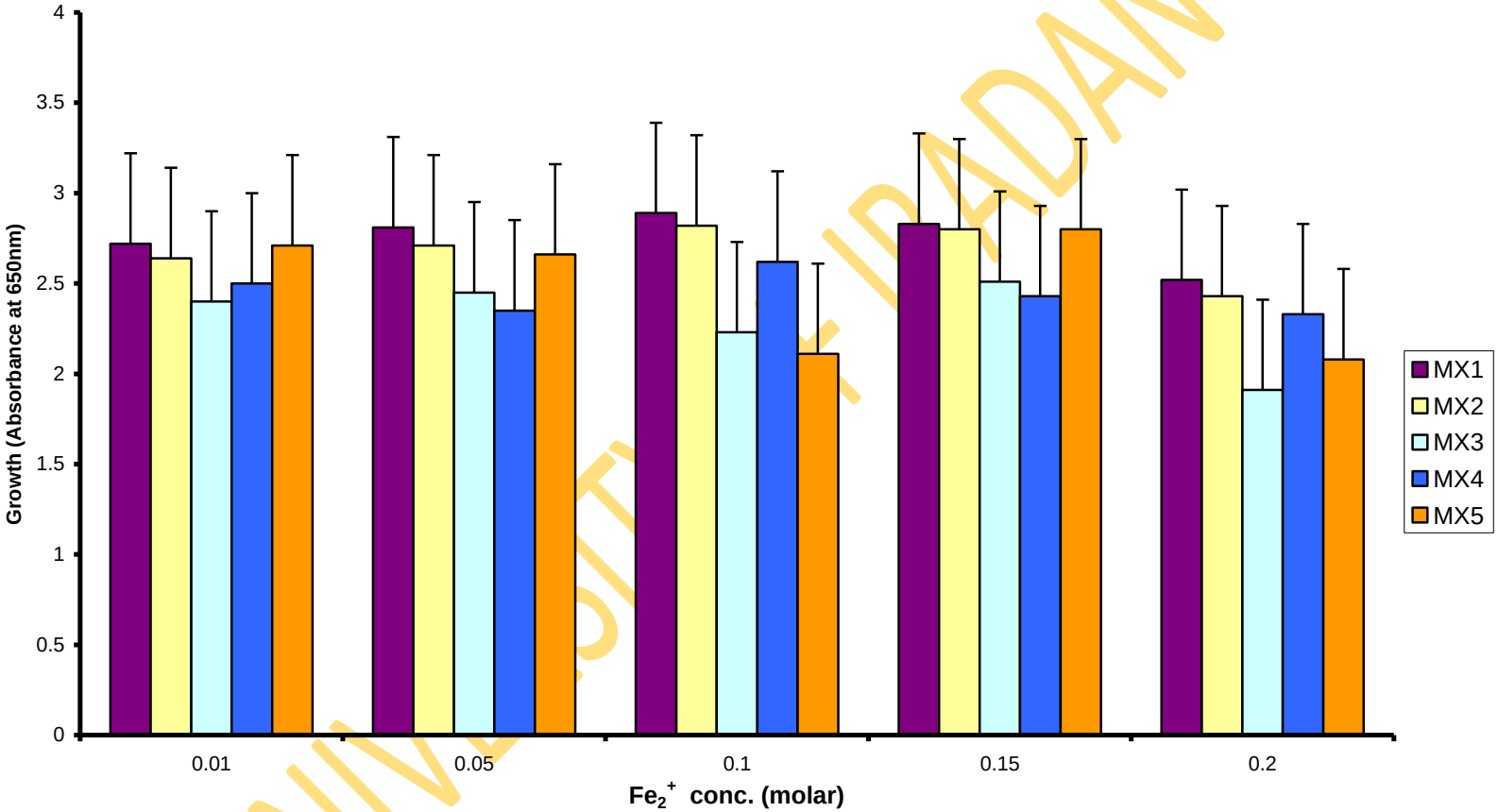


Fig. 4.42 Effect of different concentration of cation on the growth of selected yeast isolates Bar indicates standard error

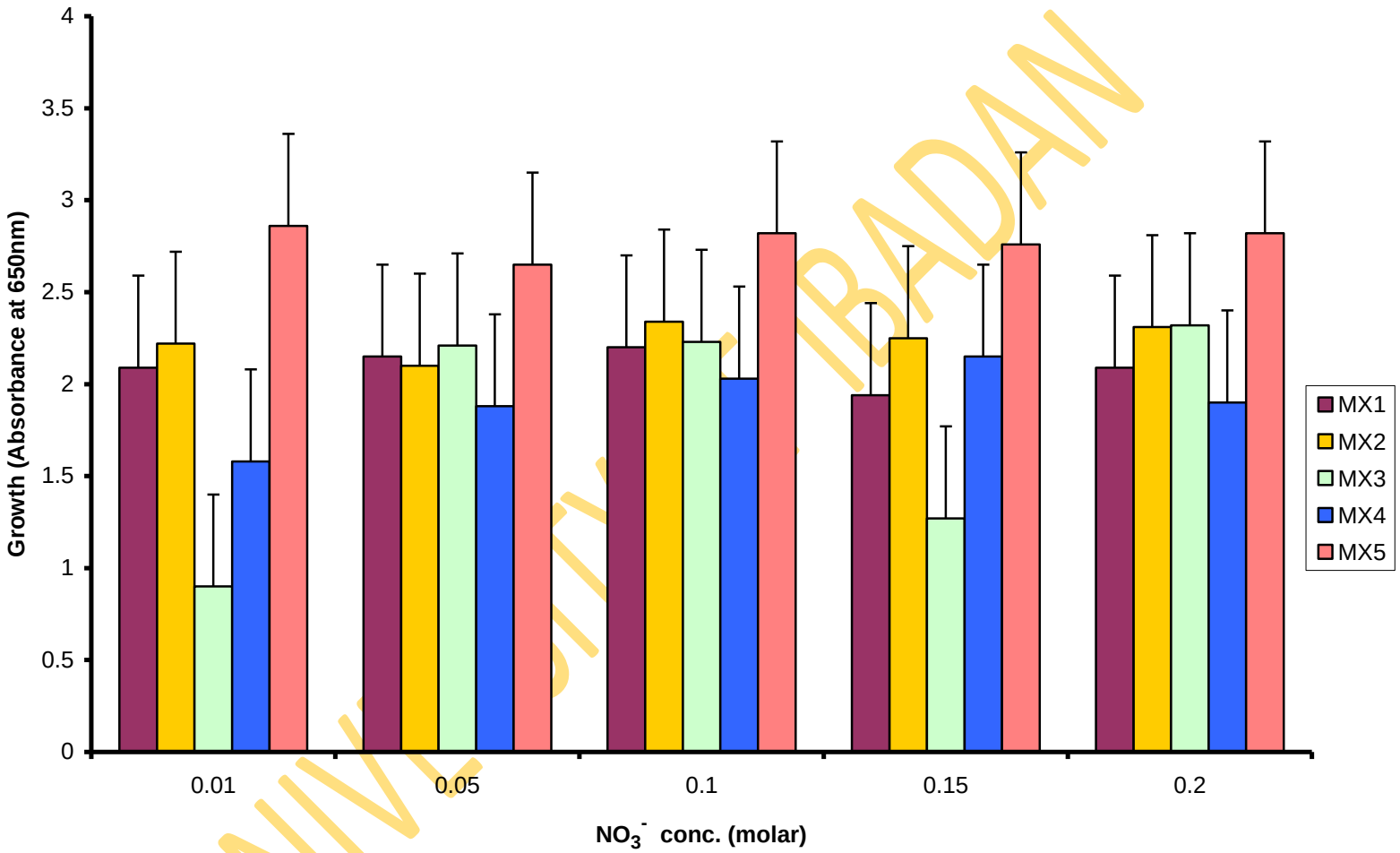


Fig. 4.43 Effect of different conc. of anions on the growth of selected yeast isolates
Bar indicates standard error

4.15 Proximate analysis, enzymes production and hydrolysis of CPH

The proximate composition analysis of CPH reveals that the substrate is heterogeneous in composition as shown in Table 4.6. It has a lignin content of 14.19%, cellulose 18.55%, hemicellulose content of 13.83% amongst others.

The cellulolytic ability of cellulase enzyme produced by *Trichoderma hamatum* using CPH and carboxylmethyl cellulose (CMC) as carbon sources and their cellulolytic activities is shown in Fig.4.44a CMC gave the highest cellulase enzyme production after incubation for 7 days in the medium giving the highest cellulolytic activity of 0.608 unit/ml than CPH which produces 0.542 unit/ml.

Fig. 4.44b presents the result of experiments to compare the production of xylanase enzyme by *T. hamatum* in two carbon sources and the activities of the enzymes produced. After seven days of incubation, xylan was observed to be more suitable substrate for the generation of xylanase producing enzyme activity of 0.819 unit/ml compared to CPH – medium which produce 0.566 unit/ml of xylanase activity.

The effect of different enzyme concentrations on the cellulolytic activity of *T. hamatum* is represented in Fig. 4.45. It was observed that increase in enzyme concentration leads to corresponding increase in enzyme activity.

Table 4.6 Proximate composition of Cocoa pod husk

Substrate Content	Values (%)
Moisture	16.4
Ash	15.9
Protein	2.5
Ether extract	2.1
Crude fibre	49.3
Carbohydrate	13.8
Total soluble solid	38.0
Calcium (mg/100g)	195
Iron (mg/100g)	8.5
Sodium (mg/100g)	225
Phosphate (mg/100g)	160
Acid detergent fibre (ADF)	32.74
Neutral detergent fibre (NDF)	46.57
Lignin	14.19
Cellulose	18.55
Hemicellulose	13.83

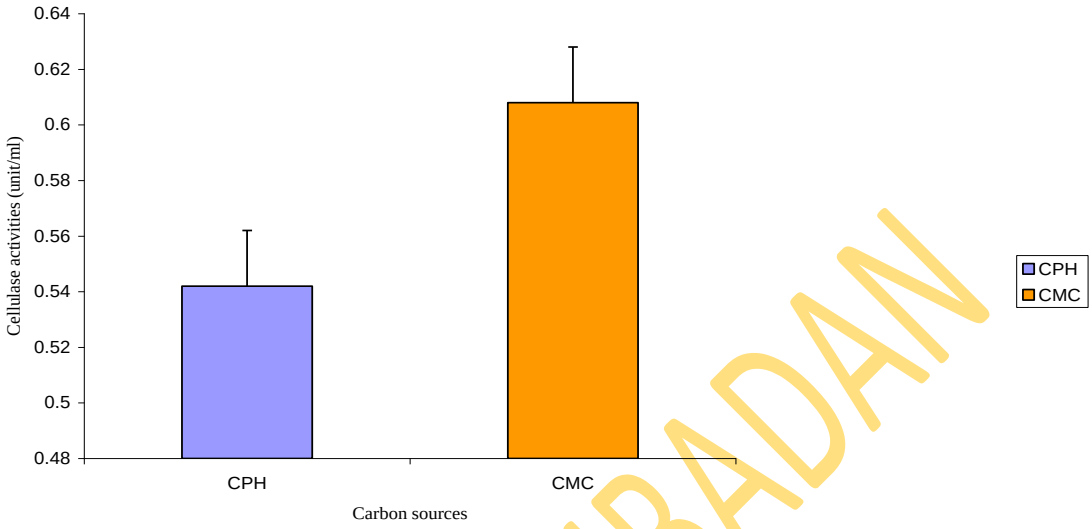


Fig. 4.44a Cellulolytic activities of *T. hamatum* in two carbon sources
Bar indicates standard error

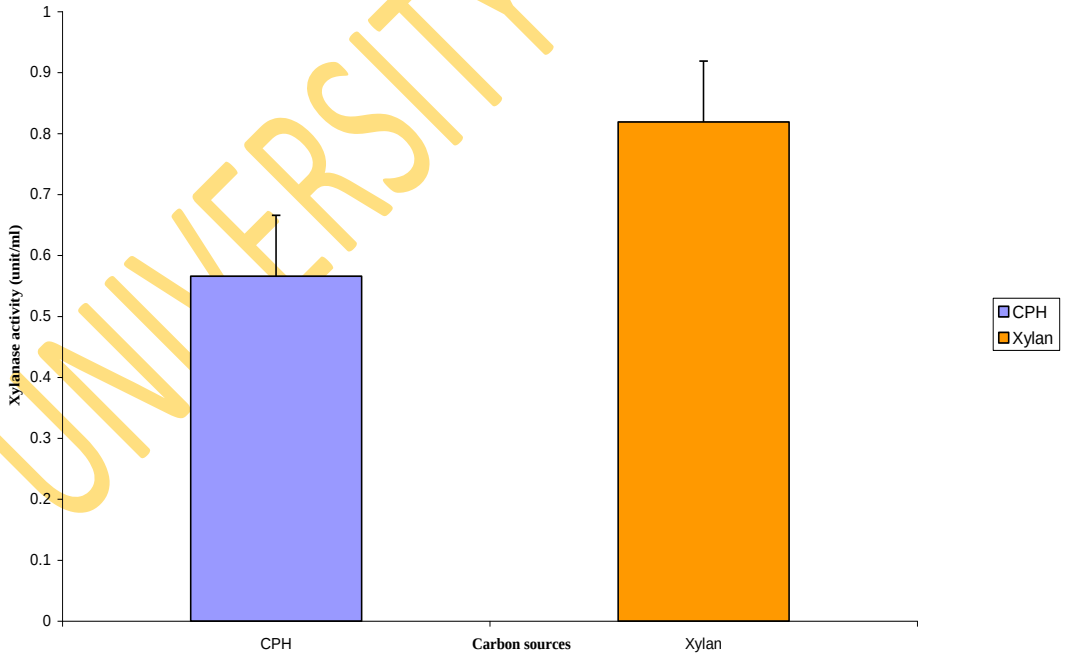


Fig. 4.44b Xylanase activity of *T. hamatum* in different carbon sources
Bar indicates standard error

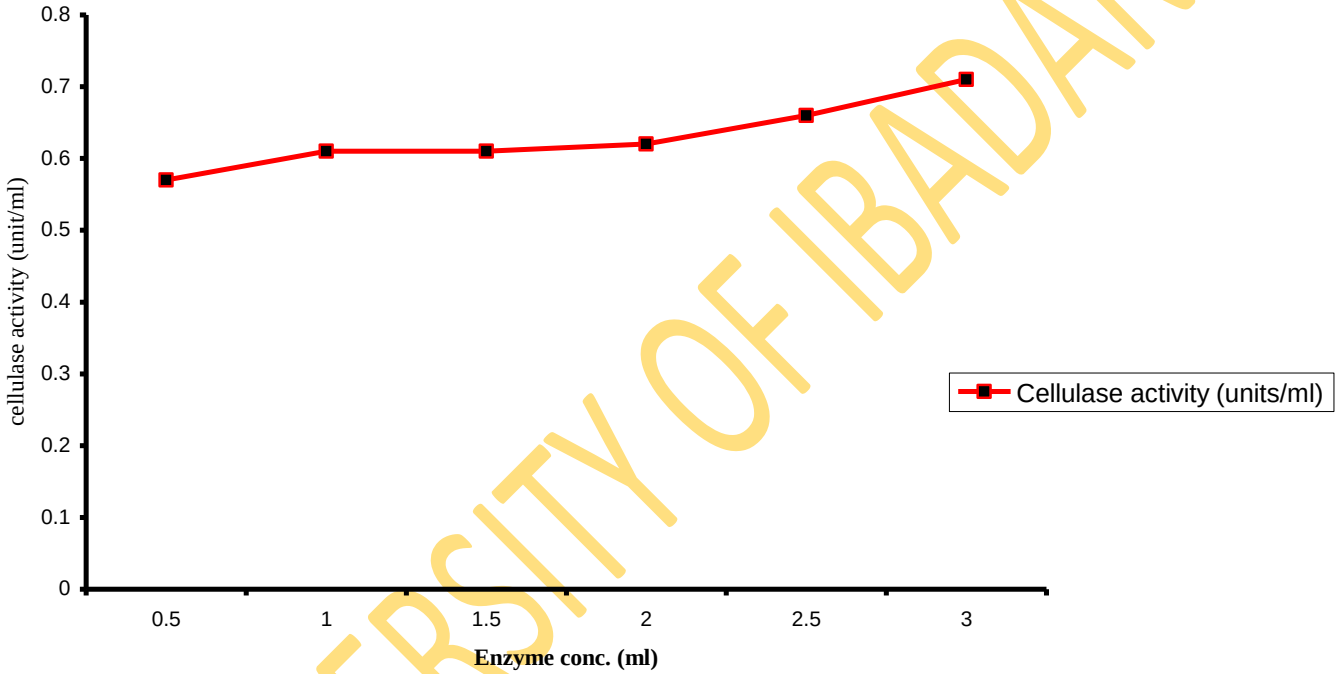


Fig. 4.45 Effect of different enzyme concentration on the cellulase activity of *Trichoderma hamatum*

Figure 4.46 shows the effect of substrate (CMC) at different concentrations on the cellulolytic activities of *T. hamatum.* From the results obtained, it was observed that the reaction velocity increases gradually with increase in substrate concentration until it reached a maximum at substrate concentration of 1%. It stabilized at 1.5%, beyond which a gradual decrease in activity of the enzyme was noticed. At concentration of 2.5% enzyme activity had reduced to 0.55 unit/ml.

The effect of different xylanase enzyme concentrations on xylanase activity of *Trichoderma hamatum* is depicted in Fig. 4.47. It was observed that xylanase activity increases with increase in enzyme concentration. Maximum activity was achieved at 1.5ml of enzyme concentration beyond which there was a decrease and later increases again.

Figure 4.48 represents the effect of different substrate concentrations (xylan) on xylanase activity of *T. hamatum.* The reaction velocity of the enzyme increases gradually from 0.5% of substrate concentration to a peak at 1.0% substrate concentration, it stabilizes at that level up to 2.0% and a gradual decrease was noted from 2.5% to 3.0% substrate concentration.

4.16 Sugar analysis with HPLC

Figure 4.49 presents the data and absorption spectra of HPLC analysis of sugars derivatives in pre-treated Acid hydrolysed CPH hydrolysate. Different concentrations of dilution factor (df) were used in order to establish the best dilution factor for acid hydrolysis of the pretreated CPH. The absorption spectra of the HPLC with standard sugar of 0.2% and df of 10 reveals that 50.10% of glucose was produced and eluted at a retention time of 1.86min with peak area of 105.96, Xylose sugar gave 11.97% and it was eluted at a retention time of 2.29 mins with a peak area of 173.56, while 11.18% of mannose was also obtained at a retention time of 2,45min with a peak area of 145.37 respectively. Galactose was not detected. The least sugar obtained was fructose; this was in trace amount of 0.002%, eluted at a retention time of 4.15mins respectively.

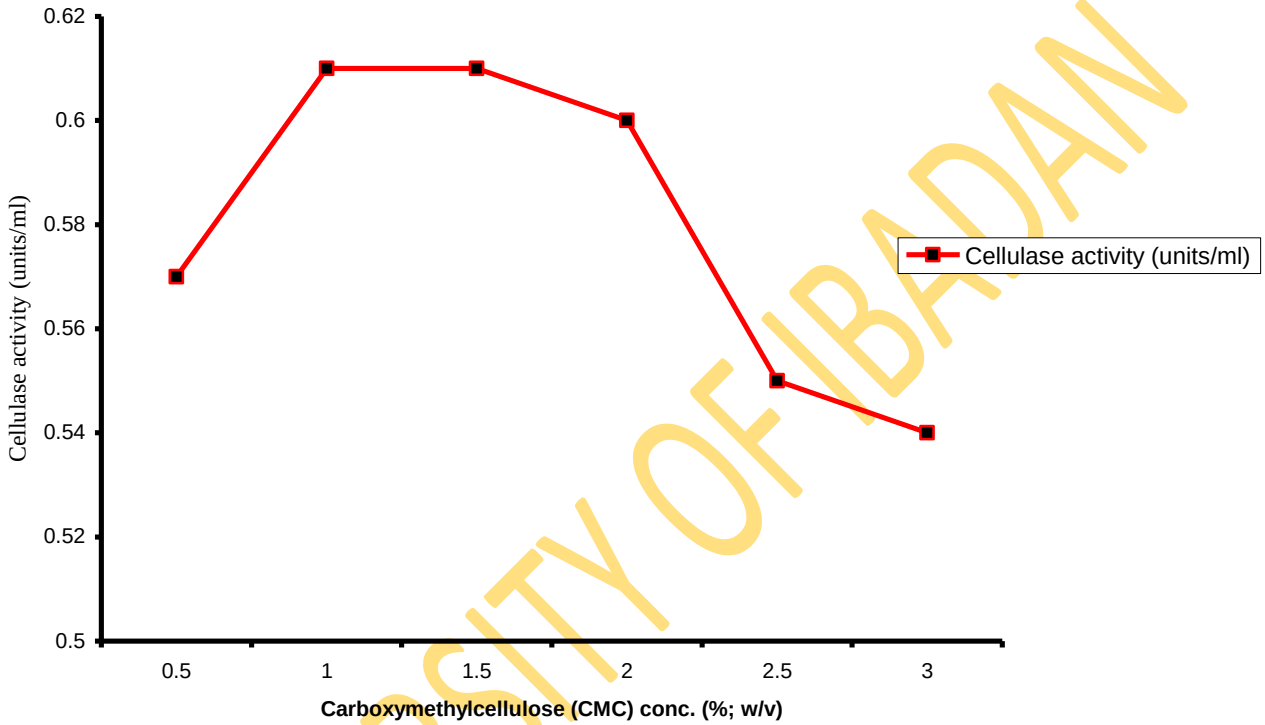


Fig.4.46 Effect of substrate concentration on cellulase activity of *T. hamatum*

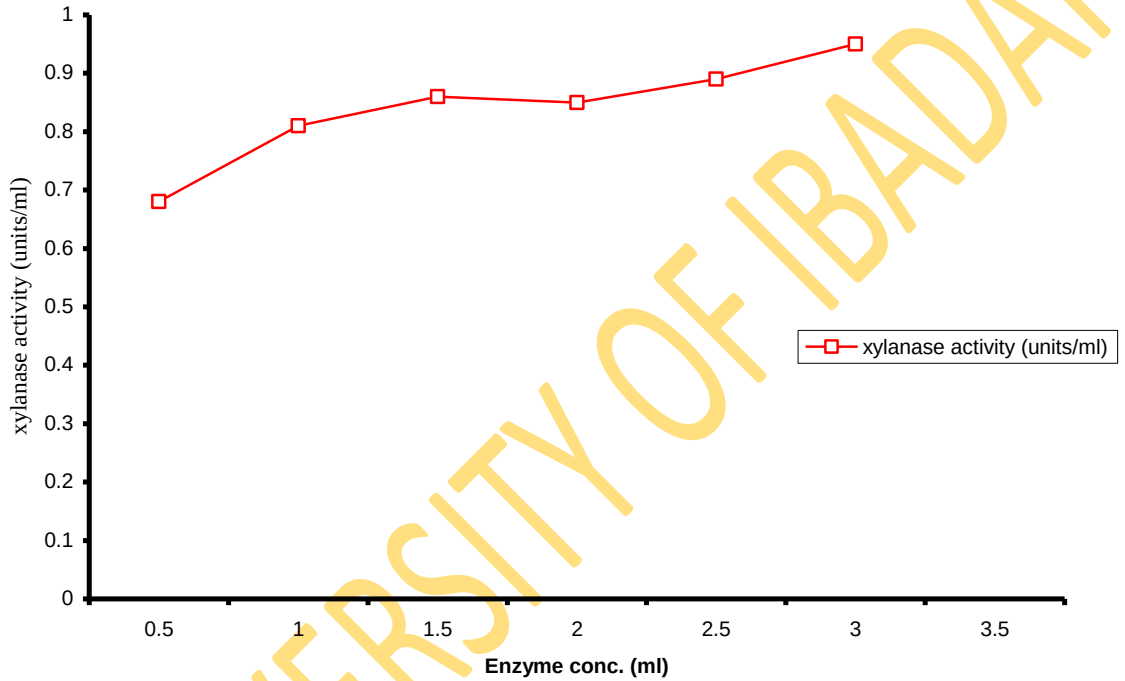


Fig. 4.47 Effect of enzyme concentrations on xylanase activity of *Trichoderma hamatum*

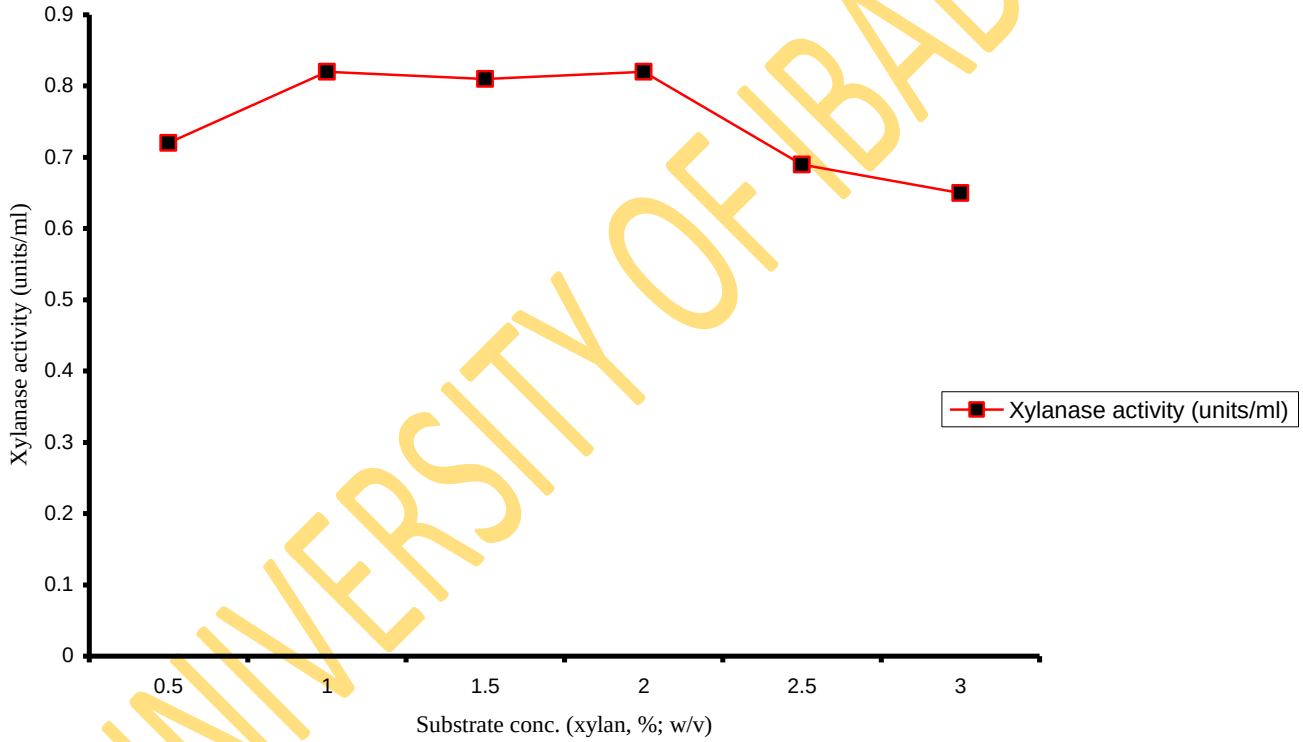


Fig. 4.48 Effect of substrate conc. on xylanase activity of *Trichoderma hamatum*

Fig. 4.49: HPLC Analysis of sugar derivatives in pretreated acid hydrolysed CPH hydrolysate

The HPLC absorption spectrum of sugar derivatives in pretreated, enzymatic hydrolysed CPH hydrolysate is presented in Fig. 4.50. HPLC spectrum with standard sugar of 1% and dilution factor of 6 reveals that 31.68% of glucose was produced by the enzyme at a retention time of 1.193 minutes with a peak area of 3332.19, galactose was produced in 16.70% within a retention time of 1.288 minutes giving a peak area of 2055.48. Small quantity of mannose sugar (4.79%) was detected at 1.658 minutes with a peak area of 5107.78. Fructose sugar was produced in trace amount of 0.33% with a retention time of 1.346 minutes.

The HPLC analysis of sugars derivatives in pretreated enzymatic hydrolysis of CPH hydrolysate without Tween 80 is presented in Fig. 4.51, with a standard sugar concentration of 1% and dilution factor of 6, the HPLC analytes reveals glucose sugar concentration of 28.50% produced with a retention time of 1.18 minutes and a peak area of 2997.48. Mannose sugar was the next in proportion of 4.42% and it was produced within a retention time of 1.67 minutes with a peak area of 4712.68. Fructose sugar was detected in trace amount of 0.43% with a retention time of 1.27 minutes.

Fig. 4.50 HPLC Absorption spectrum of sugar derivatives in pretreated Tween-80 Enzymatic hydrolysed CPH hydrolysate

Fig. 4.51 HPLC Analysis of sugar derivatives in pretreated enzymatic hydrolysed CPH hydrolysate

4.17 Fermentation of enzymatic CPH hydrolysate

The time course of sugar utilization by the different yeast isolates in enzymatic CPH hydrolysate is represented in Fig. 4.52. From the trend of sugar depletion, it was observed that the modified yeasts E1 (MX1 and MX2) depleted the sugar in the medium faster than the other isolates due to the XR and XDH genes in their genomes. At 96 hour of fermentation the amount of sugar left in the E1 medium was 7 ± 0.57g/l while the other isolates recorded the following; 9 ± 0.99g/l for isolate E2, sugar concentration of 9 ± 0.42g/l for isolate E3 and 9 ± 0.28g/l for isolate E4 respectively. It was however observed that some residual sugar of 3.0g/l of sugar was left in all the fermentation media of the different wild yeast isolate, except the modified yeast medium (E1) which gave 2.0 ± 0.28g/l of residual sugar after fermentation for 120 hours.

The corresponding amount of ethanol that was produced during fermentation in the enzymatic hydrolysed medium by the different yeast isolates is shown in Fig.4.53.While Table 4.7. a, b, c and d present the summary of fermentation performance of the different yeasts in the enzymatic hydrolysed CPH hydrolysate. It was observed that at 48 hour of fermentation, the modified yeast E1 produced ethanol at a concentration of 8.0 ± 0.28g/L with a volumetric productivity of 0.167g of ethanol/hour. Yeast isolate E2 produced 6.0 ± 0.42g/L, E3 produced 6.0 ± 0.28g/l while E4 gave 7.0 ± 0.42g/l of ethanol concentrations respectively. The maximum ethanol concentration was reached after 120 hours of fermentation with the modified yeast E1 producing the highest amount of ethanol at a concentration of 32.0 ± 0.42g/l, with yield of 0.711g/g and volumetric productivity of 0.27g of ethanol/1 hour. The other three yeasts produced virtually the same amount of ethanol at 120 hours of fermentation with E2 strain producing 27 ± 0.99g/l of ethanol with a productivity of 0.23g of ethanol/hour while E3 and E4 produced 26 ± 0.14g/l and 26 ± 0.42g/l of ethanol concentration respectively.

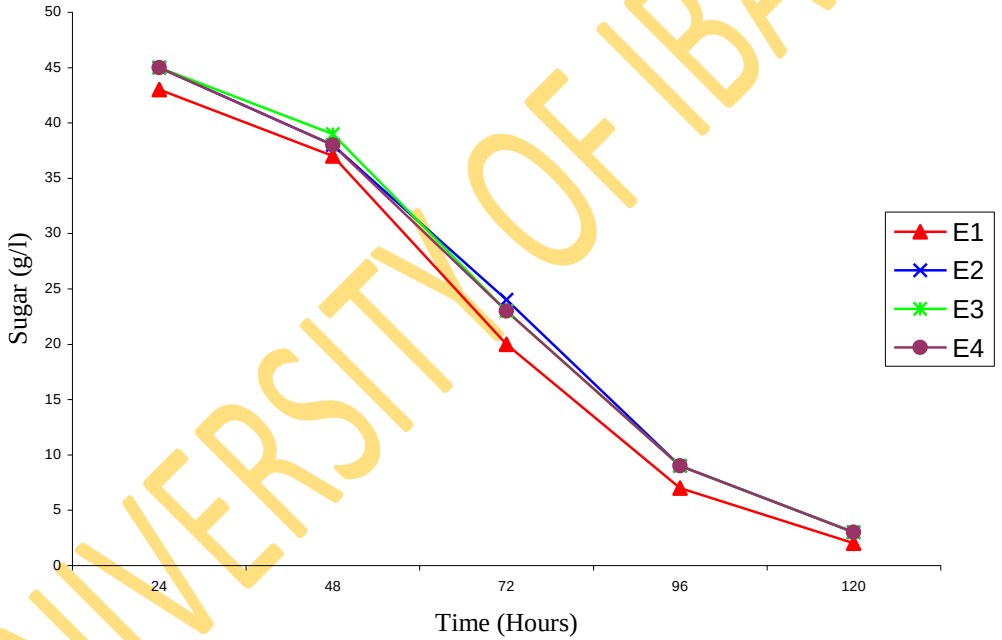


Fig.4.52 Sugar utilization during fermentation of CPH enzymatic hydrolysate by selected yeast isolates

E1 = genetically modified *S. cerevisiae* yeasts; E2 = *S. uvarum* ; E3 = *S. cerevisiae;* E4 = *S. cerevisiae*

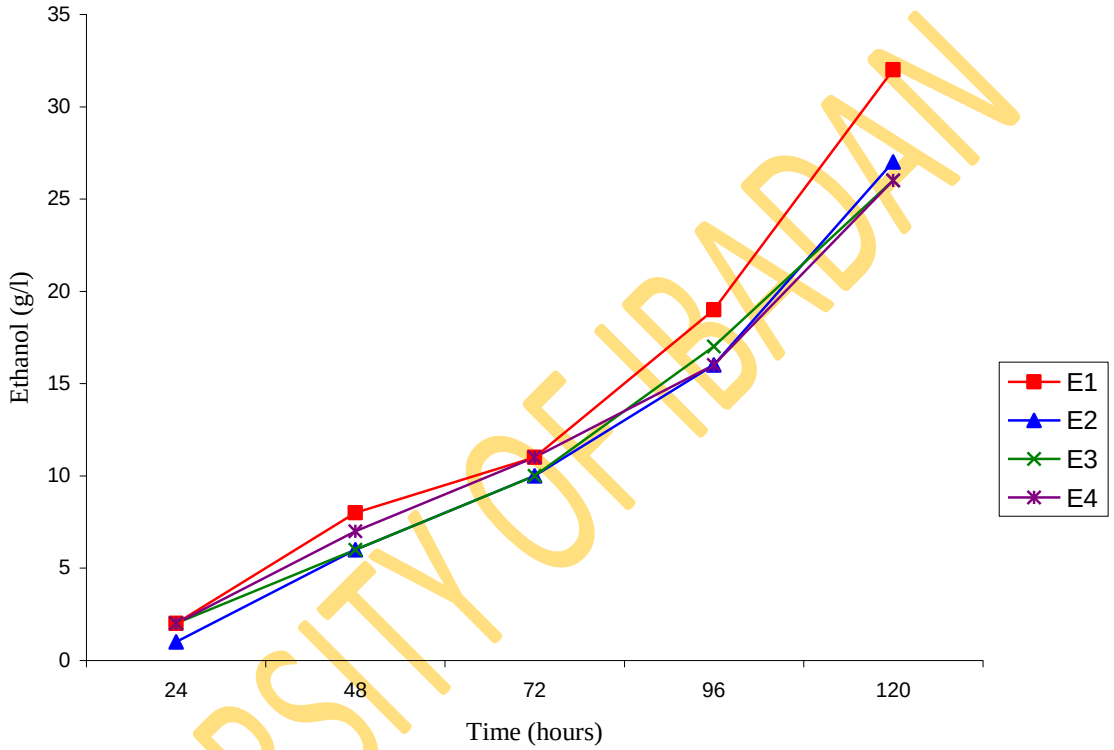


Fig.4.53 Ethanol production from fermentation of CPH Enzymatic hydrolysate by selected yeasts

E1 = genetically modified S. cerevisiae yeasts; E2 = S. uvarum; E3 = S. cerevisiae; E3 = S. cerevisiae

Table 4.7 a: Summary of fermentation performance of Genetically-modified *S. cerevisiae* yeasts E1 (MX1-XR and MX2-XDH) in Enzyme hydrolysed CPH hydrolysate medium

Sugar conc. (g/l)	Max. ethanol conc. (g/l)	Time (hour)	Ethanol yield (g/g)	Vol. productivity (g of ethanol/hr)
45	2	24	0.044	0.083
38	8	48	0.178	0.167
22	11	72	0.244	0.153
8	19	96	0.422	0.198
3	32	120	0.711	0.267

Table 4.7b : Summary of fermentation performance of *S. uvarum* yeast (strain E2) in Enzyme hydrolysed CPH hydrolysate medium

Sugar conc. (g/l)	Max. ethanol conc. (g/l)	Time (hour)	Ethanol yield (g/g)	Vol. productivity (g of ethanol/hr)
45	1	24	0.022	0.042
38	6	48	0.133	0.125
24	10	72	0.222	0.139
9	16	96	0.356	0.167
3	27	120	0.600	0.225

Table 4.7 :c Summary of fermentation performance of *S.cerevisiae* yeast (strain E3) in Enzyme hydrolysed CPH hydrolysate medium

Sugar conc. (g/l)	Max. ethanol conc. (g/l)	Time (hour)	Ethanol yield (g/g)	Vol. productivity (g of ethanol/hr)
45	2	24	0.044	0.083
39	6	48	0.133	0.125
23	10	72	0.222	0.139
9	17	96	0.378	0.178
3	26	120	0.578	0.217

Table 4.7 d: Summary of fermentation performance of *S.cerevisiae* yeast (strain E4) in Enzyme hydrolysed CPH hydrolysate medium

Sugar conc. (g/l)	Max. ethanol conc. (g/l)	Time (hour)	Ethanol yield (g/g)	Vol. productivity (g of ethanol/hr)
45	2	24	0.044	0.083
39	7	48	0.156	0.146
23	11	72	0.244	0.153
9	16	96	0.356	0.167
3	26	120	0.578	0.220

4.18 Fermentation of Acid hydrolysed CPH hydrolysate

Figure 4.54 Summarize the time course of sugar utilization by the yeast isolates in acid hydrolysed CPH medium. After 48 hours of inoculation, the initial sugar concentration of 45.0 g/l dropped to 25 ± 0.57g/l in the modified yeasts (E1) fermentation medium, 24.0 ± 0.42g/l of sugar was left in isolate E2 medium, isolate E3 medium gave 25.0 ± 0.28g/l while E4 fermentation medium gave sugar concentration of 23 ± 0.42g/l respectively. At 120hours of fermentation, the amount of residual sugar left for all the isolate dropped to 1.0g/l.

The amount of corresponding ethanol that was produced by the different yeast isolates in the acid hydrolysed fermentation medium is represented in Fg.4.55, while Table 4.8 a,b,c and d present the summary of fermentation performance of the different yeasts in acid hydrolysed CPH hydrolysate medium. After 24 hours of fermentation, the modified yeasts (E1) produced 2.0 ± 0.42g/l of ethanol concentration, ethanol yield of 0.044g/g with a productivity of 0.083g ethanol/ hour.

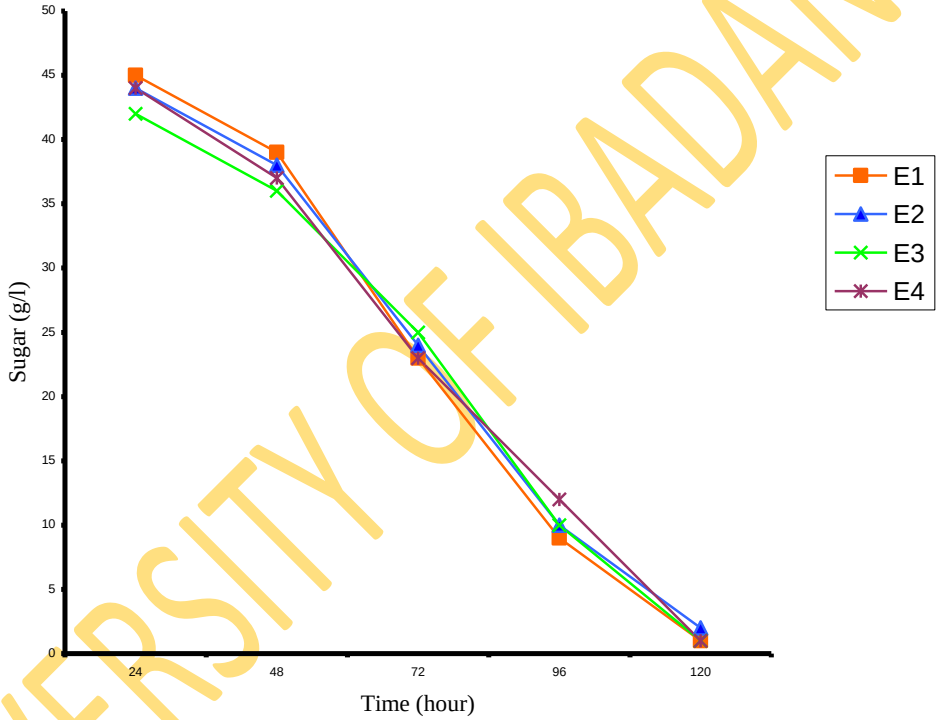


Fig. 4.54 Sugar utilization during fermentationof Acid hydrolysed CPH hydrolysate by selected yeast isolates

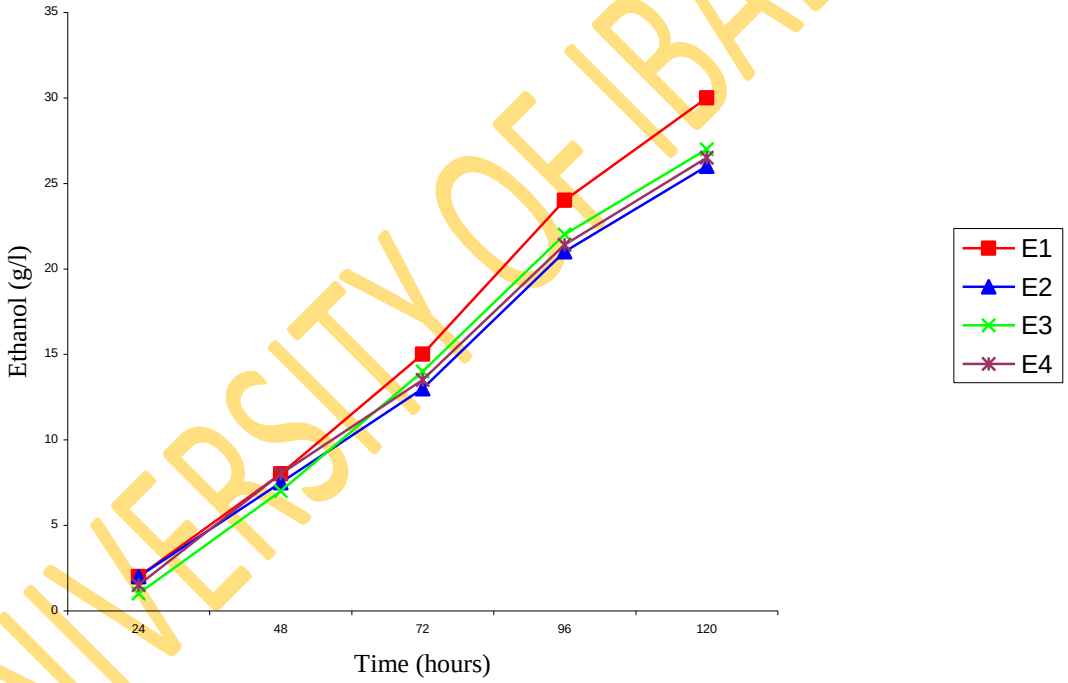


Fig. 4.55 Ethanol production during fermentation of acid hydrolysed CPH hydrolysate by selected yeast isolates

Table 4.8a: Summary of fermentation performance of modified *S. cerevisiae* yeast (strains E1) in Acid hydrolysed CPH hydrolysate medium

Sugar conc. (g/l)	Max. ethanol conc. (g/l)	Time (hour)	Ethanol yield (g/g)	Vol. productivity (g of ethanol/hr)
45	2	24	0.044	0.083
39	8	48	0.178	0.167
23	15	72	0.333	0.208
9	24	96	0.533	0.25
1	30	120	0.667	0.25

Table 4.8 b: Summary of fermentation performance of *S. uvarum* yeast (strain E2) in Acid hydrolysed CPH hydrolysate medium

Sugar conc. (g/l)	Max. ethanol conc. (g/l)	Time (hour)	Ethanol yield (g/g)	Vol. productivity (g of ethanol/hr)
45	2	24	0.044	0.083
38	7	48	0.156	0.146
24	13	72	0.289	0.181
10	21	96	0.467	0.219
1	27	120	0.600	0.225

Table 4.8 c: Summary of fermentation performance of *S. cerevisiae* yeast (strain E3) in Acid hydrolysed CPH hydrolysate medium

Sugar conc. (g/l)	Max. ethanol conc. (g/l)	Time (hour)	Ethanol yield (g/g)	Vol. productivity (g of ethanol/hr)
45	2	24	0.044	0.083
36	7	48	0.156	0.146
25	14	72	0.311	0.194
10	22	96	0.489	0.229
1	27	120	0.600	0.225

Table 4.8d: Summary of fermentation performance of *S. cerevisiae* yeast (strain E4) in Acid hydrolysed CPH hydrolysate medium

Sugar conc. (g/l)	Max. ethanol conc. (g/l)	Time (hour)	Ethanol yield (g/g)	Vol. productivity (g of ethanol/hr)
45	2	24	0.044	0.083
37	8	48	0.178	0.167
23	14	72	0.311	0.194
12	21	96	0.467	0.219
1	26	120	0.578	0.217

The other yeasts, E2, E3 and E4 produced ethanol at a concentration of 2.0 ± 0.28g/l, 1.0 ± 0.28g/l and 1.5 ± 0.57g/l respectively at 24 hours of fermentation. However, at 96 hours of fermentation the modified yeast (E1) produced 24 ± 0.42g/l of ethanol in the acid hydrolysate medium with ethanol yield of 0.533g/g and a volumetric productivity of 0.25g of ethanol/hour. This value was higher than the ethanol concentration yield and productivity of the other yeast isolates at same time of fermentation.

The maximum ethanol concentration was produced in 120 hours of fermentation by the modified strains (E1), producing the highest ethanol concentration of 30.0 ± 0.84g/l, giving a yield of 0.70g/g of ethanol and a productivity of 0.25g ethanol/hour while the other strains of yeast produced ethanol at a concentration of 27 ± 0.84g/l and 26 ± 0.28g/l respectively.

4.19 Fermentation of standard sugars with yeast

The utilization of refined pure sugars (without inhibitors) by the selected yeasts was investigated. The sugar utilization during fermentation by the different yeast isolates is presented in Fig. 4.56 (as reference medium). In this medium it was observed that all the yeast isolates utilized the sugars better than the rest fermentation media as it did not contain inhibitors. At 72 hours of fermentation there was a high utilization of sugar with 16 ± 0.42g/l of sugar left in the medium containing the modified yeast E1, whereas there was 14.0g/l of sugar left in the media containing other yeast isolates.

At 96 hours of fermentation, the amount of sugar in the fermentation media of yeast E4 and the modified yeast E1 medium dropped to 2.0 ± 0.28g/l while the other two yeast medium containing isolate E2 and E3 dropped to 4.0 ± 0.28g/l respectively. At 120 hours of fermentation, the sugar has been completely utilized in all the fermentation media with residual sugar concentration of 1.0g/l left in the medium.

The corresponding amount of ethanol that was produced by the different yeasts tested in the reference fermentation medium is represented in Fig. 4.57, while Table 4.9a, b, c and d present the summary of fermentation performances of the different yeasts in the reference sugar medium. It was observed that in the reference medium that ethanol was produced at a higher concentration and at a higher rate than in the enzyme and acid CPH hydrolysed medium. At 48 hours of fermentation the modified yeast (E1) produced 13±0.99g/l of ethanol with a yield of 0.29g/g of ethanol while E2 produced 10 ± 0.42g/l, yeast E3 produced 9 ± 0.14g/l and yeast E4 produced 9 ± 0.28g/l of ethanol concentration

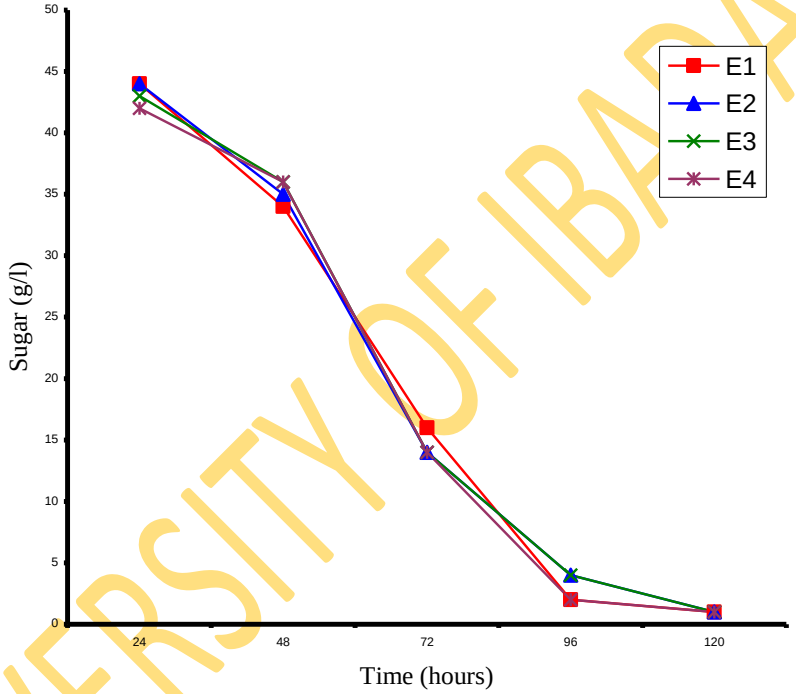


Fig.4.56 Sugar utilization in standard sugar medium during fermentation by yeast isolates

E1 = genetically modified S. cerevisiae yeasts; E2 = S. uvarum; E3 = S. cerevisiae; E4 = S. cerevisiae

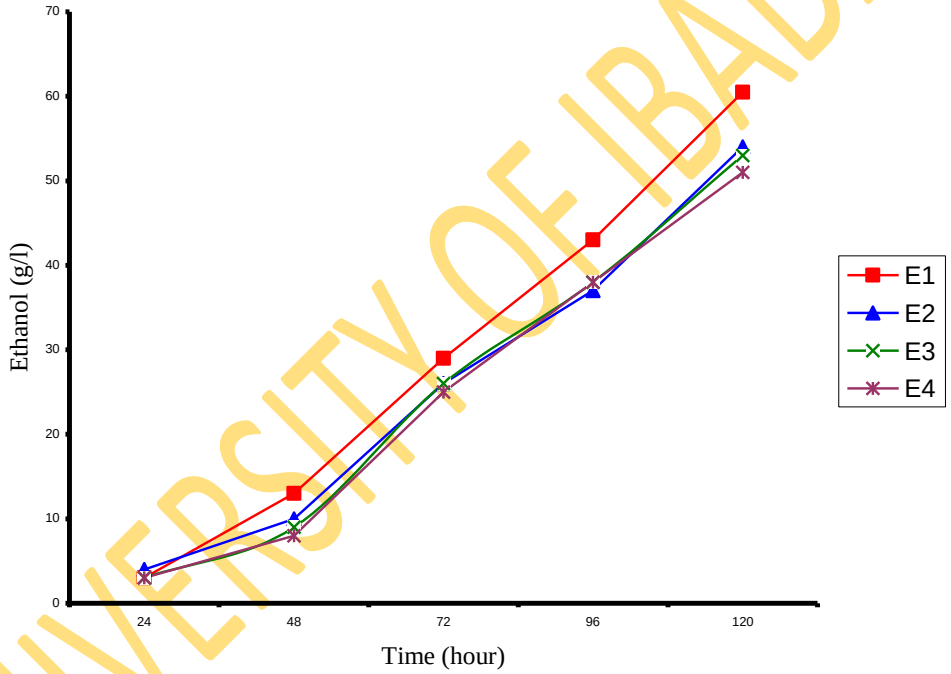


Fig. 4.57 Ethanol production from standard sugar during fermentation by selected yeast isolates

E1 = genetically modified *S. cerevisiae* yeasts; E2 = S. uvarum; E3 = *S. cerevisiae* ; E4 = *S. cerevisiae*

respectively. The amount of ethanol yield increases with the fermentation time in all the yeast isolates. The maximum ethanol concentration was achieved in 120 hours of fermentation with the modified yeast isolate producing 60.5 ± 0.85g/l of ethanol with a productivity of 0.51g of ethanol per hour, the ethanol yield was 1.36g/g.

Table 4.10 shows the summary of the statistical analysis of total ethanol formed by the selected yeast isolates in all the different fermentation medium tested. It could be observed that the genetically modified yeast produced the highest ethanol in all the fermentation medium.

Table 4.9a: Summary of fermentation performance of genetically modified *S. cerevisiae* yeast (strains E1) in standard sugar medium

Sugar conc. (g/l)	Max. ethanol conc. (g/l)	Time (hour)	Ethanol yield (g/g)	Vol. productivity (g of ethanol/hr)
44	3	24	0.067	0.125
34	13	48	0.289	0.271
16	29	72	0.644	0.403
2	43	96	0.955	0.445
1	61	120	1.356	0.508

Table 4.9 b: Summary of fermentation performance of *S. uvarum* yeast (strain E2) in standard sugar medium

Sugar conc. (g/l)	Max. ethanol conc. (g/l)	Time (hour)	Ethanol yield (g/g)	Vol. productivity (g of ethanol/hr)
44	4	24	0.089	0.167
35	10	48	0.222	0.208
14	26	72	0.578	0.361
4	37	96	0.822	0.386
1	54	120	1.200	0.450

Table 4.9c: Summary of fermentation performance of *S. cerevisiae* yeast strain (E3) in standard sugar medium

Sugar conc. (g/l)	Max. ethanol conc. (g/l)	Time (hour)	Ethanol yield (g/g)	Vol. productivity (g of ethanol/hr)
43	3	24	0.067	0.125
36	9	48	0.209	0.188
14	26	72	0.578	0.361
4	38	96	0.844	0.396
1	53	120	0.178	0.442

Table 4.9d: Summary of fermentation performance of *S. cerevisiae* yeast (strain E4) in standard sugar medium

Sugar conc. (g/l)	Max. ethanol conc. (g/l)	Time (hour)	Ethanol yield (g/g)	Vol. productivity (g of ethanol/hr)
42	3	24	0.067	0.125
36	9	48	0.200	0.188
14	25	72	0.556	0.347
2	38	96	0.844	0.396
1	51	120	1.133	0.425

Table 4.10: Statistical analysis of total ethanol formed by the selected yeast isolates in different fermentation medium tested.

Yeast isolate	Total ethanol formed (g/l)	Fermentation medium
E1	29.70	Reference sugar medium
E2	25.80	
E3	26.20	
E4	25.00	
E1	15.80	Acid hydrolysed medium
E2	14.10	
E3	14.20	
E4	14.10	
E1	14.40	Enzyme hydrolysed medium
E2	12.00	
E3	11.20	
E4	12.40	

E1 = Genetically modified *S. cerevisiae* yeasts;

E2 = *S. uvarum*;

E3 = *S. cerevisiae*;

E4 = *S. cerevisiae*

CHAPTER FIVE

5.0 DISCUSSION

The modified and unmodified starter cultures of yeasts used in this research work were isolated from induced, submerged spontaneously-fermenting Cocoa Pod Husk (CPH) derived ecosystem. The ability of the isolates to break down CPH cellulose and grow in it might be considered to be of importance for their existence in such substratum and this aided the selection of the microorganisms with the desired properties. Guidici *et al*. (2005) stated in his work on bioethanol that the first selection of commercial starter culture must always be based on a few basic features such as adaptation to growth on a specific substrate, rapid acidification or ethanol production. This observation was also corroborated with the findings of Leroy and De Vuyst (2004) who stated that a functional starter culture must achieve a sufficiently high and active cell biomass for any preparation. Thus, this process initiated without the use of starter inoculum aided the selection of organisms with desirable properties (Holzapfel, 2002).

The dominance of *S. cerevisiae* yeast in the population of yeasts isolated is of prime importance. This indicates their relevance as starter cultures for the conversion of CPH biomass to ethanol. The preponderance of this organism in alcoholic fermentation has been widely reported (Nevoigt, 2008; Ostergaad *et al*., 2000; Sandra and Carlo, 2009). This was attributed to their high ethanol tolerance as well as the ability to out-compete other microorganisms including other yeasts not capable of tolerating high ethanol concentration to carryout the last stages of alcoholic fermentation. Furthermore, *S. cerevisiae* is known to possess less stringent nutritional requirements, greater resistance to contamination (Jefferies, 2006) and the ability to grow both aerobically and anaerobically (Nevoigt, 2008). It has a broad substrate range, produce ethanol with high yield and specific productivity and also exhibit high tolerance to inhibitors present in lignocellulosic hydrolysate (Olsson and Hahn-Hagerdal, 1993, Aristidou and Penttila, 2000, Klinke *et al*., 2003). Hence it is suitable for fermentation of lignocellulosic materials (Olsson and Hahn-Hagerdal, 1993; Hahn-Hagerdal, 1994).

The strains of yeasts selected as agents for ethanol production in the present work were based on their preliminary ethanol tolerance and performance in the submerged

spontaneously-fermenting cocoa pod husk (CPH) medium during preliminary isolation. High ethanol concentration is known to reduce cell vitality as well as increase cell death (Birch and Walker, 2000). Tolerance of yeast isolates was based on Casey and Ingledew, (1986) definition of ethanol tolerance as the concentration of ethanol which will completely suppress batch growth. The simplicity of the above method, however, made it suitable for screening the strains of yeast isolates obtained in the current work for their ability to tolerate ethanol. Aside the fact that the genus *Saccharomyces* is known to have a relatively high ethanol tolerance (Aristidou and Penttila, 2000). Casey and Ingledew (1986) proposed that ethanol tolerance is an intrinsic ability of the different strains of *Saccharomyces*. Furthermore, in the work of Devantier *et al.* (2005), it was stated that the extent of yeast tolerance to ethanol determines its ethanol productivity and final titre, which is especially relevant in very-high-gravity fermentations. The efficiency of these processes in any given yeast determines its robustness and, to a large extent, ability of a given strain to perform well in industrial processes (Stanley *et al*., 2010). The above criteria were used for the selection of two yeast strains (MX1 and MX2) for further genetic improvement in order to develop recombinant *Saccharomyces* strains that can effectively ferment xylose and, in particular, co-ferment glucose and other pentose sugars present in lignocellulosic biomass of CPH hydrolysate to ethanol.

The construction of yeast strains (*Saccharomyces cerevisiae*) capable of growing and fermenting pentose sugars found in lignocellulosic materials has been the object of intense research efforts (Kotter *et al*., 1990; Kotter and Ciriacy, 1993; Tantirungkij *et al*., 1993; Walfridsson *et al*., 1995; Ho *et al*., 1999; Eliasson *et al.*, 2000). There have been a number of experimental observations indicating that wild type strains of *Saccharomyces cerevisiae* cannot utilize pentose sugars (xylose) present in lignocellulose (Chang *et al.*, 2007; Traff *et al*., 2002; Toviari *et al*., 2004; Jin *et al*., 2004; Karhumaa *et al*., 2005; Kuyper *et al.*, 2005) and it does not recognize xylose as a fermentable carbon source (Jin *et al*., 2004; Salusjarvi *et al*., 2008) because the genes encoding xylose reductase (YHR104w) (Traff *et al.*, 2002) and xylitol dehydrogenase (YLR070c) (Richard *et al*., 1999) present in its genome have low expression level that is too low to allow for xylose expression. Other observations include poor transport of xylose, redox cofactor imbalances and insufficient flux through the pentose phosphate pathway (Kòtter and Ciriacy, 1993; Jeffries, 2006).

As a result of these limitations, the present research work was focused on the production of recombinant yeast strains from the selected starter cultures through chromosomal integration of genes involved in the metabolism of pentose sugars from *Pichia stipitis* (CBS 6054) into selected MX1 and MX2 starter cultures. The introduction of xylose reductase (XR) encoded by the gene XL1 and xylitol dehydrogenase (XDH) encoded by the gene XL2 pathways are necessary for the selected starters of *S. cerevisiae*, to ferment the complex sugars in CPH hydrolysate to ethanol due to insufficient activity of these enzymes in the wild type strains. The work of Eliasson (2001) gave credence to this. This, inturn will improve xylose fermentation in lignocellulosic biomass and decrease xylitol formation (Aristidou and Penttila, 2000).

Thus, to exploit lignocellulosic material like CPH for ethanol production with maximum yield, the producting organisms should be capable of utilizing all pentoses and hexoses present in the lignocellulose. Furthermore, it is important that the producing organism should have a high inhibitor tolerance, since the lignocellulose hydrolysates contain some inhibitory substances. *Pichia stipitis* could not be used in the fermentation of the CPH hydrolyaste due to its sensitivity to inhibitors that are present in lignocellulosic hydrolysate (Linden and Hahn-Hagerdàl, 1989; Hahn-Hagerdàl *et al*., 1994). This yeast has low ethanol tolerance and equally requires a well controlled oxygenation for optimal productivity (Skog and Hahn-Hagerdàl, 1990). All these factors taken together severely limit its use in industrial exploitation. Hence, the recombinant yeast strains were developed by integrating genes for XR and XDH from *Pichia stipitis* (CBS 6054) into the yeast (S*accharomyces cerevisiae*) chromosome with the application of single copy yeast expression vectors pGAPZA and pVT100–U respectively. This method of chromosomal integration used in this work is in agreement with that of Bothast *et al.* (1999) which reported chromosomal integration of genes as being a pre-requisite to render industrial production organisms genetically stable, and this contrasts the use of multicopy, plasmid-carrying strains (Zhang *et al.*, 1996; Ugolini *et al*., 2002). The expression vectors that were used in this work have equally been designed for high level constitutive expression. It was observed that the expression of both genes (XR and XDH) in the selected yeasts has proven to be more stable and successful in the recombinants yeasts that were developed. Kotter *et al.* (1990) observed that the expression of 2 similar genes in the yeast has proven to be more successful in xylose metabolism since *S. cerevisiae* and *P. stipitis* from which the genes

were isolated are both eukaryotic and that the codon usage in *Pichia* is believed to be similar to *S. cerevisiae.* This finding is also in conformity with the work of Jefferies (2006) who demonstrated that both genes (XR and XDH) have coding regions composed of the preferred codons for highly expressed *S. cerevisiae* genes. Furthermore, the availability of highly efficient transformation methods and the susceptibility of *S. cerevisiae* to genetic modification by recombinant DNA technology in addition to the availability of the complete yeast genome sequence have aided the applicability of *S. cerevisiae* to genetic engineering (Ostergaard *et al*., 2000; Gietz and Wood, 2001).

Another unique property of the recombinant yeast strains developed in this work was the use of single copy expression vectors (pGAPZA and pVT100-U) for the cloned XR and XDH genes in the recombinant yeast strains respectively. The single copy vector method used is, however, in accordance with the recent findings of some workers who demonstrated that strains carrying multicopy plasmids are generally not suitable for industrial applications due to their instability (Futcher and Cox, 1984; Zhang *et al*., 1996). Multicopy plasmids require auxotrophic or antibiotic resistance markers to be retained in the cell, both of which are unsuitable for growth in industrial media which contain complex nutrients and are used in large volumes. In addition to inhibitor tolerance, strain stability is also an essential factor for designing recombinant industrial strains. Furthermore, the resulting recombinant yeasts also have additional extraordinary properties. For example, the synthesis of the xylose-metabolizing enzymes directed by the cloned genes in these recombinant yeasts does not require the presence of xylose or glucose for induction as the genes are constitutively expressed, nor is the synthesis repressed by the presence of glucose in the medium (Sedlak and Ho, 2004). These properties make the recombinant yeasts able to efficiently ferment xylose to ethanol and also to efficiently co-ferment glucose and xylose present in the same medium to ethanol simultaneously without a lag period.

However, the naturally-occurring xylose-fermenting yeasts, such as *P. stipitis* and *C. shehatae*, which ferment xylose, are not able to ferment xylose at all when glucose is present in the medium, even though they effectively ferment xylose in the absence of glucose (Nancy *et al*., 1998).

The growth of the recombinant yeast strains as observed in their respective media - YPD medium supplemented with zeocin and complete synthetic drop out (Ura3- medium) respectively showed that the transformed cultures grew less in plates than the untransformed

cultures. This observation strengthens the notion that the maintenance of extra-chromosomal elements inside the cell per se is a costly event requiring more energy than the maintenance of the genome alone. Hence, the importance of optimizing the construction of artificial plasmids, to avoid titration of cellular resources and interference with the normal host growth rate and metabolism (Brownlie *et al*., 1990; Summers, 1991; Bergstrom *et al*., 2000). This finding is also in agreement with the report of Smits *et al.* (2000) who previously observed an identical phenomenon in transformants containing genes that encode glycolytic enzymes and which were ascribed to a potential burden effect (Sneop *et al*., 1995).
Similar observation was also made by Gorgens *et al*. (2001) in their work "metabolic burden" on the cell. They attributed this gene effect on the cell to increase energy demand, dilution of molecular factors required for transcription and translation (e.g., proteins, RNAs, cofactors, and precursors), or simply limited cellular space. When enzyme-encoding genes are expressed, this can trigger complex metabolic changes detrimental for cells (e.g., cofactor depletion)

The physiological characterization of the yeasts (modified and unmodified) isolates were carried out based on growth, since the growth and product formation of microorganism depend to a large extent on the nature of the isolate as well as other physiological factors such as temperature, pH, carbon source, nitrogen source, different ethanol concentration and others.

The optimal growth temperature for all the yeast isolates was within 30°C to 40°C, this being same with ethanol fermentation is an advantage, as this will remove the cost of providing a different temperature environment for both phases during fermentation of the CPH-hydrolysate. The optimum temperature for yeast growth is around 30°C. However different workers have reported different optimum temperatures for ethanol production from the co-fermentation of glucose and xylose. Rudolf *et al*. (2008) have demonstrated that more xylose was consumed by TMB3400 strain at 32°C than at 37°C during SSF of sugar cane bagasse, while Olofsson *et al*. (2008) found that a temperature of 34°C was prefer in SSF of wheat straw. Differences abound in the physiology of species and strains of microorganisms. Furthermore, the good growth level exhibited by the yeasts within 24 hours of incubation within the temperature mentioned above is relevant to inoculum development since a build up of the biomass is required before microorganisms are transferred to the fermentation medium.

The yeasts exhibited a wide range of pH (4.0- 5.5) for growth, this corroborate the inference that yeasts prefer acidic medium and grow well at a pH values between 3 and 5. This low pH (acidophilic environment) gave an added advantage to the yeasts since the risk of contamination in industrial fermentation is lowered in acidic environments (Jeffries, 2006; Olofsson *et al*., 2008).

The recombinant yeasts were observed to tolerate the effect of acetic acid to an extent since they grew compared to the unmodified strains. The ability of the recombinant strains to grow in acetic acid to an extent could be ascribed to the XR and XDH genes in their genome. Various reports have shown that yeast strain transformed with these genes can detoxify inhibitors that are normally present in lignocellulosic hydrolysate by reduction of these toxic compounds to alcohols (Larsson *et al*., 2001; Liu *et al*., 2004; Petersson *et al*., 2006;). Acetic acid is one of the most important weak acids of the fermentation inhibitors, as its pK_a is close to the pH of fermentation, and therefore a significant amount is in the un-dissociated form (Palmqvist and Hahn-Hàgerdal, 2000). The wild strains of the yeast could not tolerate this acid due to the inhibitory effect of acetic acid that has been ascribed to uncoupling and intracellular anion accumulation (Palmqvist and Hahn-Hàgerdal, 2000; Almeida *et al*., 2007). The un-dissociated form is liposoluble and can diffuse through the plasma membrane, and may dissociate inside the cell, depending on the pH, thus decreasing the cytosolic pH. In addition, intracellular accumulation of anionic species may contribute to weak acid toxicity (Russel, 1992; Helle *et al.*, 2004). To avoid the drop in intracellular pH, the cell must pump out protons by the action of the plasma membrane ATPase (Rusell, 1992; Verduyn *et al*., 1992). This implies that more ATP has to be generated to maintain the intracellular pH (Pampulha and Loureiro-Dias, 1989; Taherzadeh *et al*., 1997; Palmqvist and Hahn-Hagerdàl, 2000). However, at higher concentrations of acetic acid (Larsson *et al*., 1998), the ethanol yield is decreased due to the toxic effect. If the anionic form of acetic acid is captured in the cells, undissociated acid in the medium will diffuse into the cell until equilibrium is reached (Hasunuma *et al.*, 2011)

The wild type isolates were able to utilize the different carbon sources to a good extent. The ability of microorganisms to metabolise various carbon sources differently is based on the specific activities of the enzymes involved in carbohydrate degradation (Jill and Bonita, 1998). The various sugars that were tested are present in the hydrolysate of CPH as shown by HPLC analysis. This explains to an extent, the suitability of the CPH wastes as

substrate for ethanol fermentation. All the yeast isolates- both the modified and the non-modified *Saccharomyces cerevisiae*, grew well in glucose medium. Glucose plays a regulatory role in yeast metabolism in addition to its importance as a nutrient. Studies have indicated that when glucose is available in mixture of sugars, that glucose is used preferentially to other carbon sources (Ling *et al*., 1999). This is achieved in part, by transcriptional repression of genes that are required for respiratory metabolism and utilization of other carbon sources. (Gancedo, 1998).

Furthermore, it has been shown that a low glucose concentration is needed in most yeast growth medium for efficient xylose uptake (Meinander *et al.,* 1999). This has been explained by a need for glucose in the expression of glycolytic enzymes and intermediates (Boles *et al.*, 1996) as well as generation of intermediary metabolites for the initial steps of xylose metabolism and the pentose phosphate pathway (Meinander *et al.*, 1999).

Cultivation of the different yeast strains in pentose media shows that the genetically-modified strains grew better in xylose and arabinose carbon sources than the unmodified strains due to the presence of XR and XDH genes in their genome. The ability of yeast with XR to utilize xylose has been attributed to the dual NADPH and NADH co-factor specificity of XR gene (Bruinenberg *et al.*, 1984), XR catalyses this forward reaction via an ordered bi – bi mechanism, with the cofactor binding first to the enzyme followed by the substrate (Hung, 1998). However, the natural strains of *S. cerevisiae* selected were also capable of growing on the pentose sugars though far less than the genetically-modified strains. This finding is, however, contrary to the earlier notion that *S. cerevisiae* does not recognize this pentose sugar as carbon source (Barnette, 1976; Chu and Lee, 2007). The growth observed with the non-modified strains further strengthens the finding of some workers that there is abundant natural, genetic and phenotypic variation within *S. cerevisiae* and closely-related species (Dunn *et al*., 2005; Fay and Benavides, 2005; Carreto *et al*., 2008; Kvitek *et al.*, 2008; Liti *et al.*, 2009; Schacherer *et al*., 2009). Furthermore, report of Chu and Lee (2007) that under intense selection pressure, *Saccharomyces cerevisiae* mutant will spontaneously arise and grow slowly on xylose, lends more credence to this observation.

The recombinant yeast strains were able to utilize all the sugars tested than the unmodified strains. This observation may reflect the possible function of the heterologous XR and XDH gene in stress response. Similar observation has earlier been noted in aldose reductase in *Saccharomyces cerevisiae* (Chang *et al.*, 2003; Traff *et al.*, 2004).

The effects of different concentration of ethanol on the growth of the yeast strains shows that their specific growth rate were affected at different concentrations, with the recombinant strains showing more tolerance than the unmodified strains. Ethanol at higher concentrations has been shown to reduce cell viability and increase cell death (Birch and Walker, 2000). *Saccharomyces uvarum* (unmodified) was highly susceptible to ethanol even at a level as low as 5% concentration. The susceptibility of yeasts to the toxic effects of ethanol appears to depend on the lipid composition of the membrane systems of the yeast cell. When yeasts or other microorganisms are grown in the presence of ethanol, the lipid composition of their membranes changes and some evidence indicates that this may represent an adaptive response (Stanley *et al.,* 2010). However, the specific rate of microbial growth is depressed by ethanol, and it has been suggested that membrane lipid composition is a function of the specific rate of growth and appear to be a predominant target of ethanol (Stanley *et al*., 2010). Exposure of yeast to ethanol results in increased membrane fluidity and consequential decrease in membrane integrity (Mishra and Prasad, 1989). A decrease in water availability due to the presence of ethanol causes the inhibition of key glycolytic enzymes and these may led to the denaturation of the protein (Hallsworth *et al*., 1998).

The production of cellulase enzyme by *Trichoderma hamatum* using different carbon sources –Carboxymethylcellulose (CMC) and cocoa pod husk (CPH) shows that CMC was the best carbon source for induction of cellulase enzyme. The highest amount of cellulase enzyme (0.608unit/ml) was produced with CMC as the carbon source compared to (0.542 unit/ml) produced using CPH as carbon source.

The use of CMC for cellulase synthesis is in agreement with the results of Narasimha *et al.* (2006) who reported high cellulase production with cellulose as carbon source in the basal medium for enzyme production by *A. niger*. CMC was also used to produce cellulase in *Bacillus sp.* (Khayami-Horani, 1996). Huang and Monk (2004) reported that more cellulase was produced by *Caldibacillus cellulovorans* using cellulose as substrate than when grown on cellobiose or xylan. Xylanase enzyme production by *Trichoderma hamatum* using pure xylan as carbon source was also higher than using CPH. In many organisms, higher xylanase production has been reported on pure xylans than on lignocelluloses (Chandra and Chandra, 1995; Kumar and Ramon, 1996; Gupta *et al.,* 2001; Rizzatti *et al.,* 2001) These differences in the production of cellulolytic enzymes from a variety of substrate and CPH-lignocellulose could be attributed to various factors including variable cellulose content in

lignocelluloses derived from plant sources and heterogeneity of structure and the cellulolytic ability of the organism (Narasimha *et al.,* 2006).

Cellulose and hemicellulose are found in close association with lignin in the lignocellulosic complex. This makes it difficult for hemicellulolytic enzymes to break down cellulose and xylan due to difficulty encountered during the course of lignin breakdown (Singh *et al.,* 1988). Hence in the present study, pre-treatment of CPH was effected using particle size reduction and liquid hot water (LHW) before hydrolysis. The importance of pretreatment (particles size reduction and LHW) and hydrolysis in the release of simple and fermentable sugars from CPH may have been established in this study. Pretreated lignocellulosic materials are much more susceptible to digestion by enzymes (Galbe and Zacchi, 2002) and often lead to high and improved yield of sugars (Hamelinck *et al.,* 2005). The mechanical reduction of CPH to smaller particle sizes help to decrease the crystallinity leading to an increase in available surface area for microbial action as well as a reduction in the degree of polymerization (Palmowski and Muller, 1999). Particle size reduction is also known to cause shearing of the lignocellulosic biomass (Hendrik and Zeeman, 2009).

The liquid hot water pretreatment method at 130°C for 1 hour that was employed in combination with particle size reduction of the CPH has major advantages. The results obtained give credence to this. Liquid hot water pretreatment solubilizes hemicellulose and lignin resulting in reduced concentration of risk of degradation products like furfural, HMF due to higher water input (Hendrik and Zeeman, 2009). The condensation and precipitation of lignin compounds is also reduced (Weil *et al*., 1998). This gives rise to 2 – or 5-fold increase in enzymatic hydrolysis of the substrate after LHW pretreatment (Hendriks and Zeeman, 2009). LWH causes ultrastructural changes in the surface area of the cellulose making it more accessible and susceptible to hydrolytic enzymes (Zeng *et al.,* 2007).

Increasing the pretreatment time of CPH in LHW up to one hour could be assumed to have led to an increase in the concentration of all the sugars as observed with the enzymatic hydrolysis yielding 31.7% of glucose. This finding is evidently in agreement with the work of Martin *et al.* (2002) who observed that stronger pretreatment condition led to a more complete hydrolysis of hemicellulose and favoured a more efficient enzymatic hydrolysis of cellulose.

Addition of Tween-80 surfactants to the pretreated medium prior to enzymatic hydrolysis was another factor that could be responsible for the increase in sugar concentration (31.7%)

that was obtained compared to (28.5%) in the medium without Tween-80. Taherzadel and Karimi (2007) had reported similar results and suggested that addition of surfactants during enzymes hydrolysis can modify the cellulose surface properties and lower enzymes loading. Among all the surfactants that have been used in enzymatic hydrolysis, Tween-80 was among the best and the most effective surfactants reported for enzyme hydrolysis (Alkasrawi *et al.*, 2003; Kim *et al.*, 2006; Borjesson *et al.* 2007). One reason that has been adduced for this effect is the adsorption of surfactants to lignin which prevent unproductive binding of enzymes to lignin resulting in higher productivity of the enzymes (Eriksson *et al.*, 2002).

The acid hydrolysed CPH was subjected to detoxification process through overliming with $Ca(OH)_2$ prior to fermentation in order to detoxify the inhibitors that are release into the hydrolysate, during acid hydrolysis several inhibitors from sugars degradation products such as 5-hydroxylmethylfufural (HMF), furfural (degradation products of hexoses and pentoses), weak organic acids and phenolic compounds from lignin degradation are formed. These compounds can inhibit both yeasts (Sanchez and Bautista, 1988) and enzymes (Tengborg *et al.*, 2001). It can also reduce ethanol yield and the specific ethanol productivity, extend the lag phase, and reduce the growth rate and viability of microorganisms (Almeida, 2009). It could be deduced that during the detoxification process the simultaneous addition of sodium sulphite and calcium hydroxide to the acid hydrolysate removed some toxic inhibitors through the precipitation reaction that was observed. Similar observation has been made by Barbosa *et al*. (1992); Lawford and Rousseau, 1992. The hydrolysate was centrifuged and filtered to remove the precipitate containing the toxic components associated with the calcium ions giving rise to better detoxified hydrolysate (Amartey and Jeffries, 1996).

Many authors have observed improvements in the fermentative performance of microorganisms in over-limed (detoxified) hydrolysate both in terms of yield and productivity, showing that the treatment of hydrolysates is fundamental in enhancing the conversion efficiency (Lawford and Rousseau, 1992; Amartey and Jefferies, 1996; Dien *et al*., 1999)

The HPLC analysis of the sugar compositions of CPH in both acid and enzymatic hydrolysis shows that CPH-hydrolysed medium is very heterogeneous in composition. The hydrolysate contains mainly glucose, mannose, xylose, galactose and arabinose in different

proportions. This broad spectrum of sugars found in CPH hydrolysate contrasts with bagasse hydrolysate, which contain only glucose, xylose and very small amount of arabinose and mannose (Martins *et al*., 2001; Martin *et al*., 2002)

Sugar of glucose concentration of 28.5% and 31.7% with Tween-80 were obtained in enzymatic hydrolysis compared to the amount of (50.1%) glucose that was obtained from CPH using acid hydrolysis. The high disparity in the amount of glucose released from both processes could be attributed to incomplete enzymatic hydrolysis of the CPH, probably due to the inhibition of β-glucosidase enzyme by the released sugars. This observation correlates with the demonstration of the competitive inhibition of β-glucosidase enzyme by glucose (Lee and Fan, 1983). Ojumu *et al*. (2003) had earlier demonstrated that the cumulative effect of reducing sugars, glucose and xylose are known to inhibit cellulase and xylanase respectively.

Furthermore, the lignin content of CPH could be another factor attributable to the low amount of sugar that was released in enzymatic process compared to the acid hydrolysis, as lignin is less sensitive to enzymatic attack, which may limit access of the biomass to hydrolytic enzymes.

The reaction of enzymes to different concentration of their appropriate substrate showed that cellulase enzyme has optimum activity with 1 - 2% carboxymethylcellulose (CMC) while xylanase enzyme has 2% with xylan. For a given reaction, an enzyme catalysed reaction increases with the substrate concentration (Prescott *et al*., 1999; Taylor *et al*., 2002) but there comes a point when any further increase in the concentration produces no significant changes in the reaction rate. At high substrate concentration, the active site of the enzyme is saturated and operating at maximal velocity (V_{max}).

The ethanol fermentation performance of the recombinant yeasts and the non-modified yeasts were monitored in the CPH hydrolysate along with a reference (mixture of pure sugars as in the hydrolysate). Sugar consumption and ethanol formation during this procedure shows that ethanol was produced at a higher concentration and at a higher rate in the reference sugar medium than in the CPH hydrolysates by all the yeasts. This is possibly due to the absence of inhibitors in the reference sugar medium. However, a significant production of ethanol (29.7g/L) within 120 hours and a concomitant volumetric productivity of 0.51g of ethanol/1h by the recombinant *Saccharomyces cerevisiae* compared to the unmodified strains which produced ethanol at a concentration of 26.2g/l and 25.8g/l

respectively. The depletion of fermentable sugars was detected after 120 hours of the fermentation. It has been reported in literature that the metabolism of sugars by the recombinant yeast occurs through facilitated diffusion of xylose through the hexose transporter encoded by the HXT (hexose transporter) gene family (Kruckeberg, 1996). Once inside the yeast cell, xylose is converted by a two-step oxidoreductive isomerization to xylulose (Webb and Lee, 1990). Xylose is first reduced to xylitol by a NADH – or NADPH – dependent xylose (aldose) reductase. Xylitol is then further oxidized to xylulose by NAD-dependent xylitol dehydrogenase (XDH). Xylulose is phosphorylated at the Carbon 5-OH position by xylulokinase (XK) to yield xylulose-5-phosphate which is further channeled into glycolytic intermediates via the pentose phosphate pathway (PPP) (Chu and Lee, 2007).

After fermentation, it was observed that some residual sugars were still left in the fermentation media. The work of Martins *et al.* (2002) supported this observation indirectly. It pointed to the fact that in a fermentative media composed of mixtures of sugars, the utilization of different carbon sources is sequentially due to the repression of several yeast enzymes by glucose (Gancedo and Serrano, 1989; Gancedo, 1992). Glucose repression usually constitutes a problem in several industrial fermentative processes that utilizes feedstock like molasses or wort, which are composed of mixtures of sugars (Olsson and Nielsen, 2000).

In the acid hydrolysate, the recombinant yeast produced maximum ethanol concentration of 15.8g/L (ethanol yield being 0.67g /g) in 120 hours with a volumetric productivity of 0.25g of ethanol/l h. The ability of the recombinant yeast to ferment acid hydrolysate without inhibition is due to the XR and XDH gene. This is supported by conclusion of some workers that yeast strain transformed with these genes can detoxify the inhibitors that are normally present in lignocellulosic hydrolysate through reduction of these toxic compounds to alcohols (Larsson *et al*., 2001; Liu *et al*., 2004; Petersson *et al*., 2006). Furthermore, Almieda *et al*. (2008) demonstrated that yeast strain with these genes (XR and XDH) will not only convert xylose to ethanol, but also display higher tolerance to lignocellulosic hydrolysate inhibitors especially to furfurals and 5 hydroxymethyl furfurals.

On average, there were significant differences in the ethanol yield of the three non-modified yeast isolates in the acid CPH hydrolysate. These observed differences might be due probably to the presence of residual inhibitory compounds such as acetic acid, phenolics and furan derivatives. It could further be attributed to inefficient xylose uptake in the medium as

reported by Kotter and Ciriacy (1993), a redox imbalance generated in the first two steps of xylose metabolism (Bruinenberg, 1986) and the inefficient PPP in the non-modified yeasts (Walfridsson *et al.*, 1995). Furthermore, the inability of pentose sugar to activate the lower part of glycolysis has been suggested to be the reasons for the inability of wild strain of *Saccharomyces cerevisiae* to grow on xylose (Boles *et al.*, 1993; Muller *et al.*, 1995). However, the inhibition of fermentation was not as strong as in bagasse hydrolysate as reported by Martins *et al*. (2001). This fact might be due to the detoxification process that was performed on the CPH hydrolysate prior to fermentation.

In the enzymatic hydrolysate, the recombinant yeast produced maximum ethanol concentration of 14.4 g/l and the ethanol yield was 0.71g of ethanol/g of sugar in 120 hours with a volumetric productivity of 0.28g of ethanol/l h while the non-modified yeasts produced ethanol concentration of 12.20 g/l, 12.40 g/l and 11.20 g/l respectively.

During fermentation of the hydrolysate, microscopic examination of the yeast cells in the hydrolysate was carried out. It was observed that there was an adaptation of the yeast cells to the lignocellulosic hydrolysate through the formation of petite mutants which probably help to protect essential cell components and reduced the level of apoptosis of the yeast cell in the medium, similar observation has been reprted by (Tosato and Bruschi, 2007). The presence of CPH hydrolysate is believed to have affected the activity of the yeast cells in this environment before acclimatization, by inducing the expression of genes related to the repair of cellular damage (Tosato and Bruschi, 2007). This experimental observation is corroborated by our laboratory experience. Other researchers have equally shown that yeast survival under stress conditions is achieved through a series of stress responses that depend on a complex network of sensing and signal transduction pathway leading to adaptation in cell cycle, and adjustments in gene expression profiles and cell metabolic activities (Hohmann and Mager, 2003).

With the level of fermentation that was observed with the non-modified selected starter yeasts used in this work, it can be proposed that some naturally-occurring strains of *Saccharomyces cerevisiae* are capable of utilizing pentose sugars to some appreciable extent and by extension lignocellulose, through careful selection.

This strange observation is however corroborated with the findings of Attfield and Bell, (2006) whose recent work has shown that natural genetic variation for xylose utilization does exist and that natural selection and breeding can improve xylose utilization in natural

strains of *Saccharomyces cerevisiae*. They obtained microscopic *Saccharomyces cerevisiae* colonies on xylose containing minimal media after several weeks of incubation. This strain represents the first conclusive evidence that a non-genetically modified *Saccharomyces cerevisiae* strain can be evolved to utilize xylose by relying upon expression of native xylose metabolizing genes (Attfield and Bell, 2006).

In conclusion, this work has therefore attempted to bridge the missing gap by presenting a report on how to generate energy need for the increasing human population from renewable energy source in waste biomass using indigenous organism and genetically modified starters. The adaptation of this renewable energy technology will no doubt, reduce the over dependence on the dwindling fossil fuel and prevent the perils of global warming and the looming energy crisis.

CONCLUSION AND RECOMMENDATIONS

The results of this study give novel information to a large extent on bio-ethanol production especially as regards the use of waste biomass – in this case cocoa pod husk and the indigenous starter yeasts cultures. The improvement strategies could be used to improve yeast performance in ethanolic fermentation of lignocellulosic hydrolysates. The successful integration of xylose fermentation pathways from *Pichia stipitis* into *Saccharomyces cerevisiae* yeast was a critical step towards enabling economic biofuel production from lignocellulose.

As there is no documented information hitherto, the result of this study can comfortably serve as a baseline and adopted for the production of bio-ethanol - a renewable form of fuel in our environment where lots of agricultural residues and wastes biomass abound. Apart from the economic gains of serving as an alternative fuel for cleaner environment, it is sustainable, has a considerable environmentally-friendly potential while the problem of waste management would to a reasonable extent, be handled as the study confirms the phrase "WASTE to WEALTH CREATION". CPH does not compete with food for humans in its production.

Energy is the pivot on which all other developments of a society are hinged. Thus, increased use of bio-ethanol for energy generation will provide means of energy independence and sustainability. This in turn will equally offer a new avenue for employment possibilities for the teeming unemployed youth of Nigeria. In order to create

this energy sufficiency for the nation, there should be a marriage of research collaboration between the agricultural sector and scientists. With this we would be able to harness and appreciate the divine endowment of what God has given to Nigeria.

It is evident that this research work has been able to establish the conversion technology for the production of bioethanol from renewable cellulosic biomass of cocoa pod husk using indigeneous cultures of genetically modified and wild yeasts cultures. This technology will help to solve the problem of using food based materials like maize, cassava, sugar cane etc. as the federal government initially proposed the utilization of cassava and maize for the production of bioethanol in Nigeria which will be counter productive in future and can lead to competition in the production of food for the teeming populace. Furthermore, this technology can as well be extended for ethanol production from other agricultural residues that are abound in Nigeria.

World wide, the production of bioethanol from cellulosic biomass will enhance sustainability and its continual use will affect positively the stability of the ecosystems and global climate as well as global oil reserves.

Conclusively, the adaptation of this technology in developing nations like Nigeria is a far sighted vision that will make a mile stone in the history of Nigeria transformation agenda. Ethanol has many desirable features as a petroleum substitute and could obviously help in making a smoother transition from a monolithic petroleum based energy source to a diversed biobased chemical economy.

However, the recovery of anhydrous ethanol from the fermentation medium is an area open for thorough investigation especially to obtain higher and more purified grade of ethanol.

REFERENCES

Adler, E. 1977. Lignin chemistry – past, present and future. *Wood Sci. Technol.* **11:** 169-218.

Alfani A, Gallifuoco, F., Saporosi A., Spera A., Cantarella M. 2000. Comparison of SHF and SSF process for bioconversion of steam-exploded Wheat straw. *J. Ind. Microbiol. Biotechnol.* **25**: 184 – 192.

Alkasrawi, M., Eriksson, T., Borjesson, J., Wingren, A., Galbe, M., Tjerneld, F. And Zacchi, G. 2003. "The effect of Tween-20 on simultaneous saccharification and fermentation of softwood to ethanol", *Enzyme Microb. Tech.* **33(1),** 71-78.

Almeida João RM, Tobias Modig**,** Anja Röder**,** Gunnar Lidén and Marie-F Gorwa-Grauslund 2008**.** *Pichia stipitis* xylose reductase helps detoxifying lignocellulosic hydrolysate by reducing 5-hydroxymethyl-furfural (HMF) *Biotechnol. Biofuels,* **1:**12

Almeida, J.R 2009**.** Improving the Response of Saccharomyces cerevisiae to Lignocellulosic Hydrolysate Inhibitors in Ethanolic Fermentation. University dissertation from Division of Applied Microbiology, Lund, Sweden.
(http://www.dissertations.se/about/Lunds+Universitet)

Almeida, I.R.M., Modig, T., Peterson, A., Hahn-Hagerdai, B., Liden, G., Gorwa-Grauslund, M.F. 2007. Increased tolerance and conversionofiinhibitors in lignocellulosic hydrolysates by *Saccharomyces cerevisiae. J. Chem. Technol. Biotechnol.* **82:** 340-349.

Amartey, S. and Jeffries, T. 1996. An improvement in *Pichia stipitis* fermentation of acid-hydrolysed hemicellulose achieved by over-liming (calcium hydroxide treatment) and strain adaptation. *World J. Microbiol. Biotechnol.* **12:** 281-283.

Amore,R., Kotter,P., Kuster,C., Ciriacy,M. and Hollenberg,C.P.1991. Cloning and expression in Saccharomyces cerevisiae of the NAD(P)H-dependent xylose reductase-encoding gene (XYL1) from the xylose-assimilating yeast *Pichia stipitis J. Gene* 109 (1), 89-97

Ando, S., Arai, I., Kiyoto, K., Hanai, S. 1986. Identification of aromatic monomers in steam exploded poplar and their influence on ethanol fermentation. *J. Ferment. Technol.* **64**: 567-570.

Angspanneforeningen 1994. *Report: P23332-1*. NUTEK, Stockholm, Sweden.

A.O.A.C. 1990. Official methods of analysis (15th edn.) Virginia. Ass. Official analytical chemists Wash. D.C.

Argueso, J.L., Carazzolle, M.F., Mieczkowski, P.A., Duarte, F.M., Netto, O.V. *et al.*, 2009. Genome structure of a *Saccharomyces cerevisiae* strain widely used in bioethanol production. *Genome Res*.. **19:** 2258-2270.

Aristidou, A. and Pentlila, M. 2000. Metabolic engineering applications to renewable resource utilization. *Curr. Opin. Biotechnol.*, **11**: 187 - 198

Atkins, P.W., 1990. Physical Chemistry, Fourth ed. Oxford University Press, Oxford.

Attfield, P. V., and P. J. L. Bell. 2003. Genetics and classical genetic manipulations of industrial yeasts, p. 17-55. *In* J. H. de Winde (ed.), *Functional genetics of industrial yeasts*. Springer, Berlin, Germany.

Attfield, P.V., Bell, P.J. 2006. Use of population genetics to derived non-recombinant *Saccharomyces cerevisiae* strains that grow using xylosde as a sole carbon source. FEMS *Yeast Res*, **6:** 862-8.

Axe, D.D., Bailey, J.E. 1995. Transport of lactate and acetate through the energized cytoplasmic membrane of *Escherchia coli. Biotechnol. Bioeng.*. 47, 8-19.

Azhar, A.F., Bery, M.K., Colcord, A.R., Roberts, R.S., Corbitt, G.V. 1981. Factors affecting alcohol fermentation of wood acid hydrolysate. *Biotechnol.Bioeng.*. Symp., 293-300.

Bailey, J. E**.** 1991. Toward a science of metabolic engineering. *Sci*. **252:**1668-1675.

Bailey, M.J., Beily, P., Poutanen, K. 1992. Interlaboratory testing and methods for assay of xylanase activity. *J. Biotechnol*. **23**: 257 – 70.

Bailey, J. E., S. Birnbaum, J. L. Galazzo, C. Khosla, and Shanks, J. V. 1990. Strategies and challenges in metabolic engineering. *Ann. N. Y. Acad. Sci.* 589:1-15

Balat, M. and Havva, B. 2009. Recent trends in global production and utilization of bio-ethanol fuel. *Appl. Energy*, **86**: 2273 – 2282.

Ballesteros I., Negro M., Oliva JM, Manzanares P., Ballesteros M., 2006. Ethanol production from steam-explosion pretreated wheat straw. *Appl. Biochem. Biotechnol* **130**: 496 – 508.

Barbosa, M.F.S., Beck, M.J., Fein, J.E., Potts, D. and Ingram, L.O. 1992. Efficient fermentation of *Pinus* sp. acid hydrolysates by an ethanologenic strain of *Escherichia coli. Appl. Environ. Microbiol.* **58:** 1382-1384.

Barnes, A.R., Amega, W.K.1998. Utilization of cocoa pod husk by growing-finishing pigs. 9th *International Cocoa Research Conference*, Lome, Togo, pp 449-454.

Banerjee, N., Bhatnager, R., Viswanathan, L., 1981. Inhibition of glycolysis by furfural in *Saccharomyces cerevisiae. Eur. Journal of Appl. Microbiol Biotechnol.*. **11**, 226-228.

Bardet, M., Robert, D.R., Lundqvist, K. 1985. On the reactions and degradation of the lignin during steam hydrolysis of Aspen wood. Sven. Papperstidn. 6, 61-67.

Barnett, J.A. 1976. The utilization of sugars by yeasts, *Adv Carbohyd. Chem Biochem.* **32:** 125-234.

Barnett, J.A., Payne, R.W and Yarrow, D. 1983. Yeasts characteristics and Identification, 1st Edition. Cambridge University Press, London.

Barnett, J.A., Payne, R.W. and Yarrow, D. 1990. *Yeast characteristics and Identification* 2nd edition. Cambridge University Press London.

Basso, L.C., de Amorim, H.V., de Oliveira, A.J., Lopes, M.L. 2008. Yeast selection for fuel etanol production in Brazil. *FEMS Yeast Res*., **8:** 1155-1163.

Batt, C.A, Carvallo, S., Easson, D.D., Akedo, M. And Sinskey, A.J. 1985. Direct evidence of a xylose metabolic pathway in *Saccharomyces cerevisiae*. Biotechnol Bioeng. 27: 549 553.

Beech, F.W. and Daveport, R.R. , Goswell, R.W. and Burnett, J.K. 1968. Two Simplified schemes for identifying yeast cultures. In Identification Methods for Microbiologists part B (B.M., Gibbs and D.A Shapton, Eds) pp 151 – 175. Academic Press, London.

Beech, F.W. and Daveport, R.R. 1971. Isolation, Purification and Maintenance of yeasts In: Methods in Microbiology, Booth, C.A (ed) pp. 153- 189 Academic press London.

Bergstrom, C.T., Lipsitch, M., and Levin, B.R. 2000. Natural selection, infectious transfer and existence conditions for bacterial plasmids. *Genetics* **15**, 51505 – 51519.

Bertilsson, M., Andersson, J., Liden, G. 2008. Modeling simultaneous glucose and xylose uptake in *Saccharomyces cerevisiae* from kinetics and gene expression of sugar transporters, *Bioprocess and biosystems Engineering*, doi: 10.1007/s00449-007-0169-1.

Birch, R.M. and Walker, G.M. 2000. Influence of magnesium ions on heat shock and ethanol stress responses of *Saccharomyces cerevisiae*. *Enzyme Micfrob. Technol.* **26:** 678-687.

Boles, E., Lehnert, W., Zimmermann, F.K. 1993. The role of the NAD- dependent glutamate dehydrogenase in restoring growth on glucose of a *Saccharomyces cerevisiae* phosphoglucose isomerase mutant. *Eur J. Biochem*, **217:** 469-77.

Boles, E., ller, S., Zimmermann, F.K. 1996. A multi-layered sensory system controls yeast glycolytic gene expression. *Mol. Microbiol*, **19:** 641-642.

Boles, E. and Hollenberg, C.P. 1997. The molecular genetics of hexose transport in yeasts, *FEMS Microbiol. Rev*, **21:** 85-111.

Bolen, P. L., and Detroy, R.W. 1985. Induction of NADPH-linked D-xylose reductase and NAD-linked xylitol dehydrogenase activities in *Pachysolen tannophilus* by D-xylose, L-arabinose, or D-galactose. *Biotechnol. Bioeng*. **27:**302-307

Borjesson, J., Peterson, R. and Tjerneld, F. 2007. "Enhanced enzymatic conversion of softwood lignocellulose by poly(ethylene glycol) addition", *Enzyme Microb. Tech,* **40,** 754-762.

Bothast R.J., Nichols N.N and Dien B.S. 1999. Fermentations with new recombinant organisms. *Biotechnol. Prog.* **15**: 867-875.

Bothast, R.J and Saha, B.C 1997. Ethanol production from Agricultural Biomass substrates. *Adv. Appl. Microbiol.*, **44**: 261 –285.

Boyer, L.J., Vega, K., Klasson, K.T., Clausen, E.C., Gaddy, J.L. 1992. The effect of furfural on ethanol production by *Saccharomyces cerevisiae. Biomass Bioeng.* **3 (1)**, 41-48.

Brat, D. Boles, E. and Wiedemann, B. 2009. Functional expression of a bacterial xylose isomerase in saccharomyces cerevisiae. *Appl. Environ. Microbiol.* **doi: 10**. 1128/AEM.02522-08.

Brown, T.A. 1995. Gene Cloning: an introduction. Chapman and Hall, London. ISBN 0412622408.

Brownell, H.H and Saddler J.N. 1984. Steam-explosion pretreatment for enzymatic hydrolysis. In: *United States.* Pages*:* 55-68

Brownell, H.H., Yu E.K.C., Saddler, J.N. (1986). Steam-explosion pretreatment of wood: Effect of chip size, acid, moisture content and pressure drop. *Biotechnol. Bioeng.* **28:** 792-801.

Brownlie, L., Stephenson, J.R., and Cole, J.A. 1990. Effect of growth rate on plasmid maintenance by *Escherichia coli* HB101 (pAT153). *J. Gen. Microbiol*. **136:** 2471 – 2480.

Brown, M.H. and Booth, I..R., 1991. Food Preservatives, third ed. Blackie and son, Glasgow-London.

Bruinenberg, P.M., De Bot, P.H.M., Van Dijken, J.P and Scheffers, W.A. 1984. NADH linked aldose reductase: the key to anaerobic alcoholic fermentation of xylose of yeast. *Appl. Microbiol. Biotechnol.* **19**: 256 – 260.

Bruimenberg, P.M. 1986. The NADP(H) redox couple in yeast metabolism. Antomie Van Leeuwenhoek, **52:** 411-29.

Carreto, L., Eiriz, M.F., Gomes, A.C., Pereira, P.M., Schuller, D. 2008. Comparative genomics of wild type yeast strains unveils important genome diversity. *BMC Genomics* 9: 524.

Catic, I and Sokele, R.M. 2008. Agriculture products - food for living being or for machinery. *Polimeri*, **4:**243-243.

Casey G.P. and Ingledew, W.M. 1986. Ethanol tolerance in yeasts. *Crit. Rev. Microbiol*. **13:** 219 – 280

Caulfield, D., Moore, W.E. 1974. Effect of varying crystallinity of cellulose on enzymic hydrolysis. *Wood Sci*. **6: (4),** 375-379.

Chandel, A.K., Chan, E.S., Ravinder, R.,Narasau, M.L., Rao, V. and Pogaku, R. 2007. Economics and environmental impact of bioethanol production technologies: an appraisal. *Biotech. Molecul. Biol. Rev.* **2: (1),** 014 – 032.

Chandra, R.K. and Chandra 1995. A cellulose-free xylanase from alkali tolerant *Aspergillus fischeri* Fxnl. *Biotechnol. lett.* **17**:309 – 314.

Chang V.S. and Holtzapple M.T. 1997. Lime pretreatment of switch grass. *Appl Biochem Biotechnol*, **63/65**: 3-19.

Chang, V.S. and Holtzapple, M.T. 2000. Fundamental factors affecting enzymatic reactivity. *Appl. Biochem. Biotechnol*. 5-37.

Chang Q., Harter T.M., Rikimaru L.T. and Petrash J.M. 2003. Aldo-keto reductases as modulators of stress response. *Chem Biol. Interact* 143-144: 325-332.

Chefetz, B., Keren, Z., Chen, Y. And Hadar, Y. 1998. Laccase from composted municipal solid waste purification and Characterization. *Soil Biol. Biochem*. **30**: 1091 – 1098.

Christensen, W.B. 1946. Urea decomposition as a means of differentiating *Proteus* and Paracolon organisms from each other and from *Salmonella* and *Shigella* types. *J. Bacteriol*. **52:** 461.

Chiang, L.C., Grong, C.S., Chen, L.F., Tsao, G.T., 1981. D-xylulose Fermentation to Ethanol by *Saccharomyces cerevisiae*. *Appl. Environ. Microbiol.*. **42:** 284-289.

Chu, B.C and Lee, H. 2007. Genetic Improvement of *Saccharomyces cerevisiae* for xylose fermentation. *Biotechnol. Adv.*. 25: 425 – 441

Chung, I.S. and Lee, Y.Y. 1984. Ethanol fermentation of crude acid hydrolyzate of cellulose using high-level yeast inocula. *Biotechnol. Bioeng.* **27:** 308-315.

Chum H.L., Johnson D.K., Black S.K., Overend R.P 1990. Pretreatment-catalyst effects and the combined severity parameter. *Appl. Biochem Biotechnol*, 24/25: 1-14.

Claassen PAM, van Lier JB, Contreras AML, van Niel EWJ, Sijtsma L, Stams AJM, de Vries SS and Weusthuis RA. 1999. Utilisation of biomass for the supply of energy carriers. *Appl. Microbiol. Biotechnol.* , 6:741-755.

Clark, T. and Mackie, K.L. 1984. Fermentation inhibitors in wood hydrolysates derived from the softwood *Pinus radiate. J. Chem. Biotechnol*. B. 34, 101-110.

Clark, T.A., Mackie, K.L. (1987). Steam explosion of the softwood *pinus radiata* with sulphur dioxide addition I: Process optimisation. *J. Wood Chem. Technol.* **7:** 373-403.

Clark, T.A., Mackie, K.L., Dare, P.H., McDonald, A.G. (1989). Steam explosion of the softwood *Pinus radiata* with sulphur dioxide addition II: Process charactrisation. *J. Wood Chem. Technol.* 9: 135-166.

Committee on Ozone-forming Potential of Reformulated Gasoline (1999) *Ozone-forming potential of Reformulated Gasoline*, National Academy Press, Washington, DC, U.S.A.

Cowling E.B. 1975. Physical and chemical constraints in the hydrolysis of cellulose and lignocellulosic materials. *Biochemical and Bioengineering Symposium 5*.

Crawford, R.L. 1981. Lignin biodegradation and transformation. New York: John Wiley and Sons. ISBN 0-471-05743-6.

CTA 2006. In Search of Green Gold. *Spore, Issue* 123, Pp 4 - 5.

Dale B.E. and Moreira M.J. 1982. Freeze-explosion technique for increasing cellulose hydrolysis. In: *United States.*: Pages: 31-43.

Deanda, K., Zhang, M., Eddy, C. and Picataggio, S. 1996. Development of an Arabinose-Fermenting *Zymomonas mobilis* strain by metabolic pathway engineering. *Appl. Environ. Microbiol.* **62:** 4465-4470.

Deguchi, S., Kaoru, T. and Koki, H. 2006. Cooking cellulose in hot and compressed water. *Chem. Commun.*, 3293 – 3295, doi:10.1039/b605812d.

Delgenes, J.P., Moletta, R. and Navarro, J.M. 1996. Effects of lignocellulose degradation products on ethanol fermentations of glucose and xylose by *Saccharomyces cerevisiae. Pichia stipitis*, and *Candida shehatae Enz. Microb. Technol*. 19. 220-225.

Dekker, R.F.H. 1988. Inhibitors of *Trichoderma reesei* B-glucosidase activity derived from autohydrolysis – exploded *Eucalyptus regnans. Appl. Microbiol. Biotechnol.* **29:** 593-598.

Devantier, R., S. Pedersen, and L. Olsson. 2005. Characterization of very high gravity ethanol fermentation of corn mash. Effect of glucoamylase dosage, pre-saccharification and yeast strain. *Appl. Microbiol. Biotechnol.*. **68:**622-629

De Moraes, L.M.P., Astolfi-filho, S. and Oliver, S.G. 1995. Development of yeast strains for the efficient utilization of starch: evaluation of constructs that express α-amylase and glucoamylase separately or as bifunctional fusion proteins. *Appl. Microbiol. Biotechnol.* **43:** 1067-1076

Diaz de Villegas, M.E., Villa, P., Guerra, M., Rodriguez. E., Redondo, D. and Martinez, A., 1992. Conversion of furfural into furfuryl alcohol by *Saccharomyces cerevisiae. Acta Biotechnol.* 12, 351-354.

Dien, B.S., Iten, L.B. and Bothast, R.J. 1999. Conversion of corn fibre to ethanol by recombinant E. coli strain FBR3Enzyme microb. Technol. 23, 366-371. *J. Ind. Microbiol. Biotechnol.* **22**: 575 – 581.

Dobereiner, J. and Baldani, V.L.D. 1988 Novas technolgias: biocombustivel. *Biotechnolgia. Ciencia e Desenvolvimento* **1** : 16-18.

Donaldson, L.A., Wong, K.K.Y. and Machie, K.L. 1988. Ultrastructure of steam-exploded wood. *Wood Sci. Technol.*. **22:** 103-114.

Drawert, F. and Klisch, W. 1987. Ethanol – Industrial applications. *Crit. Rev. Biotechnol*. **13**:57 – 98.

Dunlop, A.P., 1948. Furfural formation and behaviour. *Ind. Eng. Chem.* 40 (2), 204-209.

Duncan D.B. 1955. Multiple range and Multiple F-test. *Biometrics* 11 (**1**): 1-42.

Dunn, B., Levin, R.P, Sherlock, G. (2005). Microarray karotyping of commercial wine yeast strains reveals shared, as well as unique, genomic signatures. *BMC Genomics*. 6: 53.

Du Preez, J.C., Bosch, M., Prior, B.A. 1986. The fermentation of hexose and pentose sugars by *Candida shehatae* and *Pichia stipitis. Appl Microbiol Biotech.* **23:** 228-233.

"Ethanol and the Environment Q & A", Renewable Fuels Association, www.ethanolrfa.org/factfic_envir.html

Eken-Saracoglu, N., and Arslan, Y. 2000. Comparison of different pretreatments in ethanol fermentation using corn cob hemicellulosic hydrolysate with *Pichia stipitis* and *Candida shehatae*. *Biotechnol. Lett*. 22, 855-858.

Ekunsami, T.J and Odunfa, S.A. 1990. Ethanol tolerance, sugar tolerance and invertase activities of some yeasts strains isolated from steep water of fermenting cassava tubers. *J. Appl. Bacteriol*. **69**: 672 – 675.

Eliasson, A. Christensson, C., Wahlborn, C.F. and Hahn-Hagerdal, B. 2000. Anaerobic xylose fermentation by recombinant *Saccharomyces cerevisiae* carrying XYL1, XYL2 and XKS1 in mineral medium chemostat cultures. *Appl. Environ.Microbiol.* **66:** 3381-3386.

Eliasson, A., Hofmeyr, J.H.S., Pedler, S. and Hahn-Hagerdal, B. 2001. The xylose reductase/xylitol dehydrogenase/xylulokinase xylose-utilising *Saccharomyces cerevisiae*. *Enzyme Microbiol. Technol*. **29:** 288 – 297.

Eriksson, T., Borjesson, J., and Tjerneld, F. 2002. "Mechanism of surfactant effect in enzymatic hydrolysis of lignocellulose", *Enzyme Microb. Tech.* **31**, 353-364.

Esterbauer, H., Steiner, W., Labudova, I. Hermann, A., Hayn, M. 1991. Production of *Trichoderma* cellulose in laboratory and pilote scale. *Bioresour Technol.* **36:** 51-65.

Ethiraj, S., Onkarayya, H and Suresh, E.R. 1980. A note on the nature and sequence of yeasts during fermentation of apples grown in India. J. of Appl. Bacterial. **48**: 97 – 100.

Fay, J.C. and Benavides, J.A. 2005. Evidence for domesticated and wild populations of *Saccharomyces cerevisiae*. *PloS Genet*. 1:e5. doi: 10.137/journal.pgen.0010005.

Farrell, A. E., R. J. Plevin, B. T. Turner, A. D. Jones, M. O'Hare, and D. M. Kammen. 2006. Ethanol can contribute to energy and environmental goals. *Science* 311**:**506-508

Feng, T., Du, Y., Yang, J., Li, J. And Shi, X. 2006. "Immobilization of a nonspecific chitosan hydrolytic enzyme for application in preparation of water-soluble low-molecular-weight chitosan", *J. Appl. Polym. Sci.* **101(3)**, 1334-1339.

Figueira, J; Janick, J and Miller, J.N. 1993. New products from *Theobroma cacao*: seed pulp and pod gum. In J. Janick and J.E Simon (eds), New crops. Wiley, New York P. 475 – 478.

Fireoved, R.I., Mutharasan, R., 1986. Effect of furfural and ethanol on the growth and energetics of yeast under microaerobic conditions. Ann. N.Y.Ac. Sci., 433-447.

Futcher, A.B., Cox, B.S. 1984. Copy number and the stability of 2-μm circle-based artificial plasmids of *Saccharomyces cerevisiae*. *J. Bacteriol* **157:** 283-290.

Gaffney, J.S. and Marley, N. A. 1997. Potential Air Quality Effects of using Ethanol-Gasoline fuel blends: A field Study in Albuquerque, New Mexico *Environ. Sci. Technol.* **(11) 31**: 3053 – 3061

Galbe, M. and Zacchi, G. 2002. "A review of the production of ethanol from softwood".*Appl. Microbiol. Biotechnol.*. 59(6), 618 – 628.

Gancedo, J.M. 1998. Yeast carbon catabolite repression. *Microbiol. Molecul. Bio. Rev.*, **62**: 334 – 361.

Gancedo, J.M. 1992. Carbon catabolite repression in yeast. *Eur. J. Biochem.* **206:** 297-313.

Gancedo, C. and Serrano, R. 1989. Energy-yielding metabolism. In: *The Yeasts*. Vol. 3, 2nd edn. Eds. Rose. A.H. and Harrison, J.S. London: *Academic Press*. ISBN 0125964137

Gharpuray M.M., Lee, Y.H., Fan L.T. 1983. Structural modification of lignocellulosics by pretreatments to enhance enzymatic hydrolysis. *Biotechnol. Bioeng.*, **25**: 157-172.

Ghose, T.K. 1987. Measurement of cellulase activities. *Pure and Appl. Chem*. 59 **(2):** 257-268.

Gibbs, M., DeMoss, R.D. 1954. Anaerobic dissimilation of C14-labeled glucose and fructose by *Pseudomonas lindneri, J. Biol. Chem* **207:** 689-694.

Gietz, R. D., and R. A. Woods. 2001. Genetic transformation of yeast. *BioTechniques* 30: 816 -820, 822 – 826, 828.

Gong, C.S., Claypool, T.A., McCracken, L.D., Maun, C.M., Ueng, P.P. 1983. Conversion of pentoses by yeasts. *Biotechnol. Bioeng.* **25:** 85-102.

Gorgens, J. F., W. H. van Zyl, J. H. Knoetze, and Hahn-Hagerdal, B. 2001. The metabolic burden of the *PGK1* and *ADH2* promoter systems for heterologous xylanase production by *Saccharomyces cerevisiae* in defined medium, *Biotechnol. Bioeng.* **73:**238-245

Gray, W.D. 1945. The sugar tolerance of four strains of distillers yeast. *J. Bacteriol*. **49**: 445 – 452.

Gregg, D. and Saddler, J.N. 1986. A techno-economic assessment of the pretreatment and fractionation steps of a biomass-to-ethanol process. *Appl. Biochem. Biotechnol*. 711-727.

Grous, W.R., Converse, A.O., Grethlein, H.E. 1986. Effect of steam explosion pretreatment on pore size and enzymatic hydrolysis of poplar. *Enzyme Microbiol. Technol.* **8**, 274-280.

Grethlein H.E. 1985. The effect of pore size distribution on the rate of enzymatic hydrolysis of cellulosic substrates. *Nat. Biotechnol.* **3**:155-160.

Grootjen, D.R.J., van der Lans, R.G.J.M., Luyben, KC.A.M. 1990. Effects of the aeration rate on the fermentation of glucose and xylose by *Pichia stipitis* CBS 5773, *Enzy. Microbiol. Technol*. **12:** 20-23.

Gueldener, U., J. Heinisch, G. J. Koehler, D. Voss, and J. H. Hegemann 2002. A second set of loxP marker cassettes for Cre-mediated multiple gene knockouts in budding yeast. *Nucleic Acids Res*. 30:e23

Goffeau, A., B. G. Barrell, and Bussey, B. 1996. Life with 6000 genes. *Sci*. **274**:546-567

Giudici, P., Solieri, L., Pulvirenti, A.M and Cassanelli, S. 2005. Strategies and perspectives for genetic improvement of wine yeasts. *Appl. Microbiol. Biotechnol*. **66:** 622 – 628.

Gupta, S., Kuhad, R.C. Bhushan, B. and Hoondal, G.S. 2001. Improved xylanase production from a haloalkalophilic Staphylococcus sp. SG-13 using inexpensive agricultural residues. *World J. Microbiol. Biotechnol.* **17**: 5 – 8.

Hahn-Hagerdal, B. 1996. Ethanolic fermentation of lignocellulose hydrolysates. *Appl. Biochem. Biotechnol*.**57/58**, 195-199.

Hahn-Hägerdal, B., M. Galbe, G. Gorwa-Grauslund, G. Lidén, and G. Zacchi**.** 2006. Bio-ethanol: the fuel of tomorrow from the residues of today. *Trends in Biotechnol.* **24**:549-556.

Hahn-Hagerdal, B., Jeppson, H., Skoog, K., and Prior, B. A. 1994. Biochemistry and physiology of xylose fermentation by yeasts. *Enzyme Microb. Technol.* **16**, 933-943.

Hahn-Hagerdal, B.,, C.F. Wahlbom, M. Gardonyi, W.H. van Zyl, R.R. Cordero Otero, and L.J. Jonsson B. 2001. Metabolic engineering of *Saccharomyces cerevisiae* for xylose utilization. *Adv. Biochem. Eng. Biotechnol.* **73**: 53 – 84.

Hahn-Hagerdal, B., Karhumaa, K., Fonseca, C., Spencer-Martins, I., Gorwa-Grauslund, M 2007. Towards industrial pentose-fermenting yeast strains. *Appl Microbiol Biotech*, **74:** 937-953.

Hahn-Hagerdal, B., Jeppsson, H., Olsson, L., Mohagheghi, A. 1994. An interlaboratory comparison of the performance of ethanol-producing micro-organisms in a xylose-rich acid hydrolysate. *Appl. Microbiol. Biotech.* **41:** 62-72.

Hallsworth, J.E., Nomura, Y. and Iwahara, M. 1998. Ethanol-induced water stress and fungal growth. *J. ferment. Bioeng.* **86:** 451 – 456.

Hamacher, T., Becker, J., Gardonyi, M., Hahn-Hagerdal, B., Boles, E. 2002. Characterization of the xylose-transporting properties of yeast hexose transporters and their influence on xylose utilization. *Microbio.* **148:** 2783-2788.

Hamelinck, C.N., Van Hooijdonk, G. and Faaij, A.P.C. 2005. Ethanol from lignocellulosic biomass: techno-economic performance in short-, middle- and long-term. *Biomass Bioenergy*, 28: 384 – 410.

Hamelinck, C.N. 2005. Ethanol from lignocellulosic biomass: techno-economic performance in short-, middle- and long-term, *Biomass and Bioenergy* **28**: 384 – 410.

Hammerschlag, R. 2006. Ethanol's energy return on the investment: a survey of the literature 1990-present. *Environ. Sci. Technol*. **40:**1744-1750

Harrigan, W.F. and McCance, M.E. 1966. *Laboratory Method in Microbiology.* Academic Press London. New York pp. 342.

Hasunuma, T., Tomoya, S., Ryosuke, Y., Kazuya, Y., Jun, I. and Akihiko, K. 2011. Metabolic pathway engineering based on metabolomics confers acetic and formic acid tolerance to a recombinant xylose-fermenting strain of *Saccharomyces cerevisiae*, *Microbial cell factories*, 10: 2 (http://www.microbialcellfactories.com/content/10/1/2)

Haug, R.T. 1993. *The Practical Handbook of Compost Engineering.* Lewis Publishers, Boca Raton, Florida, USA.

Heipieper, H.J., Weber, F.J., Sikkema, J., Kewelo, H., de Bont, J.A.M., 1994. Mechanism of resistance of whole cells to toxic organic solvents. *TIBTECH* **12**, 209-415.

Helle, S.S., Murray, A., Lam, J., Cameron, D.R., Duff, S.J.B. 2004. Xylose fermentation by genetically modified *Saccharomyces cerevisiae* 259ST in spent sulfite lilquor. *Bioresource Technol.* **92:** 163-171.

Hendriks, A.T.W.M and Zeeman, G. 2009. Pretreatment to enhance the digestibility of lignocellulosic biomass. *Biores. Technol.* **100**: 10 – 18.

Hinman, N.D., Wright, J.D., Hoagland, W., and Wyman, C.E. 1989. Xylose fermentation: an economic analysis. *Appl. Biochem. Biotechnol.* **20/21**, 391-401.

Hirosawa, I., K. Aritomi, H. Hoshida, S. Kashiwagi, Y. Nishizawa, and R. Akada**.** 2004. Construction of a self-cloning sake yeast that overexpresses alcohol acetyltransferase gene by a two-step gene replacement protocol. *Appl. Microbiol. Biotechnol*. **65:**68-73.

Ho, N.W., Chen, Z., Brainard, F.P. and Sedlak M. 1999. Successful design and development of genetically engineered *Saccharomyces* yeast for effective co-fermentation of glucose and xylose from cellulosic biomass to fuel ethanol. *Adv. Biochem. Eng. Biotechnol.* **65**, 163-192.

Hohmann, S. and Mager, W.H. 2003. Y*east stress Responses*. Berlin: Springer – Verlag.

Holzapfel, W.H. 2002. Appropriate starter culture technologies for small-scale fermentation in developing countries. *Int. J. Food Microbiol*. **75**: 197 – 212.

Holtzapple, M.T., Jun, J.H., Ashok, G., Patibandla, S.L. and Dale, B.E. 1991. "The ammonia freeze explosion (AFEX) process – A practical lignocellulose pretreatment", *Appl. Biochem. Biotech* 29-9, 59-74.

Huang, X.P. and Monk, C. 2004. Purification and characterization of a cellulose (CMCase) from a newly isolated thermophilic, aerobic bacterium *Caldibacillus cellulovarans* gen. nov., sp. nov. *World J. Microbiol.Bbiotechnol.* **20** : 85 – 92.

Hung, L. 1998. The structure and function of yeast xylose (Aldose) reductase. *Yeast*, 14: 977 – 984.

Imai, T., Ohono, T. 1995. The relationship between viability and intracellular pH. In the yeast *Saccharomyces cerevisiae. Appl. Environ. Microbiol.* **61**, 3604-3608.

Ingram, L.O., Gomez, P.F., Lai, X., Moniruzzaman, M., Wood, B.E., Yomano, L.P. and York, S.W. 1998 Metabolic engineering of bacteria for ethanol production. *Biotechnol. Bioeng.* **58:** 204-214.

Iyer, P.V. WZW, Kim, S.B., Lee, Y.Y. 1996. Ammonia-recycled percolation process for pretreatment of herbaceous biomass, *Appl. Biochem Biotechnol***. 57/58**: 121-132

Janke, C., M. M. Magiera, N. Rathfelder, C. Taxis, S. Reber, H. Maekawa, A. Moreno-Borchart, G. Doenges, E. Schwob, E. Schiebel, and Knop, M. 2004. A versatile toolbox for PCR-based tagging of yeast genes: new fluorescent proteins, more markers and promoter substitution cassettes. *Yeast* **21**:947-962.

Jeffries, T.W. and Jin, Y.S 2004. Metabolic engineering for improved fermentation of pentoses by yeasts. *Appl. Microbiol. Biotechnol.* **63:** 495 – 509.

Jeffries, T. W. 1985. Emerging technology for fermenting D-xylose. *Trends in Biotechnol.* **3:**208-212.

Jeffery H. Miller ,1992. A short course in Bacterial Genetics. A laboratory manual and Handbook for Escherichia coli and related bacteria. *Cold spring Harbor Laboratory Press*.

Jeffries, T. W. 1983. Utilization of xylose by bacteria, yeasts, and fungi. *Advanced Biochem. Engin. Biotechnol.* **27**:1-32

Jeffries, T.W. and Jin, Y.S. 2000. Ethanol and thermotolerance in the bioconversion of xylose by yeasts. *Adv. Appl. Microbiol.* **47:** 221-268.

Jeffries, T.W 2005. Ethanol fermentation on the move. *Nat. Biotechnol.* **23 (1)**: 40 - 41

Jeffries, T.W. 2006. Engineering yeasts for xylose metabolism. *Curr. Opin. Biotechnol.* **17:** 320-326.

Jeffries, T.W., Grigoriev, I.V., Grimwood, J., Laplaza, J.M., Aerts, A., Salamov, A., Schmutz, J., Lindquist, E., Dehal, P., Shapiro, D., Yong-Su, J., Passoth, V. and Richardson, P.M. 2007. Genome sequence of the lignocellulose-bioconverting and xylose-fermenting yeast *Pichia stipitis. Nat. Biotechnol*. **3:** doi: 10.1038/nbt1290

Josefsson, T., Lennholm, H. and Gellerstedt, G. 2001. Changes in cellulose supramolecular structure and molecular weight distribution during steam explosion of aspen wood. *Cellulose*, **8**: 289 – 296.

Jin, Y.S., Laplaza, J.M, and Jefferies, T.W. 2004 *Saccharomyces cerevisiae* engineered for xylose metabolismo exhibits a respiratory response. *Appl. Environ. Microbiol.* **70:** 6816 – 6825.

Jill, L.R. and Bonita, A.G. 1998. Response of cultures of *Propionibacterium* to acid and low pH: Tolerance and inhibition. *J. Food Protect.* **61**: 211 – 216.

Jonsson, L.J., Palmqvist. E., Nilvebrant, N.O. and Hahn-Hagerdal, B. 1998. Detoxification of wood hydrolysates with laccase and peroxidase from the white-rot fungus *Trametes versicolor. Appl.Microbiol. Biotechnol.* **49:** 691-697.

Kaiser C., Susan, M. and Aaron M. 1994. *Methods in Yeast Genetics*. A cold spring Harbor Laboratory Course Manual.

Kaisa, K., Sanchez, R.G., Hahn-Hagerdal, B. and Gorwa-Grauslund, M.F. 2007. Comparison of the xylose reductase-xylitol dehydrogenase and the xylose isomerase pathways for xylose fermentation by recombinant *Saccharomyces cerevisiae*. *Microbial cell factories*, **6:5,** doi:10.1186/1475-2859-6-5.

Karhumaa, K., Hahn-Hagerdal, B., Gorwa-Grauslund, M.F. 2005. Investigation of limiting metabolic steps in the utilization of xylose by recombinant *Saccharomyces cerevisiae* using metabolic engineering. *Yeast.* **22:** 359-368.

Katahira S., Mizuike A., Fukuda H. and Kondo A. 2006. Ethanol fermentation from lignocellulosic hydrolysate by a recombinant xylose – and cellooligosaccharide-assimilating yeast strain. *Appl. Microbiol. Biotechnol.,* doi 10.1007/s00253-006-0402-x.

Kerr A.J. and Goring D.A. 1975. Ultrastructural arrangement of the wood cell wall. *Cellul chem. Technol*, 9: 563-573.

Khayami-Horani, H. 1996. Partial purification and some properties of an alkaline cellulose from an alkalophilic *Baccillius sp. World J. Microbiol. Biotechnol.* **12**: 525 – 529.

Kilian, S.G., Uden, N. 1988. Transport of xylose and glucose in the xylose-fermenting yeast *Pichia stipitis, Appl Microbiol Biotech,* **27:** 545-348.

Kim, J.S., Lee, Y.Y., Park, S.C. 2000. Pretreatment of wastepaper and pulp mill sludge by aqueous ammonia and hydrogen peroxide. *Appl. Biochem Biotechnol.*. **84/86:** 129-139.

Kim, T.H., Kim, J.S. Sunwoo, C and Lee, Y.Y. 2003. Pretreatment of corn stover by aqueous ammonia. *Bioresour. Technol*, **90:** 39 – 47

Kim, S.B., Kim, H.J. and Kim, C.J. 2006a. "Enhancement of the enzymatic digestibility of waste newspaper using Tween", *Appl. Biochem. Biotech.* 129-132, 486-495.

Klemm, D., Brigitte, H., Hans-Peter, F. and Andreas, B. 2005. "Cellulose: Fascinating Biopolymer and Sustainable Raw material". *ChemInform 36 (36). Doi:10.1002/chin.200536238*

Klinner, U., and Schafer, B. 2004. Genetic aspects of targeted insertion mutagenesis in yeasts. *FEMS Microbiol. Rev.* **28**:201-223

Klinner, U., Fluthgraf, S., Freese, S and Passoth, V. 2005. Aerobic induction of respire-fermentative growth by decreasing oxygen tensions in the respiratory yeast *Pichia stipitis. Appl. Microbiol. Biotechnol.* **67**: 247 – 253.

Klinke, H.B, Olsson, L., Thomsen A.B., Ahring, B.K. 2003. Potential inhibitors from wet oxidation of wheat straw and their effect on ethanol production of *Saccharomyces cerevisiae* wet oxidation and fermentation by yeast. *Biotechnol. Bioeng.* **3- 81**: 738 – 747.

Kliskinen, L. L; Viikari, L and Kruss. 2002. Purification and Characterization of novel laccasse from *Ascomycetes melanocarpus albomyces*. *Appl. Microbiol. Biotech*. **59**: 198 - 204.

Knocke, C. and Vogt J. 2009. Biofuels - challenges and chances: How biofuel development can benefit from advanced process technology**.** *Eng. Life Sci.* **2**: 96 - 99.

Kotter, P., Amore, R., Hollenberg, C.P. and Ciriacy, M. 1990. Isolation and characterization of the *Pichia stipitis* xylitol dehydrogenase gene, XYL2, and construction of a xylose-utilizing *Saccharomyces cerevisiae* transformant *J. Curr. Gen.* **18 (6)**: 493-500

Kotter, P. and Ciriacy, M. 1993. Xylose formation by *Saccharomyces cerevisiae*. *Appl. Microbiol. and Biotechnol.*, **38:** 776-783.

Koullas, D.P., Christakopoulos, P., Kekos, D., Macris, B.J., Koukios, E.G. 1992. Correlating the effect of pretreatment on the enzymatic hydrolysis of straw. *Biotechnol. Bioeng*. **39,** 113-116.

Krishnan, M.S., Ho, N.W., Tsao, G.T. 1999. Fermentation kinetics of ethanol production from glucose and xylose by recombinant *Saccharomyces* 1400 (pLNH33). *Appl. Biochemist. Biotechnol.* **77-79**: 373 – 388.

Kruckeberg, A.L. 1996. The hexose transporter family of *Saccharomyces cerevisiae*. *Arch Microbiol*. **166:** 283-292.

Kumar, S. and Ramon, D. 1996. Purification and regulation of the synthesis of a B-xylosidase from Aspergillus nidulans. *FEMS Microbiol. Lett.*, 135: 287 – 293.

Kurtzman, C.P. 1990. *Candida shehatae* – Genetic diversity and phylogenic relationship with other xylose –fermenting yeast. *Antonie Van Leeuwenhoek*, ***57:*** 215 – 222.

Kuyper, M., Aeron, A.W, Johannes, P., Van Dijken, J.P and Jack, T.P. 2004. Minimal metabolic engineering of *Saccharomyces cerevisiae* for efficient anaerobic xylose fermentation: a proof of principle.

Kuyper, M., Hartog, M.M., Toirkens, M.J., Almering, M.J., Winkler, A.A., Van Dijken, J.P., Pronk, J.T. 2005a. Metabolic engineering of a xylose-isomerase-expressing *Saccharomyces cerevisiae* strain for rapid anaerobic xylose fermentation. *FEMS Yeast Res*. **5:** 399-409.

Kuyper M., Toirkens, M.J., Diderich, J.A., Winkler, A.A., Van Dijken, J., Pronk, J.T. 2005b. Evolutionary engineering of mixed-sugar utilization by a xylose-fermenting *Saccharomyces cerevisiae* strain. *FEMS Yeast Res*. **5:** 925-934.

Kvitek, D.J., Will, J.L, Gasch, A.P 2008. Variations in stress sensitivity and genomic expression in diverse *Saccharomyces cerevisiae* isolates. *PloS Genet*ics.4:e1000223. doi 10.137/journal.pgen.1000223.S

Lapierre, C., Rolando, C. and Monties, B. 1983. Characterization of poplar lignins acidolysis products: capillary gas-liquid and liquid-liquid chromatography of monomeric compounds. *Holzforschung* 37, 189-198.

Larsson S, Palmqvist E, Hahn-Hagerdal B, Tengborg C, Stenberg K, Zacchi G, and Nilvebrant N.O 1998. The generation of fermentation inhibitors during dilute acid hydrolysis of softwood. *Enzyme Microb Technol*, 3-4:151-159.

Larsson S, Nilvebrant NO, Jonsson, L.J. 2001. Effect of overexpression of *Saccharomyces cerevisiae* Pad1p on the resistance to phenylacrylic acids and lignocellulose hydrolysates under aerobic and oxygen-limited conditions**.** *Appl.Microbiol. Biotechnol.* **57:**167-174

Larsson S, Cassland P, Jonsson L.J. 2001. Development of a *Saccharomyces cerevisiae* strain with enhanced resistance to phenolic fermentation inhibitors in lignocellulose hydrolysates by heterologous expression of laccase**.** *Appl. Environ. Microbiol.* **67:**1163-1170.

Larsson, S., Palmqvist, E., Hahn-Hagerdal, B., Tengborg, C., Stenberg, K., Zacchi,m G. and Nilvebrant, N.O. 1999. The generation of fermentation inhibitors during dilute acid hydrolysis of softwood. *Enzy. Microbial Technol.* **24**: 151-159.

Larsson, S., Reimann, A., Nilvebrant, N.O. and Jonsson, L.J. 1999. Comparison of different methods fr the detoxification of lignocellulosic hydrolysates of spruce. *Appl. Biochem. Biotechnol.* **77**-79, 91-103.

Larsson, S., Quintana-Sainz, A., Reimann, A., Nilvebrant, N.O. and Jonsson, L.J. 2000. Influence of lignocellulose-derived aromatic compounds on oxygen-limited growth and ethanolic fermentation by *S. cerevisiae. Appl. Biochem. Biotechnol.* **84**-86, 176-182.

Laureano-Perez, L., Taymouri, F., Alizadeh, H., Dale, B.E. 2005. Understanding factors that limit enzymatic hydrolysis of biomass. *Appl. Biochem. Biotechnol*., 1081-1099.

Lawford, H.G. and Rousseau, J.D. 1992. Effect of acetic acid on xylose conversion to ethanol by genetically engineered. *Escherichia coli. Appl. Biochem. Biotechnol.* **34-35:** 185-204.

Lee, J. 1997. Biological conversion of lignocellulosic biomass to ethanol. *J. Biotechnol.* **56:** 1-24.

Lee, Y.Y., Iyer, P. and Torget, R.W. 1999. "Dilute-acid hydrolysis of lignocellulosic biomass", *Advanced Biochemistry. Eng./Biotechnol.* **65** *(Recent Progress in Bioconversion of Lignocellulosics*), 93-115.

Lee, G.W., and Chang, H.N. 1997. Kinetics of ethanol fermentations in membrane cell recycle fermentors. *Biotechnol. Bioeng.* **24**: 1105-1112.

Lee, Y.H. and Fan, L.T. 1983. Kinetic studies of enzymatic hydrolysis of insoluble cellulose. (II) Analysis of extended hydrolysis times. *Biotechnol. Bioeng.*. **25:** 939-966.

Lee, W.J., Kim, M.D., ryu Y.W., Bisson, L.F., Seo, J.H.: 2002. Kinetic studies on glucose and xylose transport in *Saccharomyces cerevisiae, Appl Microbiol Biotech*, **60:** 186-191.

Lee, J.M. and Woodward, J. 1983. "Properties and application of immobilized β-glucosidase coentrapted with *Zymomonas mobilis* in calcium alginate". *Biotech. Bioeng.* 15(1), 1-24.

Lee, H., Biely, P., Latta, R.K., Barbosa, M.F.S. and Schneider, H. 1986. Utilization of xylan by yeasts and its conversion to ethanol by *Pichia stipitis strains. Appl. Environ. Microbiol*. **52:** 320 – 324.

Leroy, F. and De Vuyst, L. 2004. Lactic acid bacteria as functional starter cultures for the food fermentation Industry. *Trends Food sci. Technol*. **15:** 67 – 78

Ligthelm, M.E., Prior, B.A., Preez, J.C. 1988. The oxygen requirements of yeasts for the fermentation of d-xylose and d-glucose to ethanol. *Appl. Microbiol. Biotech* **28:** 63-68.

Linden, T., Peetre, J., and Hahn-Hagerdal, B. 1992. Isolation and characterization of acetic acid tolerant galactose fermenting strains of *Saccharomyces cerevisiae* from a spent sulfite liquor plant. *Appl.Environ. Microbiol.* **58**: 1661-1669.

Linden, T. and Hahn-Hagerdal, B. 1989. fermentation of lignocellulose hydrolysate with yeast and xylose isomerase. *Enzyme Microbiol. Technol*. **11:** 583 - 589

Lin, K.W., Ladisch, M.R., Voloch, M., Patterson, J.A., Noller, C.H.1985. Effect of pretreatment and fermentation on pore size in cellulosic materials. *Biotechnol. Bioeng.*, **27**: 1427-1433.

Ling, Y., Aurthur, L.K., Jan, A.B. and Van Dam, K. 1999. Growth and glucose repression are controlled by glucose transport in *S. cerevisiae* cells containing only one glucose transporter. *J. Bacteriol.* (15), 181: 4673 – 4675.

Liti, G., Carter, D.M., Moses, A.M., Warringer, J., Parts 2009. Population genomics of domestic and wild yeasts. *Nat.* **458**: 337 – 341.

Liu, Z.L, Slininger P.J, Dien, B.S., Berhow MA, Kurtzman C.P. and Gorsich S.W. 2004. Adaptive response of yeasts to furfural and 5-hydroxymethylfurfural and new chemical evidence for HMF conversion to 2,5-bis-hydroxymethylfuran.*J. Ind. Microbiol. Biotechnol.* **31**:345-352.

Lodder, J and Kreger-Van rij, N.J.W. 1952. "The Yeast". A Taxonomic study. North Holland Publishing Co. Amsterdam.

Lynd, L.R., Weimer, P.J., van Zyl, W.H., Pretorius, I.S. 2002. Microbial Cellulose Utilization: Fundamentals and Biotechnology. *Microbiol Mol. Biol. Rev.* **66:** 506-577.

Lynd, L. R., M. S. Laser, D. Bransby, B. E. Dale, B. Davison, R. Hamilton, M. Himmel, M. Keller, J. D. McMillan, J. Sheehan, and C. E. Wyman**.** 2008. How biotech can transform biofuels. *Nat. Biotechnol.* **26**:169-172.

Lyer P.V. WZW, Kim S.B., Lee Y.Y. 1996. Ammonia-recycled percolation process for pretreatment of herbaceous biomass, *Appl. Biochem Biotechnol*, **57/58**: 121-132.

Lyons, T. F., Kelsall, D. and Murtagh, J. eds 1995. The Alcohol textbook, Nottingham University Press, Nothingham, U.K.

MacDonald D.G. Bakhshi N.N, Mathews J.F. Roychowdhury A., Bajpai, P. and Murry M.Y.1983. Alkali treatment of corn stover to improve sugar production by enzymatic hydrolysis. *Biotechnol. Bioeng.* **25**: 2067-2076.

Malek, V.S. 1989. Biotechnology – The golden Age. *Adv. Appl. Microbiol.* **34**: 264 – 307.

Majumdar, P. and Chanda, S. 2001. Chemical profile of some lignocellulosic crop residues. *Indian J. Agric. Biochemist.* 14 (1 & 2), 29-33.

Mandels, M and Reese, E.T. 1956. Induction of cellulose in *Trichoderma viride* as influenced by carbon sources and metals. Biology branch, Pioneering Research Division, U.S Army Quartermaster Research and Development Center, Natick, Massachusetts, **73**: 269 - 278

Martinez, A., Rodriguez, M.E., York, S.W., Preston, J.F., and Ingram, L.O. 2000. Effect of $Ca(OH)_2$ treatments (over-liming) on the composition and toxicity of bagasse hemicellulosic hydrolyzates. *Biotechnol. Bioeng.* **69**: 526-536.

Martin C., Galbe M., Wahlbom C.F., Hahn-Hagerdal B. and Jonsson L.J. 2002. Ethanol production from enzymatic hydrolysates of sugarcane bagasse using recombinant xylose-utilizing *Saccharomyces cerevisiae. Enz. Microbiol. Technol*. **31**: 274-282.

Martin C., Wahlbom, F., Galbe M., Jonsson L.J and Hahn-Hagerdal B., 2001. Preparation of sugarcane bagasse hydrolysates for alcoholic fermentation by yeasts. *In Proceeding of sixth Brazillian Symposium on the Chemistry of Lignins and other wood components*, vol. 7, eds Teixeira da Silva, F., Ferraz, A & Paiva, T.C.B. pp. 361 – 367.

Martin-Carlo, Fernandez, T., Garcia, R., Carrillo, E., Marcet, M., Galbe, M and Jonsson, L.F. 2002. Preparation of hydrolysates from tobacco stalks and ethanolic fermentation by *Saccharomyces cerevisiae*, *World J. Microbiol. Biotechnol*. **18:** 857 – 862

Martins, L.O; Soares, C.M; Pereora, M., Teixeira, T., Costa, G.H. Jones and A.O Henriques. 2002. Molecular and Biochemical characterization of a highly stable bacterial laccase that occur as a structural component of the *Bacillius subtilis* endospore coat. J. Biol. Chem., **277**: 18849 – 18859.

McMillan, J.D. 1994. "Pretreatment of lignocellulosic biomass", in ACS Symposium Series (*Enzymatic Conversion of Biomass for Fuels Production),* Himmel, M.E., Baker, J.O. and Overend, R.P. (eds.), 292-324.

McMillan, J.D. 1997. Bioethanol production: status and prospects. *Renewable Energy*, **2-3:**295-302

Meinander, N.Q., and Hahn-Hagerdal, B. 1977. Influence of cosubstrate concentration on xylose conversion by recombinant, XYL1-expressing *Saccharomyces cerevisiae:* a comparison of different sugars and ethanol as cosubstrates, *Appl. Environ. Microbiol*, **63:** 1959-1964.

Meinander, N.Q,, Boels, I., Hahn-Hagerdal, B. 1999. Fermentation of xylose/glucose mixtures by metabolically engineered *Saccharomyces cerevisiae* strains expressing XYL1

and XYL2 from *Pichia stipitis* with and without overexpression of TAL1, *Biores. Technol*, **68 (1):** 79-87.

Melake, T., Passot, V.V. and Klinner, U. 1996. Characterization of the genetic system of the xylose-fermenting yeast *Pichia stipitis. Curr. Microbiol.* **33**: 237 – 242

Mes-Hartree, M., and Saddler, J.N. 1983. "The nature of inhibitory materials present in pretreated lignocellulosic substrates which inhibit the enzymic hydrolysis of cellulose," *Biotechnol. Lett.* 5(8), 531-536.

Meynell, G.C and Meynell E. 1970. Theory and Practice in experimental bacteriology. 2nd ed. Cambridge Uni. Press pg. 34.

Mikulaasova, M., Vodny, S. and Pekarovicova. A. 1990. Influence of phenolics on biomass production by *Candida utilis* and *Candida albicans. Biomass* 23, 149-154.

Mills, C., Child, J.J. and Spencer, J.F.T., 1971. The utilization of aromatic compounds by yeasts, A. van Leeuw. 37, 281-287.

Miller,G.L 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal. Chem***. 31**: 426 – 8.

Miller, J.H. 1992. "A short course in bacterial genetics: A Laboratory Mannual and hand book for *Escherichia coli* and related bacteria" Cold Spring Harbour Laboratory Press, Cold Spring Harbour, N.Y.

Mishra, P., and Singh, A. 1993. Microbial pentose utilization. *Advanced and Applied Microbiology.* 39**:** 91-152.

Mishra, P. and Prasad, R. 1989. Relationship between ethanol tolerance and fatty acyl composition of *Saccharomyces cerevisiae. Applied Microbiology and Biotechnology* 30: 294 – 298.

Mohanty, A.K. 2000. Biofibres, biodegradable polymers and biocomposites: An overview. *Macromolecular Matter. Eng.*276/277, 1-24.

Mosier, N., Wyman, C., Dale, B., Elander, R., Lee, Y.Y., Holtzapple, M. and Ladisch, M. 2005. Features of promising technologies for pretreatment of lignocellulosic biomass, *Bioresources Technology*. 96: 673-686.

Moniruzzaman M., Dien B.S., Skory C.D., Chen Z.D., Hespell R.B., Ho N.W., Dale B.E. and Bothast R.J. 1998. Fermentation of corn fibre sugars by an engineered xylose utilizing *Saccharomyces* yeast strain. *World J. Microbiol. Biotechnol.* **13**:341-346.

Muller, S., Boles, E., May, M., Zimmermann, F.K. 1995. Different internal metabolites trigger the induction of glycolytic gene-expression in *Saccharomyces cerevisiae*. *J. Bacteriol*, **177:** 4517-9

Myers, H.M., Montgomery, D.C. 1991. Response surface methodology: process and product optimization using designed experiments. Wiley-Interscience. New York.

Nair, G.S., Sindhu, R. and Shashidhar, S. 2008. Fungal xylanase production under solid state and submerged fermentation conditions. African. *J. Microbiol. Res.* **2***: 082 – 086.*

Nancy W. Y. Ho, Zhengdao Chen, and Brainard, A.P. 1998. Genetically Engineered Saccharomyces Yeast capable of effective co-fermentation of glucose and xylose. *Appl. Environ. Microbiol*. ***(5)*** **64**:1852 – 1859.

Navarro, A.R. 1994. Effects of furfural on ethanol fermentation by *Saccharomyces cerevisiae:* mathematical models. *Curr. Microbiol*. **29**, 87-90.

Narasimha, G., Sridevi, A., Buddolla, V., Subhosh, C.M., and Rajasekhar, R.B. 2006. Nutrient effects on production of cellulolytic enzymes by *Aspergillus niger*. *African J. Biotechnol.* **5**:472 – 476.

Nardi, J.B. 2006. Communities of microbes that inhabit the changing hindgut landscape of a subsocial beetle. *Arth. Struct. Dev*. **35:** 57 – 68.

Nevoigt, E. 2008. Progress in Metabolic Engineering of *Saccharomyces cerevisiae*. *Microbiology and Molecular Biology Review*. 72: 379-412

Nguyen, Q.A. 1993. Economic analyses of integrating a biomass-to-ethanol plant into a pulp/saw mill. In *Bioconversion of Forest and Agricultural Residues.* Ed. Saddler, J.N. pp. 321-334. Wallingford: CAB International. *ISBN 051897982*.

Nguyen, Q.A., Tucker, M.P., Boynton, B.L., Keller, F.A., Schell, D.J. 1998. Dilute acid pretreatment of softwoods. *Appl. Biochem Biotechnol***. 70/72:** 77-87.

Nilvebrant, N.O., Reimann, A., Sousa, F., Kleen, M., Palmqvist, E., 1997. Identificationof fermentation inhibiting compounds from acid hydrolysis of wood. In: Proceedings of the Nineth International Symposium on Wood and Pulping Chem., Montreal, 79, pp. 1-4.

Nilvebrant, N.O., Reimann, A., Kleen, M., Larsson, S., Palmqvist, E., in preparation. Chemical characterization of fermentation inhibiting dilute acid hydrolysates from spruce.

Novak, M., Strehaiano, P., Moreno, M and Goma, G. 1981. Alcoholic fermentation: on the inhibitory effect of ethanol. *Bacteriol and Bioeng.* **23**: 201

Obi, K.C.S. 1993. Alternative energy sources through Biotechnology. *Proceeding of the National Workshop on Biotechnology in National Development*. Festac '77 Durban Hotel, Lagos, 25th - 28th April, 1993.

Ogier, J.C., Ballerini, D., Leygue, J.P., Rigal, L. and Pourquie, J. 1999. "Ethanol production from lignocellulosic biomass," *Oil / Gas Science and Technology/Revue de l'lnstitut Francais du Petrole* **54(1**). 67-94.

Ohgren K., Bengtsson O., Gorwa-Grauslund M.F., Galbe M., Hahn-Hagerdal B., Zacchi G. 2006. Simultaneous saccharification and co-fermentation of glucose and xylose in steam-pretreated corn stover at high fiber content with *Saccharomyces cerevisiae* TMB3400. *J. Biotechnol*. Dio 10.1016/j.jbiotec.2006.05.001.

Ohgren K., Rudolf, A., Galbe, M. and Zacchi, G. 2006. Fuel ethanol production from steam-pretreated corn stover using SSF at higher dry matter content. *Biomass Bio-eng.* **30**: 863 – 869.

Ohgren, K., Gelbe, M., Zacchi, G. 2005. Optimization of steam pretreatment of SO_2-impregnated corn slover for fuel ethanol production. *Appl. Biochem. Biotechnol.* **121/124**: 1055-1067.

Olofsson K., Rudolf A. and Liden, G. 2008. Designing simultaneous saccharification and fermentation for improved xylose conversion by a recombinant strain of *Saccharomyces cerevisiae. J. Biotechnol*, **134**: 112 – 120.

Olsson, L. and Hahn-Hagerdal, B: 1993. Fermentative performance of bacteria and yeasts in lignocellulose hydrolysates. *Process Biochem.* **28:** 249-257.

Olsson, L. and Hahn-Hagerdal, B. 1996. Fermentation of lignocellulosic hydrolysates for ethanol production. *Enz.yMicrobial Technol*. **18**: 312-331.

Olsson, L., Hahn-Hagerdal, B., and Zacchi, G. 1995. Kinetics of ethanol production by recombinant *Escherichia coli* KO11.*Biotechnol. Bioeng.* **45**, 356-365.

Olsson, L. and Nielsen, J. 2000. The role of metabolic engineering in the improvement of *Saccharomyces cerevisiae*: utilization of industrial media. *Enzy. Microbial Technol.* **26**: 785 – 792.

Olsson, K., Magnus, B. and Gunnar, L. 2008. A short review on SSF – an interesting process option for ethanol production from lignocellulosic feedstocks. *Biotechnology for Biofuels*, 1: 7 doi: 10.1186/1754 – 6834 -1-7.

Ojumu, T.V., Solomon, B.O., Betiku, E., Layokun, S.K. and Amigun, B. 2003. Cellulase production by *Aspergillus flavus* Linn isolate NSPR 101 fermented in sawdust, bagasse and corncob. *African J. Biotechnol.* **2:** 150-152.

Ortega, N., Busto, M.d., and Perez-Mateos, M. 2001. "Kinetics of cellulose saccharification by *Trichoderma reesei cellulases," Int. Biodeterior. Biodegrad.* **47(1)**, 7-14.

Ostergaad, S., Olsson, L. and Nielsen, J. 2000. Metabolic Engineering of *Saccharomyces cerevisiae. Microbiol. Molecular Biol. Rev.* **64**: 34 – 50.

Overend R.P. and Chornet E. 1987. Fractionation of lignocellulosics by steam-aqueous pretreatments. *Phil. Trans. Roy. Soc. Lond.* **321**: 523-536.

Oura, E. 1977. Reaction products of yeast fermentations. *Process Biochem.* 19-35.

Palmowski, L. and Muller, J. 1999. Influence of the size reduction of organic waste on their anaerobic digestion. In: II International Symposium on Anaerobic Digestion of Solid Waste. *Barcelona* 15 – 17 June, pp 137 – 144

Palmqvist, E., Hahan-Hagerdal, B., Szebgyel, A., Zacchi, G., and Reczey, K. 1997. Simultaneous detoxification and enzyme production of hemicellulose hydrolysates obtained after steam pretreatment. *Enzy. Microbial Technol.* **20**: 286-293.

Palmqvist, E., Galbe, M., and Hahn-Hagerdal, B. 1998. Evaluation of cell recycling in continuous fermentation of enzymatic hydrolyzates of spruce with *Saccharomyces cerevisiae* and on-line monitoring of glucose and ethanol. *Appl. Biochem.Biotechnol.* **50**, 545-551.

Palmqvist, E., Almeida, J. and Hahn-Hagerdal, B. 1999. Influence of furfural on anaerobic glycolytic kinetics of *Saccharomyces cerevisiae* in batch culture. *Biotechnol. Bioeng.* **62**: 447- 454.

Palmqvist. E., Meinander, Q., Grage, H. and Hahn-agerdal, B. 1999. Main and interaction effects of acetic acid, furfural and phydroxybenzoic acid on growth and ethanol productivity of yeasts. *Biotechnol. Bioeng.* **63**: 46-55.

Palmqvist, E. and Hahn-Hägerdal, B. 2000. Fermentation of lignocellulosic hydrolysates, II: inhibitors and mechanisms of inhibition**.** *Bioresource Technol.* **1:**25-33.

Palonen, H., Thomsen, A.B., Tenkanen, M., Schmidt, A.S., Vilkari, L. 2004. Evaluation of wet oxidation pretreatment for enzymatic hydrolysis of softwood. *Appl. Biochem. Biotechnol.* **117**, 1-17.

Pampulha, M.E. and Loureiro-Dias, M.C., 1989. Combined effect of acetic acid, pH and ethanol on intracellular pH of fermenting yeast. *Appl. Microbiol. Biotechnol*. **31**, 547-550.

Parent, S. A., and K. A. Bostian 1995. Recombinant DNA technology: yeast vectors, p. 121-178. *In* A. E. Wheals, A. H. Rose, and J. S. Harrison (ed.), The yeasts, vol. 6. Yeast genetics. *Academic Press, London, United Kingdom.*

Parisi, F. 1989. Advances in lignocellulosic hydrolysis and in the utilization of the hydrolysates. *Advan. Biochem.l Eng.* **38**:53-87.

Panchal, C. J. 1990. Yeast strain selection. Marcel Dekker, Inc., New York, NY.

Passoth,V., Hansen, M., Klinner, U. and Emeis. 1992. The electrophoretic banding pattern of the chromosomes of *Pichia stipitis* and *Candida shehatae*. *Curr. Genet.* ***22:*** 429 – 431

Passoth, V., Cohn, M., Schafer, M., Hahn-Hagerdal, B. and Klinner, U. 2003. Analysis of the hypoxia-induced ADH2 promoter of the respiratory yeast *Pichia stipitis* reveals a new mechanism for sensing of oxygen limitation in yeast. *Yeast* **20**: 39 - 51

Pelczar, M.J., Chan, E.C.S and Krieg, N.R. 1993. Microbiology: Concepts and Applications. International Eds. McGraw Hill Inc., N.Y.

Percival Zhang, Y.-H., M. E. Himmel, and Mielenz, J.R. 2006. Outlook for cellulase improvement: screening and selection strategies. *Biotechnol. Adv.* ***24****:452-481*

Perez, Perez J., Munoz, d., Munoz-Dorado, J., de la R., Rubia Tdl, Martinez, Martinez, J. 2002. Biodegradation and biological treatments of cellulose, hemicellulose and lignin: an overview. *Int. Microbiol.* **5**:53-63.

Petersson A, Almeida JRM, Modig T, Karhumaa K, Hahn-Hägerdal B, Gorwa-Grauslund MF, Liden G (2006) : A 5-hydroxymethyl furfural reducing enzyme encoded by the *Saccharomyces cerevisiae* ADH6 gene conveys HMF tolerance. *Yeast* **23**:455-464.

Picataggio, S., and Zhang, M. 1996. Biocatalyst development for bioethanol production from hydrolyzates. *In* "Handbook on Bioethanol: Production and Utilization" (C.E. Wyman, ed.), pp 21-35. Taylor and Francis, Washington, DC

Pitkanen, J.P., Aristidou, A., Salusjarvi, L., Ruohonen, L., Penttila M. 2003. Metabolic flux analysis of xylose metabolism in recombinant *Saccharomyces cerevisiae* using continuous culture, *Metab. Eng.*, **5:** 16-31.

Popoff, T., Theander, O., 1976. Formation of aromatic compounds from carbohydrates part III. Reaction of D-glucose and D-fructose in slightly acidic, aqueous solution, *Acta Chem. Scand. B* 30, 397-402.

Prescott, L.M; Harley, J.P and Klein, D.A. 1999. Microbiology. Metabolism: Energy, Enzymes and regulations. ISBN 0-07-112259-1. Fifth edition, New York. McGraw-Hill press.

Price, E.C. 1985: Microbiology of Anaerobic *Digestion in Biotechnology Application and Research.* PN. Cheremisinoff and R.P. Quellette, Eds. Lancaster, P.A.: *Technomic Pub.* Co., Inc. pp. 52-59.

Prior, B.A., Kilian S.G., du Preez J.C. 1989. Fermentation of D-xylose by the Yeasts Candida Shehatae and *Pichia Stipitis***.** *Process Biochem.* **24:**21-32.

Puls J., Poutanen, K., Korner, H.U. and Viikari, L.1985. Biotechnical utilization of wood carbohydrates after steaming pretreatment. *Appl.Microbiol. Biotechnol.*, **22**: 416-423.

Puri, V.P. 1984. Effect of cystallinity and degree of polymerization of cellulose on enzymatic saccharification. *Biotechnol. Bioeng*. **26,** 1219-1222.

Qiu, G. And Li, Y. 2000. "Immobilization of cellulase on magnetic agarose composite microsphere", *Jingxi Huagong* **17,** 115-117.

Qiu, G. And Li, Y. 2001. "Studies on preparation and characterization of magnetic gelatin microspheres and immobilization of cellulase", *Yaowu Shengwu Jushi* **8,** 197-199.

Ramos L.P., Mathias, A.L, Silva, F.T., Cotrim, A.R., Ferraz, A.L., Chen, C.L.1999. Characterization of residual lignin after SO_2-catalyzed steam explosion and enzymatic hydrolysis of eucalyptus viminalis wood chips. *J. Agric. Food Chemist*, **47**: 2295-2302.

Reddy, N. and Yang, Y. 2005. Biofibers from Agricultural byproducts for Industrial Applications. *Trends in Biotechnol.*, **23 (1)**: 22 – 27.

Reese, E.T., Siu, R.G.H., Levinson, H.S. 1950. The biological degradation of soluble cellulose derivatives and its relationship to the mechanism of cellulose hydrolysis. *J. Bacteriol* **59:** 485-497

Revelle, R., and Suess, H. E. 1957. Carbon dioxide exchange between atmosphere and ocean and the question of an increase of atmospheric CO_2 during the past decade, *Tellus* IX, 18-27.

Richard, P., Toivari, M.H. and Penttilà, M. 1999. Evidence that the gene *YLR070c* of *Saccharomyces cerevisiae* encodes a xylitol dehydrogenase. *FEBS letters* 457: 135 – 138.

Rizzatti, A.C.S., Jorge, J.A., Terenzi, H.F., Rechia, C.G.V and Polizeli, M.L.T.M. 2001. Purification and properties of a thermostable B-D-xylosidase produced by a thermotolerant *Aspergillus phoenicis*. *J. Ind. Microbiol. Biotechnol.* **25:**156 – 160.

Roberto, I.C., Lacis, L.S., Barbosa, M.F.S., de Mancilha, I.M. 1991. Utilization of sugar came bagasse hemicellulosic hydrolysate by *Pichia stipitis* for the production of ethanol. *Process Biochem*. **26:** 15-21.

Rogers, P.L., Lee, K.J., Tribe, D.E. 1979. Kinetics of alcohol production by *Zymomonas mobilis* at high sugar concentrations. *Biotechnol. Letts* **1:** 165-170.

Ropars, M., Marchal, R., Pourquie, J., Vandecasteele, J.P. 1992. Large-scale enzymatic hydrolysis of agricultural lignocellulosic biomass, Part 1: Pretreatment procedures, *Bioresour. Technol.* **42:** 197-204.

Rose, R.D., Winston, F and Hieter, P. 1990. *Methods in Yeast Genetics: A laboratory Course Manual*. Cold Spring Habour laboratory press, New York.

Rose, A.H. 1980. Recent research on Industrially important strains of *Saccharomyces cerevisiae*. *In Biology and activity of yeasts*. Pp 103 – 121. Edited by F.A Skinner, S.M Passmore and R.R Davenport. London Academic press.

Rudolf A, Baudel, H., Zacchi, G., Hahn-Hagerdal, B. And Liden, G. 2008. Simultaneous saccharification and fermentation of steam-pretreated bagasse using *Saccharomyces cerevisiae* TMB3400 and *Pichia stipitis* CBS6054. *Biotechnol. Bioeng.* **99:** 783 – 790.

Rusell, P.D. 1987. Fermenter and Bioreactor Design in *Food Biotechnology* l. R. King and P.S.Y. Cheethan eds. London. *Elsevier Applied Science*, pp. 1 – 48.

Russell, J.B. 1992. Another explanation for the toxicity of fermentation acids at low pH - anion accumulation versus uncoupling**.** *J. Appl. Bacteriol.*, **5:**363-370.

Saddler, J.N., Ramos, L. and Breul, C. 1993. Steam pretreatment of lignocellulosic residues. In *Bioconversion of Forest and Agricultural Residues.* Ed. Saddler, J.N. pp. 73-91, Wallingford: CAB International, ISBN 0851987982.

Saha, B.C., Freer, S.N., and Bothast, R.J. 1995. In "Enzymatic degradation of Insoluble Polysaccharides" (J.N. Saddler and M.H. Penner, eds.) pp. 197 – 207. American Chemical Society, Washington, DC.

Saka, S. 1991. Chemical composition and Distribution. Dekker, New York, pp. 3-58.

Salusjarvi, L., Kankainen, M., Soliymani, R., Pitkanen, J.P, Pentillà, M. and Ruohonen, L. 2008. Regulation of xylose metabolism in recombinant *Saccharomyces cerevisiae. Microbial Cell Factories*. 7:18.

Sandra, R. and Carlo, Z. 2009. Organisms associated with acetic acid bacteria in vinegar production. L. Solieri, P. Guidici (eds), Vinegar of the World. Springer-Verlag Italia. Pp.8, ISBN: 978-0-470-0865-6.

Sanchez, B., Bautista, J. 1988. Effects of furfural and 5-hydroxym-ethylfurfural on the fermentation of *Saccharomyces cerevisiae* and biomass production from *Candida guillermondii*. Enzyme Microb. Technol. **10:** 315-318.

Sambrook, J., Fritsch, E.F and Maniatis, T. 1989. *Molecular cloning: A laboratory Manual*, 2nd edn. Cold Spring Harbor laboratory press, Cold spring Harbor, New York.

Sassner, P., Martensson, C.G., Galbe, M., Zacchi, G. 2008. Steam pretreatment of H_2SO_4-impregnated Salix for the production of bioethanol. *Bioresour. Technol.* **99**: 137-145.

Schacherer J., Shapiro, J.A., Ruderfer, D.M. and Kruglyak, L. 2009. Comprehensive polymorphism survey elucidates population structure of *Saccharomyces cerevisiae*. *Nature*, 458: 342 – 345.

Schell, D.J., Duff, B. 1996. Review of pilot plant programs for bioethanol conversion. In: *Handbook on Bioethanol: Production and Utilization.* Edited by Wyman C.E. Taylor & Francis; 381-394.

Schubert, C. (2006). "Can biofuels finally take center stage?" *Nat. Biotechnol.* **24(7)**, 777-784.

Sears, K.D., Beelik, A., Casebier, R.L., Engen, R.J., Hamilton, J.K. and Hergert, H.L. 1971. Southern pine prehydrolyzates: characterization of polysaccharides and lignin fragments, *J. Polymer Sci.* **36**. 425-443.

Sedlak M. and Ho N.W. 2004. Production of ethanol from cellulosic biomass hydrolysates using genetically engineered *Saccharomyces yeast* capable of co-fermenting glucose and xylose. *Appl. Biochem Biotechnol.* **113-116**: 403-416

Seeley, H.W (Jnr) and Van Demark, P.J. 1972. Microbes in Action. A laboratory Manual of Microbiology 2nd Ed. W.A. Freeman and Co. San Francisco.

Service, R.F. (2007). "Cellulosic ethanol. Biofuel researchers prepare to reap a new harvest," *Science* 325(5818), 1488-1491.

Sharma S.K., Kalra K.L., Grewal H.S. 2002. Enzymatic saccharification of pretreated sunflower stalks. *Biomass Bioenergy*, **23:** 237-243.

Sheff, M. A., and Thorn, K.S. 2004. Optimized cassettes for fluorescent protein tagging in *Saccharomyces cerevisiae*. *Yeast* **21**:661-670

Sims, R, E.H. 2002. The brilliance of Bioenergy, In Business and in practice, James and James Ltd (Science Publisher) London, 316pp, ISBN: 1 902916 - 28 – X.

Simon Ostergaard, Lisbeth Olsson and Jens Nielsen 2000. Metabolic engineering of *Saccharomyces cerevisiae*. *Journal of Microbiology and Molecular Biology Reviews.* Vol. **64**, No. 1 p. 35 – 50.

Singh, A., and Mishra, P. 1995. Microbial pentose utilization: Current application in biotechnology. In "*Progress in Industrial Microbiology*" 33: 1 – 31, Elsevier Amsterdam.

Singh, A., Abidi, A.B., Darmwal, N.S. and Agrawal, A.K. 1988. Evaluation of chemical pre-treatment for biodegradation of agricultural lignocellulosic wastes by *Aspergillus niger*. *MIRCEN Journal* **4:** 473-479.

Skoog, K., Hahn-Hagerdal, B. 1990. Effect of oxygenation on xylose fermentation by *Pichia stipitis*, *Appl. Environ Microbiol.* **56:**3389-3394.

Smits, H. P., J. Hauf, S. Müller, T. J. Hobley, F. K. Zimmermann, B. Hahn-Hägerdal, J. Nielsen, and L. Olsson**.** 2000. Simultaneous over expression of enzymes of the lower part of glycolysis can enhance the fermentative capacity of *Saccharomyces cerevisiae*. *Yeast* 16:1325-1334.

Snoep, J. L., L. P. Yomano, H. V. Westerhoff, and Ingram, L. O. 1995. Protein burden in *Zymomonas mobilis*: negative flux and growth control due to overproduction of glycolytic enzymes. *Microbiology* **141**:2329-2337

Soderstrom J., Pilcher, L., Galbe M. and Zacchi G. 2002. Two-step steam pretreatment of sofwood with SO_2-impregnation for ethanol production. *Appl. Biochem Biotechnol*, **98/100**: 5-21.

Soderstrom, J., Pilcher, L., Galbe, M., Zacchi, G. 2003. Tow-step steam pretreatment of softwood by dilute H_2SO_4 impregnation for ethanol production. *Biomass Bioenergy*. **24:**475-486

Soderstrom, J., Galbe, M., Zacchi, G. 2005. Separate versus simultaneous saccharification and fermentation of two-step pretreated softwood for ethanol production. *J. Wood Chem. Technol*. **25:** 187-202.

Somerville, C. 2007. Biofuels. *Cur. Biotechnol*., **17**, R115 – R119.

Sonderegger, M., Jeppsson, M., Larsson, C., Gorwa-Grauslund, M.F., Boles, E., Olsson, L., Spencer-Martins, L., Hahn-Hagerdal, B. and Sauer, U. 2004. fermentation performance of engineered and evolved xylose-fermenting *Saccharomyces cerevisiae* strains. *Biotechnol Bioeng*. **87**: 90 – 98.

Soni, M.L., Meehrotra, R.N., Kapoor, R.C., 1982. Dissociation constant and some thermodynamic parameters of laevulinic acid. *Indian Journal of Chemistry*. A 21, 196-197.

Solomon B.D, Barnes, J.R. and Halvorsen, K.E. 2007. Grain and cellulosic ethanol: History, economics, and energy policy**.** *Biomass Bioenergy*, **6**:416-425.

Stanley, D., Bandara, A., Fraser, S., Chambers, P.J. and Stanley, G.A. 2010. The ethanol stress response and ethanol tolerance of *Saccharomyces cerevisiae. J. Appl. Microbiol.*, **109**: 13 – 24.

Stambuk, B., Dunn, B., Alves-Jr S., Duval, E., Sherlock, G. 2009. Industrial Fuel Ethanol Yeasts Contain Adaptive Copy Number Changes in Genes Involved in Vitamin B1 and B6 Biosynthesis. *Genome Res*.. **19:** 2271-2278.

Stenberg, K., Tengborg, C., Galbe, M., Zacchi, G. 1998. Otpimisation os steam pretreatment of SO_2-impregnated mixed softwoods for ethanol production. *J. Chem. Tech. Biotechnol* **71:** 299-308.

Stouthamer, A.H., 1979. The search for a correlation between theoretical and experimental growth yields. *Int. Rev. Biochem. Microb. Biochem*. 21, 28.

Subramanian, K.A., Singal, S.K., Saxena, M and Singhal, S. 2005. Utilization of liquid biofuels in automotive diesel engines: An Indian perspective. *Biomass Bioenergy*, **29**: 65- 72

Summers, D.K. 1991. The kinetics of plasmid loss. *Tibtech*. **9**, 273 – 278.

Sun, Y. and Cheng, J. 2002. "Hydrolysis of lignocellulosic materials for ethanol production: A review". *Bioresource Technol.* **83(1),** 1-11.

Suh, S.O., Marshall, C.J., McHugh, J.V and Blackwell, M. 2003. Wood ingestion by passalid beetles in the presence of xylose-fermenting gut yeasts. *Mol. Ecol*. **12**: 3137 – 3145.

Suortti, T. 1983. Identification of antimicrobial compounds in heated neutral glucose and fructose solutions. *Lebensm. Unters. Forsch*. **177**, 94-96.

Swings, J. and De Ley, J.1984. In *Bergy's Manual of Systematic Bacteriology (*eds Krieg, N.R. and Holt, J.S.), William and Wilkins, Baltimore, USA, vol. 2, pp. 576.

Swings, J., De Ley, J. 1977. The Biology of *Zymomonas. Bacteriol Rev*. **41:** 1-46.

Taherzadeh, M.J., Gustaffson, L., Niklasson, C. and Liden, G. 1999. Physiological effects of 5-hydrozymethyl-furfural (HMF) on *Saccharomyces cerevisiae,* submitted.

Taherzadeh, M.J. and Karimi, K. 2007 Enzyme-based hydrolysis processes for ethanol from lignocellulosic materials: A review. *BioResources* **2(4),** 707 -738.

Taherzadeh, M.J. and Karimi, K. 2007. Acid based hydrolysis processes for ethanol from lignocellulosic materials: *A review*, *Bio-Resources* **2(3)**, 472 – 479.

Taherzadeh, M.J., Gustafsson, L., Niklasson, C. and Liden, G. 1998. Conversion of furfural in aerobic and anaerobic batch fermentation of glucose by *Saccharomyces cerevisiae. J. Biosci. Bioeng.* **87**, 169-174.

Taherzadeh M.J, Niklasson C, Liden, G. 1997. Acetic acid - friend or foe in anaerobic batch conversion of glucose to ethanol by *Saccharomyces cerevisiae***,** *Chem Eng Sci* 15:2653-2659.

Taherzadeh M.J. 1999. *Ethanol from lignocellulose: Physiological effects of inhibitors and fermentation strategies*, Chemical Reaction Engineering, Chalmers University of Technology, Goteborg, Sweden.

Taherzadeh M.J, Gustafsson L, Niklasson C., Liden, G. 2000. Physiological effects of 5-hydroxymethylfurfural on *Saccharomyces cerevisiae*. Applied Microbiology and Biotechnology 53**:**701-708

Tan, K.T., Lee K.T., Mohamed, A.R. 2008. Role of energy policy in renewable energy accomplishment: The case of second-generation bioethanol. *Energy Policy*, **9:**3360-3365

Tani, Y., Lim, W. and Yang, H. 1988. Isolation of L-methionine enriched mutant of a methylotrophic yeast, *Candida boidinii No. 2201. J. Ferment. Technol.* **66**, *153 – 158.*

Takahashi, C.M., Lima, G.C., Takahashi, D.F. and Alterthum, F. 2000. Fermentation of sugar cane bagasse hemicellulosic hydrolysate and sugar mixtures to ethanol by recombinant *Escherichia coli* KO11. *World J. Microbiol. Biotechnol.* **16:** 829-834.

Tantirungkij, M., Makashima, N., Seki, T. and Yoshida, T. 1993 Construction of xylose assimilating *Saccharomyces cerevisiae*. *J. Ferment. Bioeng.* **75**: 83-88.

Targonski, Z. 1992. Biotransformation of lignin-related aromatic compounds by *Pichia stipitis* Pignal. *Zentralbl. Mikrobiol*. **147**: 244 – 249.

Taylor, D.J., Green, N.P.O. and Stout, G.W. 2002. Biological science *Enzymes* R. Stoper, Eds. Third Edition. United Kingdom: *Cambridge University Press*. Chapter **4:** 116-127.

Tengborg, C. 2000. *"Bioethanol Production: Pretreatment and Enzymatic Hydrolysis of Softwood,"* Ph.D. Thesis, Lund University, Sweden.

Tengborg, C., Galbe, M., Zacchi, G. 2001. Reduced inhibition of enzymatic hydrolysis of steam-pretreated softwood. *Enzyme Microb Technol.* **28:** 835-844.

Tengborg, C., Galbe, M. and Zecchi, G. 2001b. "Influence of enzyme loading and physical parameters on the enzymatic hydrolysis of steam-pretreated softwood", *Biotechnol. Prog.* **17(1)**:, 110-117.

Thompson, N.S. 1993. Hemicellulose as a biomass resource, in *Wood and Agricultural Residues*, pp. 101-115, Academic Press.

Thompson, D.N., Chen, H.C., Grethlein, H.E., 1992. Comparison of pretreatment methods on the basis of available surface area. *Bioresour. Technol*. **39**, 155-163.

Toivola, A., Yarrow D., van den Bosch, E., van Dijken, J.P., Scheffers, W.A. 1984. Alcoholic fermentation of D-xylose by yeasts. *Appl. Environ. Microbiol.* **47:** 1221-1223.

Toviari, M.H., Salusjarvi, L., Ruohonen, L. and Penttila, M. 2004. Endogenous xylose pathway in *Saccharomyces cerevisiae* . *Appl. Environ. Microbiol*, **70:** 3681 – 3686.

Tosato, V. and Bruschi, C.V. 2007. Analysis and Manipulation of the genome dynamic structure. *J. Biotechnol. Molecul. Biol. Rev*. **2(3)**: 086- 096.

Traff, K.L, Jeppsson M., Hahn-Hagerdal B., Gonwa-Grauslund M.F. 2004 Endogenous KADPH-dependent aldose reductase activity influences product formation during xylose consumption in recombinant *Saccharomyces cerevisiae. Yeast*, 21: 141-150.

Traff, K.L., Jonsson, L.J., Hahn-Hagerdal, B. 2002. Putative xylose and arabinose reductase in *Saccharomyces cerevisiae.* Yeast 19: 1233 – 1241.

Tran, A.V., Chambers, R.P., 1985. Red oak derived inhibitors in the ethanol fermentation of xylose by *Pichia stipitis* CBS 5776. *Biotechnol. Lett*. **7,** 841-846

Tu, M., Zhang, Z., Kurabi, A., gilkes, N., Mabee, W. And Saddler, J. 2006. "Immobilization of beta-glucosidase on *Eupergit* C for lignocellulose hydrolysis", *Biotechnol. Lett.* **28(3),** 151-156.

Tu, M., chandra, R.P. and Saddler, J.N. 2007. "Evaluating the distribution of cellulases and the recycling of free cellulases during the hydrolysis of lignocellulosic substrates," *Biotechnol. Prog.* **23(2),** 398-406.

Tyson, K. S., Riley, C.J., and Humphreys, K.K. 1993. Fuel cycle evaluation of biomass-ethanol and reformulated gasoline, Report No. NREL/TP-463-4950. *National Renewable Energy Laboratory, Goldon, CO,* NREL/TP-463-4950.

Ugolimi S., Tosato V., Bruschi C.V. 2002. Selective fitness of four episomal shuttle-vectors carrying HIS3, LEU2, TRP1 and URA3 selectable markers in *Saccharomyces cerevisiae.* Plasmid, 47: 94-107.

Ulbricht, R.J., Sharon, J., Thomas, J., 1984. A review of 5'hydroxymethylfurfura HMF in parental solutions. *Fundamental Applied Toxicology*. 4, 843-853.

Unten, S.,Ushijima, H., Shimizu, H. Tsuchie, H., Kitamura, T., Moritome, N and Sakagami, H., 1991. Effect of caco husk extract on human immunodefficiency virus infection. *Letters Appl. Microbiol*. **14**: 251-254.

Van Dijken, J.P., Van den Bosch, E., Hermans, J.J., de Miranda, L.R. and Scheffers, W.A. 1986. Alcoholic fermentation by 'non-fermentative' yeasts. *Yeast* **2:** 123 – 127.

Van Soest, P.J. 1994. *The Nutritional Ecology of the Ruminant.* Pp. 476-490, Cornell University Press, Ithaca, NY.

Van Walsum, G.P., Laser, M.S., Lynd, L.R. 1996. Conversion of lignocellulosics pretreated with liquid hot water to ethanol. *Appl. Biochem Biotechnol.* **57/58**: 157-170.

Van Zyl, C., Prior, B.A., and Du Preez, J.C. 1988. Production of ethanol from sugarcane hydrolysate by *Pichia stipitis, Appl. Biochem. Biotechnol.* **17**, 357-369.

Varga, E., Szengyel, Z., Reczey, K. 2002. Chemical pretreatments of corn stover for enhancing enzymatic digestibility. *Appl. Biochem Biotechnol.* **98/100:** 73-87.

Verduyn, C., Postma, E., Scheffers, W.A., Van Dijken, J.P., 1990. Energetics of *Saccharomyces cerevisiae* in anaerobic glucose-culture study on the regulation of respiration and alcoholic fermentation. *Yeast* **8**, 501-517.

Verduyn C, Postma E, Scheffers W.A, Van-Dijken, J.P 1992. Effect of benzoic acid on metabolic fluxes in yeasts: a continuous-culture study on the regulation of respiration and alcoholic fermentation. *Yeast* , **7:**501-517.

Verduyn, C., Postma, E., Scheffers, W.A., Van Dijken, J.P. 1990. Physiology of *Saccharomyces cerevisiae* in anaerobic glucose-limited chemostat cultures, *J. Gen Microbiol* **136:** 395-403.

Villa, G.P., 1992. Microbial transformation of furfural to furfuryl alcohol by *Saccharomyces cerevisiae. Acta Biotechnol.* **12**. 509 - 512.

Von Sivers M., Olsson, L., Hahn-Hagerdal, B., and Zacchi, G. 1994. Cost analysis of ethanol production from willow using recombinant *Escherichia coli*. *Biotechnol. Prog.* **10**: 555-560.

Von Sivers, M., Zacchi, G. 1996. Ethanol from lignocellulosics: a review of the economy, *Bioresour Technol* **65:** 131-140.

Walfridsson, M., Hallborn, J., Penttila, M., Keranen, S., Hahn-Hagerdal, B. 1995. Xylose-metabolizing *Saccharomyces cerevisiae* strains overexpressing the TKL1 and TAL1 genes encoding the pentose phosphate pathway enzymes transketolase and transaldolase. *Appl. Environ. Microbiol.* **61:** 4184-90.

Walfridsson, M, Anderlund, M., Bao, X., Hahn-Hagerdal, B. 1997.Expresson of different levels of enzymes from the *Pichia stipitis* XYL1 and XYL2 genes in *Saccharomyces cerevisiae* and its effects on product formation during xylose utilization, *Appl. Microbiol. Biotech*, **48:** 218-224.

Warth, A.D.1988. Effect of benzoic acid on growth yield of yeasts differing in their resistance to preservatives. *Appl. Environ. Microbiol.* **54 (8)**, 2091-2095.

Watanabe S, Kodaki T, and Makino, K. 2005. Complete reversal of coenzyme specificity of xylitol dehydrogenase and increase of thermostability by the introduction of structural zinc. *J. Biol. Chem.* **280**: 10340 – 10349.

Weast, R.C., 1975 – 1976. *Handbook of Chemistry and Physics*, Fiftysixth ed. CRC Press, Cleveland.

Webb, S.R., Lee, H., 1990. Regulation of D-xylose utilization by hexoses in pentose-fermenting yeasts. *Biotechnol Adv.*, **8:** 685-97

Weil, J.R., Sarikaya, A., Rau, S.L., Goebz, J., Lasisch, C.M., Brwer, M., Hendrickson, R., Tadisch, M.R. 1998. Pretreatment of corn fibre by pressure cooking in water. *Appl. Biochem. Biotechnol.* **73**: 1 – 17.

Weigert, B., Klein, K., Rizzi, M., Lauterbach, C., Dellweg, H., 1988. Influence of furfural on the aerobic growth of the yeast *Pichia stipitis. Biotechnol. Lett.*. **10 (12)**: 895 -900.

Wheals, A.E., Basso, L.C., Alves, D.M.G and Amorim, H.V. 1999 Fuel ethanol after 25 years. *Trend in Biotechnol.* **17**: 482 – 487.

Wickerham, L.J. 1946. A Critical Evaluation of Nitrogen Assimilation Test commonly used in classification of yeasts. *J. Bacteriol*. **52**: 295 – 298.

William, J.A. and K. Badaru 1989. Progress in Cacao Breeding., *In Progress in Tree Crop Research.* 2nd Edition Cocoa Research Institute of Nigeria, CRIN, Ibadan, Nigeria.

Wong K.K.Y., Deverell, K.F., Mackie, K.L., Clark, T.A., Donaldson, L.A. 1988. The relationship between fiber-porosity and cellulose digestibility in steam-exploded *Pinus radiate*. *Biotechnol. Bioeng.*, **31:** 447-456.

Wu, J. And Ju, L.K. 1998. "Enhancing enzymatic saccharification of waste newsprint by surfactant addition", *Biotechnol. Prog.* 649-652.

Wyman, C.E., Dale, B.E., Elander, R.T., Holtzapple, M., Ladisch, M.R. and Lee, Y.Y 2005. Coordinated development of leading biomass pretreatment technologies. *Bouresour. Technol*. **96**: 1959 – 1966.

Wyman, C. E. 1994. Alternative fuels from biomass and impact on carbon dioxide accumulation. *Appl. Biochem. Biotechnol.* 455/46, 897-915.

Wyman, C.E. 1996. *Handbook on Bioethanol: Production and Utilization*, Washington, DC, Taylor & Francis.

Zeng M. Mosier N.S., Huang, C.P., Sherman, D.M., Ladisch, M.R. 2007. Microscopic examination of changes of plant cell structure in corn stover due to hot water pretreatment and enzymatic hydrolysis. *Biotechnol. Bioeng.*, 97: 265-278.

Zhang, M., Eddy, C., Deanda, K., Finkelstein, M. and Picataggio, S.1995. *Science* **267**, 240-243

Zhang, Z., Moo-Young, M., Chisti, Y. 1996. Plasmid stability in recombinant *Saccharomyces cerevisiae. Biotechnol. Adv.* **14:** 401-435.

Zhang, Y.H.P., Lynd, L.R. 2004. Toward an aggregated understanding of enzymatic hydrolysis of cellulose; noncomplexed cellulase systems. *Biotechnol. Bioeng*. **88 (7)**, 797-824.
http://www.baff.info
http://www.jgi.doegov/pichia
http://www.ncbi.nlm.nih.gov/blast
http://www.yeastgenome.org
http://www.invitrogen.com
http://www.dualsystems.com/support/vector-information.html
http://www.jbc.org/cgi/content/full280/11
http://www.ethanolrfa.org/pr041101.html.
http://genome.ornl.gov/microbial/saro/
http://www.distill.com/berg/
http://www.cpv.org.co/irrd9/kinh91.htm
http://www.eubia.org/332.0.html#832
www.ethanolrfa.org/factfic_envir.html
http://www.en.wikipedia.org/wiki/cellulose
http://www.nature.com/nature/journal/v454/n7206/full/nature07190.html
http://www.en.wikipedia.org/wiki/lignin
http://www.en.wikipedia.org/wiki/furfural
Cassava industrial revolution in Nigeria (www.fao.org/docrep/007/y5548e/y5578e09.htm).

Appendix 1

a. Detail Information of *Pichia stipitis* (NBRC 10063)

NBRC No.	NBRC 10063
Scientific Name of this Strain	***Pichia stipitis*** Pignal
Synonymous Name	Synonym: *Yamadazyma stipitis*
Type Strain	
History	IFO 10063 <- CBS 6054 <- J. Santa Maria
Other Culture Collection No.	ATCC 58785=CBS 6054=BCRC 21777=NRRL Y-11545
Other No.	
Rehydration Fluid	703
Medium	108
Cultivation Temp.	24°C
Source of Isolation	
Locality of Source	
Country of Origin	
Biohazard Level	
Applications	Ethanol;production from xylose
Mating Type	
Genetic Marker	
Plant Quarantine No.	
Animal Quarantine No.	
Herbarium No.	
Restriction	
Comment	
References	
Sequences	LSU rDNA D1D2

b. Preparation of Growth medium for Pichia stipitis (NBRC 10063)

Medium No.	108
Medium	YM Agar
Composition	
Glucose	10 G
Peptone	5 G
Yeast extract	3 G
Malt extract	3 G
Distilled water	1 L
Agar	

c. Preparation of Rehydration fluid medium for Pichia stipitis (NBRC 10063)

Medium No.	703
Medium	YM Liquid Medium
Composition	
Glucose	10 G
Peptone	5 G
Yeast extract	3 G
Malt extract	3 G
Distilled water	1 L

d. Composition of Luria Bertani (LB) medium

Compositions:

Ingredients	Grams/Litre
Tryptone	10.0
Yeast extract	5.0
Sodium chloride	5.0
Agar	10.0

Final pH 7.2 at 37°C

Appendix 2

a. Materials used for Extraction of Genomic DNA from *P. stipitis* CBS 6054

- **Breaking buffer (compositions)**

4ml of 2% (v/v) Triton X100

2ml of 1% sodium dodecyl sulphate (SDS)

4ml of 0.1M Nacl

2ml of 10mM of Tris pH 8.0

400ul of 1mM EDTA pH 8.0

200ml of deionised water

- RNAse 1mg/ml
- 4 M Ammonium acetate

Acid washed Glass beads 425 – 600um (G8772 – 100G)

b. Reagents for Chemical method of plasmid DNA preparation

Solution I (For 100ml)

0.9% Glucose

2.0ml of 0.5M EDTA

2.5ml of 1M Tris. Hcl (pH 8.0) and

Distilled dionized water to a final volume of 100ml

Solution II (Freshly prepared) for 10ml

0.2ml of 10N NaOH

0.5ml of 20% SDS and

9.3ml of distilled deionized water

Solution III (Ice cold) for 100ml

60ml of 5M potassium acetate

11.5ml of glacial acetic acid and

28.5ml of distilled deionized water

c. Synthetic Drop – out mix (microbiology strains) compositions

Ingredients	Quantities (g)	% w/v
Bacto-yeast nitrogen base w/o amino acids	6.7	0.67
(Ammonium sulfate)*	5	0.5
Glucose	20	2
Bacto-agar	20	2
Drop-out mix	2	0.2
Distilled water	1000ml	

* Only if not already present in the Bacto-yeast nitrogen base.

Ingredients	Grams	%	mg. in 1000ml
Adenine	0.5	1.7	18
Arginine	2	7.0	76
Histidine	2	7.0	76
Leucine	10	35.1	380
Lysine	2	7.0	76
Methionine	2	7.0	76
Proline	2	7.0	76
Threonine	2	7.0	76
Tryptophan	2	7.0	76
Tyrosine	2	7.0	76
Uracil	2	7.0	76
Total	**28.5**	**100**	**1082**

For each litre of drop-out medium add ~2g of drop-out mix (in case of leucine drop-out add ~0.7g).

LIQUID MEDIA

Adenine, Threonine and Tryptophan are supposed to be thermolabile, so prepare a stock solution for each of them to add after autoclaving:

Amino acid	Stock solution	Final vol. for 1L	Storage
Adenine	0.5g/100ml	4ml	Room temperature
Threonine	4g/100ml	5ml	Room temperature
Tryptophan	1g/100ml	2ml	+4^{o}C

Appendix 3:

Buffers

a. TE Buffer

Tris – Cl (pH 8.0)	100mM
EDTA (pH 8.0)	10mM

b. Loading Buffer

80% glycerol	5ml
2% bromophenol	2ml
2% xylene cyanol	2ml
0.5M EDTA (pH 8.0)	2ml
10x loading buffer	1ml

c. Tris-Borate (TBE) 5x composition

Tris borate	54.0g
Boric acid	27.5g
EDTA	2.92g
Deionized water	1 litre

Appendix 4

HPLC Analysis

HPLC Analysis of sugar derivatives in Acid hydrolysed CPH hydrolysate

Sample	Glucose		Fructose		Galactose		Xylose		Mannose	
	P/A	%	P/A	%	P/A	%	P/A	%	P/A	%
CPH	105.96	50.10	0.04	0.002	-	-	173.56	11.96	145.37	11.18
Std	R/T	P/A	R/T	P/A	R/T	P/A	R/T	P/A	R/T	P/A
0.2%	1.86	4.23	4.15	3.74	3.84	1.42	2.29	29.0	2.45	13.0

Note *P/A = Peak area*
R/T = Retention time
Dilution factor (df) = 10

HPLC Analysis of sugar derivatives in pretreated Tween-80 Enzyme hydrolysed CPH hydrolysate .

Sample	Glucose		Fructose		Galactose		Mannose	
	P/A	%	P/A	%	P/A	%	P/A	%
CPH + Tween80	3332.19	31.68	3236.49	0.33	2055.48	16.79	5107.78	4.79
Std	R/T	P/A	R/T	P/A	R/T	P/A	R/T	P/A
1.0%	1.193	631.13	1.346	58892.5	1.288	738.39	1.658	6394.67

Note *P/A = Peak area*
R/T = Retention time
Dilution factor (df) =1: 6

HPLC Analysis of sugar derivatives in pretreated Enzyme hydrolysed CPH

Sample	Glucose		Fructose		Galactose		Mannose	
	P/A	%	P/A	%	P/A	%	P/A	%
CPH	2997.48	28.50	4197.39	0.43	-	-	4712.68	4.42
Std	R/T	P/A	R/T	P/A	R/T	P/A	R/T	P/A
1.0%	1.178	631.13	1.269	58892.5	-	-	1.665	6394.67

Note *P/A = Peak area*

R/T = Retention time

Dilution factor (df) =1: 6