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Functional Expression of Pichia Stipitis XR and XDH Genes into Starter Cultures of *Saccharomyces cerevisiae* Obtained from Degrading Cocoa (*Theobroma cacao* L.) Pod Husk Biomass

R. O. Igbinadolor[1,2,3*], C. V. Bruschi[2] and A. A. Onilude[3]

[1]Department of End-use Research, Cocoa Research Institute of Nigeria, P.M.B. 5244, Ibadan, Nigeria.
[2]Department of Microbiology, University of Ibadan, Ibadan, Nigeria.
[3]Department of Yeast Molecular Genetics, International Centre for Genetic Engineering and Biotechnology (ICGEB), Trieste, Italy.

Authors' contributions

This work was carried out in collaboration among all authors. Authors ROI, CVB and AAO designed the study, author ROI performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Authors ROI, CVB and AAO managed the analyses of the study. Author ROI managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Biobased energy and fuels are among the exercisable sustainable energy options mankind has in the not-so-distant future as issues pertaining to global warming and shortfall in fossil fuels loom dark over the planet. The environmental necessity to stop this development by switching to alternative strategies nowadays is generally undisputed. Biofuel made from biomass provide unique environmental economic strategic benefit and can be considered as safe and by and large, the cleanest liquid fuel alternative to fossil fuels. Biofuel produced from Agricultural waste biomass like cocoa (*Theobroma cacao* L) pod husk shows many potentials advantages in comparison with sugar or starch-based stocks since the latter materials are also food for human and animals. However, the

**Corresponding author: E-mail: richosa2002@yahoo.com;*

complex nature of this biomass necessitates the use of genetic techniques to produce engineered organisms that are able to transform this polymer into the desired product. With Bioinformatics tools using NCBI BLAST programme, two genes XL1 and XL2 encoding pentose utilization were isolated from the genomic DNA of *Pichia stipitis* (CBS 6054) and two primers each were designed to span the full coding region of these genes with attached enzymes restriction sites using DNA strider 1.4f7 and *Macplasmap programmes*. PCR reactions were carried out on 120ηg of the isolated genomic DNA for 30 cycles using the DNA Gotaq polymerase enzyme. The amplified PCR fragments were introduced into plasmid vectors pGAPZA and pVT100-U respectively and the constructs were then used to transform the selected ethanol-producing strain of *S. cerevisiae* (BY4743) isolated from degrading cocoa pod husk biomass meant to serve as starter for biofuel production from cocoa pod husk hydrolysate.

Keywords: Biofuel; biomass; cocoa pod husk; bioinformatics; primers; PCR.

1. INTRODUCTION

It is widely acknowledged that fuels from renewable resources are becoming increasingly important in times of dwindling crude oil supply and growing environmental concern of the public. Plant biomass, particularly when accruing as a waste product, is an attractive feedstock for bioethanol production [1]. Lignocellulosic biomass is any organic matter that is available in a renewable basis which includes energy crops, agricultural residues, aquatic plants, wood and wood residues as well as other waste materials [2].

An important requirement from such an alternative strategy would be the complete conversion of all the available sugars in the biomass hydrolysate into ethanol. Commercial production of bioethanol from lignocellulosic hydrolysate by yeasts requires strain that can ferment all sugars both hexose and pentose sugars present in the hydrolysate. The pentose sugar D-xylose is a major constituent of lignocelluloses and it is the second most abundant renewable sugar in nature, its fermentation to ethanol is considered to have great economical potential. Upon hydrolysis, lignocellulosic biomass yields mixtures of hexoses and pentoses. The eukaryotic yeast *Saccharomyces spp*. are the safest and most effective microorganisms for fermenting sugars to ethanol and tradition\ally have been used in industry to ferment glucose to ethanol. However, *Saccharomyces* spp. have been found to be unsuitable for fermenting sugars derived from cellulosic biomass. This is because most of the hydrolysates of cellulosic biomass contain two major fermentable sugars, glucose and xylose. *Saccharomyces* spp., including *Saccharomyces cerevisiae*, are not able to ferment xylose to ethanol or to use this pentose sugar for aerobic growth [3], instead it converts it primarily to xylitol with only a small fraction going into biomass or ethanol [4].

Even though *Saccharomyces* spp. are not able to metabolize xylose aerobically and anaerobically, there are other yeasts, such as *Pichia stipitis* and *Candida shehatae*, that are able to ferment xylose to ethanol and to use xylose for aerobic growth. However, these naturally occurring xylose-fermenting yeasts are not effective fermentative microorganisms, and they also have a relatively low ethanol tolerance [5]. Thus, they are not suitable for large-scale commercial production of ethanol from cellulosic biomass. By introducing the genes for xylose metabolism from *Pichia stipitis* into *Saccharomyces cerevisiae* allows the utilization of both pentoses and hexoses present in lignocellulosic biomass. Metabolic engineering has proven successful in endowing *S. cerevisiae* with the ability to consume xylose and to direct the resulting carbon flux to ethanol production.

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 [6] metabolic engineering has conferred two major advantages: (i) the directed modification of strains without the accumulation of unfavorable mutations and (ii) the introduction of genes from foreign organisms to equip *S. cerevisiae* with novel traits. The latter is particularly crucial for industrial biotechnology and this formed the crux of the present research work to provide pathways in yeast that extend the spectrum of usable waste agricultural biomass like cocoa pod husk for bioethanol production.

2. MATERIALS AND METHODS

Cryotube glass containing lyophilized *Pichia stipitis* Pignal (NBRC 10063) CBS 6054 was obtained from the National Institute of Technology and Evaluation (NITE) Biological Resource Centre, Chilba, Japan.

Rehydration fluid – YM medium consisting of 10 g glucose, 5 g peptone, 3 g Yeast extract and 3 g of malt extract per litre was prepared. 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 200 μl of the rehydration medium. 50 μl each of the *P. stipitis* rehydration medium was then used to inoculate YMPD agar medium plate and incubated at 24˚C for 48 hours. Also 50ul was used to inoculate the broth medium (YM) - rehydration fluid in an Erlenmeyer flask with baffles (for oxygenation). The flask was incubated at 24˚C in a rotatory shaker of 120 rpm for 2 days.

2.1 Microbial Strains

E.coli Xl1 Blu MRF' was obtained from the Yeast molecular Genetic lab of ICGEB, Italy, preserved in glycerol at -70˚C.

2.2 Selection of Yeast Strains

For the development of suitable starter cultures, yeast isolates with the highest frequency of ecological occurrence from degrading cocoa pod husk during isolation were selected.

2.3 Plasmid Vectors

Plasmids used in this study were Expression vectors - PGAPZαA which uses Zeocin as a selectable maker. This vector was obtained from the Department of Genetics, Tarbiat Modares University, Iran. Other expression vectors used in this study include PVT100U which uses both Ampicillin as selectable maker for bacterial and ura3 for yeast. This vector was a generous gift from the Yeast Molecular Genetic laboratory of ICGEB, Italy.

2.4 Media and Culture Conditions

E.coli was grown in Luria Bertani (LB) medium supplemented with 75 μg/ml of Ampicillin. 25 μg/ml of Zeocin was added to LB medium used for *E. coli* for transforming PGAPZαA vector and 100 μg/ml of Zeocin was added to YPD medium for yeast culture. Synthetic medium (Drop out mix) was also compounded.

2.5 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 respectively. Primers for PCR were designed using Saccharomyces Genome Database (http://www.yeastgenome.org/) and sequences were synthesized by Sigma.

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

The genomic DNA of *P. stipitis* was isolated by inoculating the yeast strain (*Pichia stipitis*) into 45ml of yeast peptone dextrose broth (YPD) and incubated in a rotary shaker overnight at 24˚C. After 24 hours, the culture broth was examined microscopically to ensure that there was no contamination and the method of [7] was employed for the isolation of yeast genomic DNA.

2.7 DNA Analysis

Restriction sites fragment generation of the plasmid vectors and the inserts genes were generated with Bioinformatics tools of Yeast Molecular Genetics laboratory of ICGEB using DNA strider 1.4f7 and Macplasmap programmes respectively. Based on the published sequence of *P. stipitis*, the genes of interest were identified using blast search programme at the NCBI website (http://www.ncbi.nlm. nih.gov/BLAST).

2.8 Primer Design

The primer for the coding sequence of the genes (Xylose reductase) XL1 and Xylitol dehydrogenase gene XL2 were designed by using the Saccharomyces Genome Database (SGD) (http://www.yeastgenome.org/)

2.9 PCR Reaction (XLI Gene) and Cloning of PsXR Gene

Based on the published sequence of *Pichia stipitis* XL1 and XL2 genes [GeneBank: X59465] [8] the forward and reverse primers were designed to span the full coding region of the genes XL1 and XL2 respectively. PCR was

carried out on 120 ŋg of the template DNA of *Pichia stipitis* using the two designed primers and Gotaq DNA polymerase enzyme in a 50 ul volume under the following reaction conditions of 30 cycles, initial denaturation of 94˚C for 4 minutes, cycling stage of 94˚C for 30seconds, 52˚C for 30 seconds (Tm of primer), 72˚C for 1 minute, extension at 72˚C for 4 minutes and hold at 16˚C.

2.10 Ligation Reaction

Ligation reaction was carried out on the XL1 gene with the plasmid vector pGAPZA while XL2 gene was ligated to pVT-100U vector. These were carried out in a reaction mixture containing the ligase buffer and the T4 ligase enzyme. The mixture was incubated at 16^{o}C overnight.

2.11 Transformation of Competent cell of *E. coli*

Competent cells of *E. coli* strain BLU MRF was prepared in 50mM $Cacl_2$ solution, this was then used to transformed the ligation mixture of XLI gene into pGAPZA vector and XL2 gene into pVT-100LU vector using the method of (14).

2.12 Genetic Transformation

Transformation of *E. coli* was performed according to the method of Sambrook (28). Yeast transformation was carried out by the modified Lithium acetate method as described in Rose *et al.,* (27).

3. RESULTS

3.1 Selected Yeast Strain from Cocoa Pod Husk

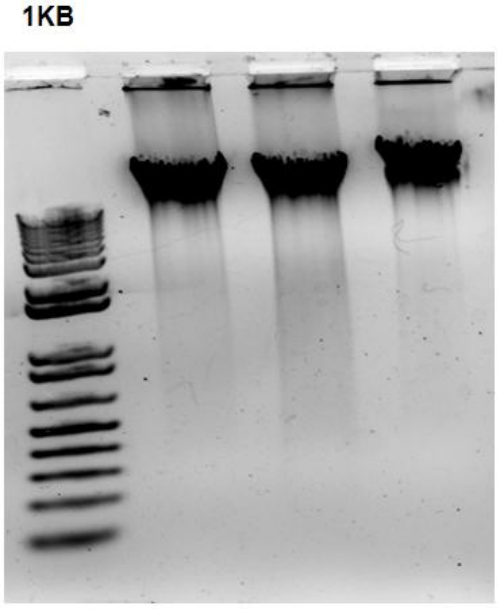
Selected yeast strain of *Saccharomyces cerevisiae* obtained as starter cultures from degrading cocoa pod husk was identified as strain BY4743 with genotype outline (Table 1).

3.2 Genomic DNA of *P. stipitis*

The genomic DNA of *P. stipitis* isolated was found to be 15.4Mbp (15,400kbp) in size when separated in agarose gel electrophoresis and consists of 8 chromosomes.

Xylose reductase gene (XL1) was found on chromosome 5 with an Open Reading Frame (ORF) of 2025bp and a coding sequence of 957bp starting at ATG codon nucleotide 356 and extending to TAA codon at nucleotide position at 1312 this encodes 319 amino acids.

Xylitol dehydrogenase gene (XL2) was found on chromosome 1 with 1963bp Open Reading Frame (ORF) and a coding sequence of 1092 bp starting at ATG codon nucleotide 319 and extending to TAA codon at nucleotide position 1410, encoding for 364 amino acids.



Genomic DNA of *P. stipitis* (15.4Mbp)

Table 1. Genotype of yeast (BY4743)

Name	Genotype
BY4743	Mata/α *his3Δ1/his3Δ1* *leu2Δ0/leu2Δ0* *MET15/met15Δ0 lys2Δ0/LYS2 ura3Δ0/ura3Δ0*

3.3 Xylose Reductase (XR) Gene Coding Sequence

Single strand Chromosome 5

DNA sequence 957 base pairs atgccttctatt ……atcttcgtctaa Linear

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atgccttctattaagttgaactctggttacgacatgccagccgtcggttt
cggctgttggaaagtcgacgtcgacacctgttctgaacagatctaccgtg
ctatcaagaccggttacagattgttcgacggtgccgaagattacgccaac
gaaaagttagttggtgccggtgtcaagaaggccattgacgaaggtatcgt
caagcgtgaagacttgttccttacctccaagttgtggaacaactaccacc
acccagacaacgtcgaaaaggccttgaacagaaccctttctgacttgcaa
gttgactacgttgacttgttcttgatccacttcccagtcaccttcaagtt
cgttccattagaagaaaagtacccaccaggattctactgtggtaagggtg
acaacttcgactacgaagatgttccaattttagagacctggaaggctctt
gaaaagttggtcaaggccggtaagatcagatctatcggtgtttctaactt
cccaggtgctttgctcttggacttgttgagaggtgctaccatcaagccat
ctgtcttgcaagttgaacaccacccatacttgcaacaaccaagattgatc
gaattcgctcaatcccgtggtattgctgtcaccgcttactcttcgttcgg
tcctcaatctttcgttgaattgaaccaaggtagagctttgaacacttctc
cattgttcgagaacgaaactatcaaggctatcgctgctaagcacggtaag
tctccagctcaagtcttgttgagatggtcttcccaaagaggcattgccat
cattccaaagtccaacactgtcccaagattgttggaaaacaaggacgtca
acagcttcgacttggacgaacaagatttcgctgacattgccaagttggac
atcaacttgagattcaacgacccatgggactgggacaagattcctatctt
cgtctaa
```

3.4 Primer Design for Xylose Reductase (XL1 GENE) and Introduction of Enzyme Sites

Using primers designed for these two region (the forward and reverse primer), we successfully amplified a coding sequence of 957 base pairs fragment of xylose reductase gene from *Pichia stipitis* genomic DNA. With attached two enzymes restriction sites for *BstBI* and *SacII* into the Xylose reductase gene at the position 5' and 3' ends respectively.

Fwd 5'----- ATGCCTTCTATTAAGTTGAAC……3'
Rev 5'…..TTAGACGAAGATAGGAATC ……..3'

5'----T T↓C G A A----3'
3'--A A G C↑T T----5'

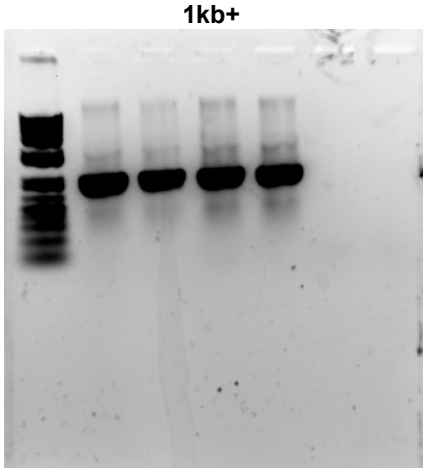
Restriction site for **BstBI** (*Bacillus stearothermophilus* B225)

5'----C C G C↓G G----3'
3'---G G↑C G C C----5'

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

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

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

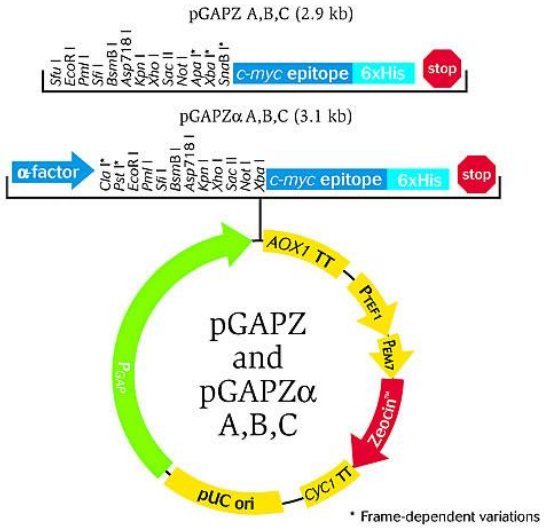


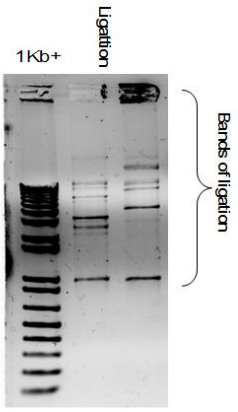
PCR of XL1 gene 957 bp

3.5 Plasmid Vector (pGAPZαA) Used for Insertion of XL1 Gene

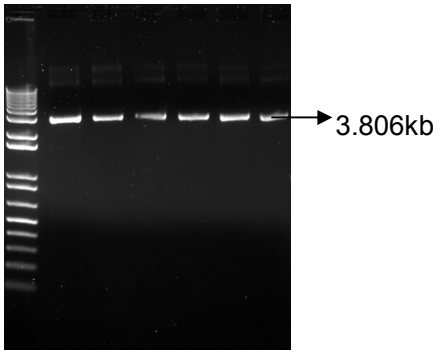
pGAPZA is a Pichia expression vector, it uses the GAP promoter to constitutively express recombinant proteins. Selection of this vector is based on the dominant selectable marker zeocin, which is bifunctional both in *Pichia stipitis* and *E. coli*.

Restriction of gel purified pGAPZαA vector (3.147kb) with *BstB1* and *SacII* restriction enzymes resulted to a plasmid size of 2.825kb and when ligated to digested gel XL1 (9.81kb) gene gave a total size of 3.806kb when transformed into the competent cell of *E. coli* cell as shown on the plate below.





Ligation of PGAPZA Vector to XL1 gene of Pichia stiptis.
Used for the transformation of E. coli XL1 BLU competent cells



Transformation of competent cell (*E. coli*) with ligation mixture

3.6 Xylitol Dehydrogenase Gene (XL2) From *P. stipitis* on chromosome 1

The gene coding for xylitol dehydrogenase (XL2) was located on chromosome 1 with a total genome sequence of 1963 base and a coding sequence of 1092bp.

3.6.1 Pichia stipitis CBS 6054 XL2 Coding region (1092bp) on Chromosome 1

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   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 ctccaagttg 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 ttggtaacgc tgctggtcca
 841 gtcagcttcc caatcaccgt tttcgccatg 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
```

3.6.2 Primers design for XL2 gene

These primers are for PCR

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

These primers will amplify a fragment of 1092 base pairs

3.6.3 Introduction of restriction sites into Xylitol Dehydrogenase (XL2) Primer

Two enzyme restriction sites for PstI and BamHI were introduced into the Xylitol dehydrogenase gene (XL2) at the 5' and 3' ends respectively.

5----C T G CA G ----3
3′---G ACG T C----5

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

5----G G A T C C----3
3′--- C C T A G G----5

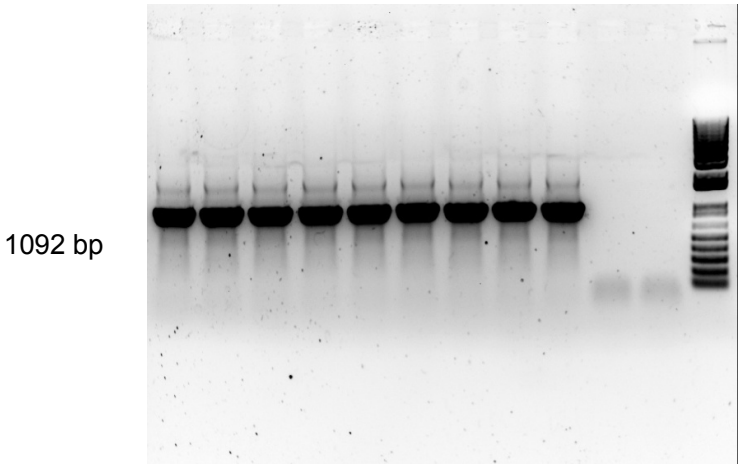
Restriction site for **BamHI** (Reverse primer)

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

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

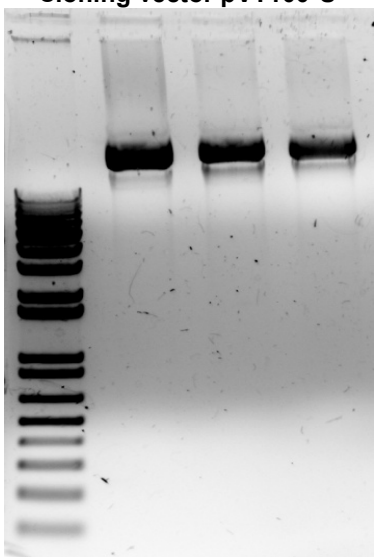
3.6.4 PCR Reaction and cloning of (XL2 Gene)

Based on the published sequence of *Pichia stipitis* XL2 gene [GeneBank: X55392] [9] the following two primers were designed to span the full coding region of XL2 gene. PCR was carried out on the template DNA of *Pichia stipitis* (120 ηg) with designed primers, using Gotaq DNA polymerase enzyme in a 50µl volume under the following reaction conditions of initial denaturation at 94˚C for 4 minutes, cycling stage at 94˚C for 30 seconds, 52˚C for 30 seconds (Tm of primer), 72˚C for 1 minute, extension at 72˚C for 4 minutes and hold at a temperature of 16˚C.



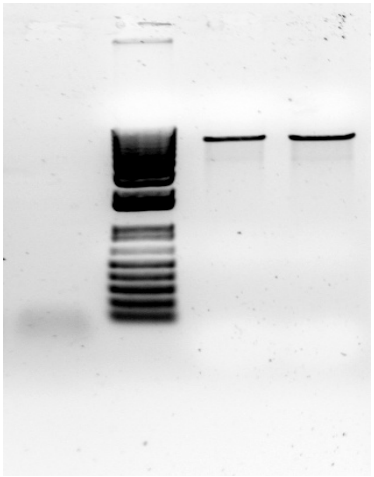
PCR of XL2 gene from *Pichia stipitis*

Cloning vector pVT100-U



3.6.5 Isolation of plasmid DNA from expression vector pVT100-U (6.9Kb)

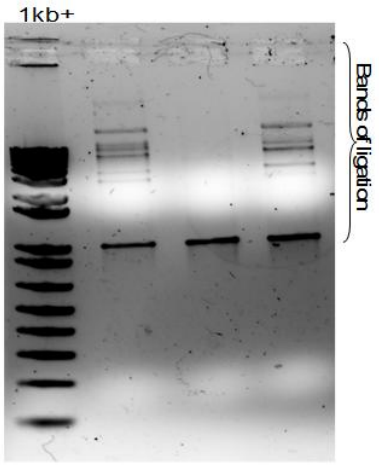
Plasmid pVT100U is a yeast expression vector with a total size of 6.9kb. The vector habours the promoter of ADC1 gene encoding alcohol dehydrogenase gene from *S. cerevisiae*. Immediately downstream from the promoter, at position – 12 upstream of the translation – initiation codon ATG, lies a polylinker sequence comprising unique restriction enzyme sites of 161bp. The plasmid has selection markers of (URA3) for *S. cerevisiae* and uses ampicillin for *E. coli*. It can replicate in *S. cerevisiae* because of the presence of the 2μ plasmid replicon.



Gel Purification of vector (pVT100-U) digested with Pst1 and BamH1

3.6.6 Ligation of digested gel purified XL2 gene to pVT100-U expression vector

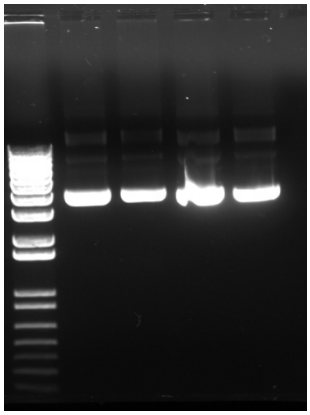
Ligation reaction was carried out on the XL2 gene with the plasmid vector pVT100-U in a reaction mixture containing the ligase buffer and the T4 ligase enzyme. The mixture was incubated at 16^{o}C overnight.



Ligation of XL2 gene with pVT100-U vector

3.6.7 Transformation of competent *E. coli* cell with construct

Transformed competent cells of *E. coli* strain XL1 BLU MRF with the ligation mixture of (XLI and the pVT100-U vector) is shown on the plate below. The ligation of the restricted plasmid 6.65kb with the insert gene XL2 of 1092bp gave a total size of 7.9kb.



Transformant Xl2 gene and the vector pVT100-U

3.6.8 Yeast Transformation: Transformation of *Saccharomyces* sp. strain BY4743 with pGAPZA and PVT100U plasmids

Strain of *Saccharomyces cerevisiae* used for Transformation (Table 2).

Table 2. GENOTYPE OF YEAST STRAIN BY4743

Name	Genotype		
BY4743	Mata/	*his3Δ1/his3Δ1*	*leu2Δ0/leu2Δ0*
	MET15/met15Δ0 lys2Δ0/LYS2 uraΔ0/uraΔ0		

Saccharomyces sp. strain BY4743, which can tolerate high temperatures and high ethanol concentrations, should be an ideal strain for producing fuel ethanol. Thus, this yeast strain was used as the host to receive the pGAPZA and PVT100U plasmids. Transformation of *Saccharomyces* sp. strain BY4743 with both plasmids and selection of transformants were carried out as described in Materials and Methods. The recombinant strains were initially identified by their ability to grow on YEPD plates containing zeocin (100 µg per ml) for pGAPZA plasmid with XL1 insert Ura3 drop out synthetic complete medium as the selectable marker for PVT100U plasmid with XL2 gene.

Each transformant colony was verified by using restriction enzyme analysis technique and also confirmed by the colony PCR test.

4. DISCUSSION

This paper describes the strategies used to develop recombinant *Saccharomyces* strains that can effectively ferment xylose and, in particular, to coferment glucose and xylose present in the same medium. This development was intended to provide a means by which wild-type yeast strains, particularly diploid or polyploid industrial strains (such as strain BY4743) that may have superior properties desirable for ethanol fermentation, can easily be transformed for industrial applications. The construction of yeast strain capable of growing and fermenting pentose sugars found in lignocellulosic materials has been the object of intense research efforts [10,11,12,13,14,15]. In the present work, it has been demonstrated that through genetic engineering central metabolism of yeast can be redirected to create new strains for commercial applications. To enable further improvement in ethanol production from lignocellulose, xylose reductase (XR) encoded by the gene XL1 and xylitol dehydrogenase (XDH) encoded by the gene XL2 pathways are necessary for *S. cerevisiae*, to ferment xylose to ethanol due to lack of genes encoding these enzymes in the yeast. This will improve xylose fermentation in lignocellulose and decreased xylitol formation, [16]. To maximize the ethanol yield, the production organism should be capable of utilizing all pentoses and hexoses present in the lignocellulose, and, furthermore, it is important that the production organism has a high inhibitor tolerance, since the lignocellulose hydrolysates contain inhibitory substances.

The recombinant yeast strains were developed from *S. cerevisae* strain (BY 4743) by integrating genes for XR and XDH from *Pichia stipitis* (CBS 6054) into the yeast chromosome with the use of yeast expression vectors pGAPZA and pVT100–U respectively which are designed for high level constitutive expression. Some authors [17] has observed that the expression of both 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 the codon usage in *Pichia* is believed to be similar to *S. cerevisiae.* Besides the metabolic engineering approach, has been employed to improve the cell performance for ethanol production and to increase the stability of the recombinant strains [18].

This is also in agreement with the work of Jeffery [19] 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 has aided *S. cerevisiae* genetic engineering [20].

Another unique property is the use of expression vectors (pGAPZA and pVT100-U) for the cloned *XR* and *XDH* genes in our recombinant yeast strains. These plasmids do not require the presence of xylose or glucose for induction, and also the expression of the cloned genes is not repressed by glucose in the cultural medium. This will allow the recombinant *Saccharomyces* strains to effectively coferment glucose and

xylose without a lag period. 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 [21]. Data comparing the ability of our recombinant yeast strains to ferment xylose and to coferment glucose and xylose with the ability to produce ethanol is being published elsewhere.

The transformant with the XR gene on PGAPZA vector was grown on YPD medium supplemented with zeocin as selectable marker while transformant with XDH gene cloned on pVT100-U plasmid was cultivated on drop out synthetic medium with Ura 3 as the selectable marker. The presence of the expression cassettes in both transformants were examined and confirmed by colony PCR using the corresponding genes primers and restriction enzyme analysis respectively (data not shown). Based on our results, it was observed that the transformants grew less than the control strains. This finding is in agreement with Some authors, [22] who previously observed an identical phenomenon in transformants containing genes that encodes glycolytic enzymes and ascribed it to a potential burden effect, [23].

Similar observations was also made by some workers [24] in his own work "metabolic burden" on the cell, and explained that this effect could increased 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)

Furthermore, the transformation of *S. cerevisiae* with both genes as observed in this work will result in significant increase in ethanol productivity from lignocellulosic materials like Cocoa Pod Husk which it is intended for, as it has been demonstrated by some workers [25] 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. These compounds have been shown to affect both the specific growth rate and fermentation of ordinary yeast [26]. Furthermore, yeast strain transformed with these genes can detoxify the inhibitors that are normally present in lignocellulosic hydrolysate by reduction of these toxic compounds to alcohols [27,28,26,29].

Conclusively, since the resulting recombinant-fermenting *Saccharomyces* strains contain genes that can effectively co-ferment glucose and xylose present in the same medium. This unique property should make the recombinant *Saccharomyces* strains particularly effective in large-scale production of bio-ethanol from fermenting mixed sugars present in cellulosic biomass hydrolysates like cocoa pod husk hydrolysate for which it is intended for. Bioethanol is already an accepted transportation fuel in many countries and can be used in its pure form or as a blend with gasoline. Ethanol has many desirable features as a petroleum substitute and could help make a smoother transition from petroleum – based to a bio-based chemical economy.

Further experimental work to use this organism in the fermentation of Cocoa pod husk hydrolysate is ongoing.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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