# Expression Of Xylose Reductase Enzyme From *Spathaspora passalidarum* In *Saccharomyces cerevisiae.*

**Yaseen I. Mamoori*, Abdul Ghani I. Yahya and Majed H. AL-Jelawi**
Department of Biotechnology, College of Science, Al- Nahrain University, Baghdad, Iraq.

## Abstract

Baker's yeast (*Saccharomyces cerevisiae*) has been genetically engineered to ferment the pentose sugar xylose present in lignocellulosic biomass. One of the reactions controlling the rate of xylose utilization is catalyzed by xylose reductase (XR).The current study describes xylose reductase from *Spathasporapassalidarum* with NADH preference. According to JGI site the gene coding for this enzyme contains 954 nucleotides and it consists of 317 amino acids. The restriction sites for the enzymes SacII and NotI located on the 5´ termini for both the forward and reverse specific primers were designed using Lasergen 9.0 program. The genomic DNA was isolated and purified from *S .passalidarum.* Polymerase chain reaction (PCR) was used to amplify this gene. The amplified gene was cloned into pSN303 plasmid resulting of the pYIM1 plasmid and then transformed into *Escherichia coli*. This plasmid was reisolated from *E. coli*, sequenced,and finally transformed into *S. cerevisiae*. The yeast transformants carrying pYIM1 plasmid named YJTY1. The specific activity of enzyme was 1.55 and 0.48 U/mg on NADH and NADPH respectively for YJTY1. This enzyme has a natural preference for NADH which makes it a good candidate for combination with $NAD^+$ dependent xylitol dehydrogenase which may enable *S. cerevisiae* to utilize xylose under anaerobic conditions and convert it to ethanol.

**Keyword**: *Xylose reductase, Spathaspora Passalidarum, Cloning.*

# كلونة وتعبير إنزيم *Xylose Reductase* من *passalidarum* في *Saccharomyces cerevisiae*

**ياسين اسماعيل المعموري، عبد الغني ابراهيم يحيى وماجد حسين الجيلاوي**
قسم التقانة الاحيائية، كلية العلوم، جامعة النهرين، بغداد، العراق.

## الخلاصة

خميرة الخبز *Saccharomyces cerevisiae* حورت وراثيا لتخمر الزايلوز السكر الخماسي الموجود في الكتلة الحيوية اللكنوسيليلوزية. احد التفاعلات المسيطرة على استغلال الزايلوز يتم بواسطة إنزيم Xylose Reductase. الدراسة الحالية توصف إنزيم Xylose Reductase من *Spathaspora passalidarum* مع تفضيل لل NADH . طبقا لموقع JGI فان الجين المشفر لهذا الانزيم يحتوي 954 قاعدة نايتروجينية ويحتوي على 317 حامض اميني. صممت مواقع القطع للانزيمين SacII و NotI الواقعة في الطرف النهائي 5 لكل من البادئ الأمامي والخلفي بواسطة برنامج Lasergen 9.0 . عزل الدنا الجينومي ونقي من *S. passalidarum*. ضخم هذا الجين بواسطة PCR . كلون الجين المضخم في البلازميد pSN303 لينتج

---

*Email: yaseenismaeel@yahoo.com

البلازميد pYIM1 وحول الى بكتيريا *Escherichia coli*. عزل البلازميد مرة اخرى من *E.coli* وحدد تعاقبه واخيرا حول الى *S. cerevisiae* . المتحولات التي تحمل البلازميد pYIM1 سميت YJTY1. الفعالية النوعية للإنزيم كانت 1,55 و 0,48 وحدة/ملغم على NADH و NADPH على التعاقب للسلالة YJTY1 . هذا الانزيم له تفضيل طبيعي ل لNADH الذي يجعله مرشح جيد لمزجه مع xylitol dehydrogenase المعتمد على $NAD^+$ الذي يمكن أن يجعل *S. cerevisiae* قادرة على استغلال الزايلوز في الظروف اللاهوائية وتحويله إلى ايثانول

## Introduction:

There is currently great worldwide interest in producing bioethanol through fermentation of lignocellulose biomass derived from forest and agricultural by-products. Utilization of this biomass does not compete with food and feed production. However, the overall conversion of lignocellulose to ethanol is more complicated than sucrose and starch-based ethanol production. A significant fraction of lignocellulose biomass may consist of xylose which comprises as much as 40% of the total carbohydrate content [1]. Xylose is not utilized by native *S. cerevisiae*, which is the organism of choice in ethanol industries. This yeast produce ethanol with high efficiency as well as it exhibits high resistance to ethanol, by-products, and other inhibiting substances that are present in lignocellulose hydrolysates[2,3]. Fermentation of xylose to ethanol has consequently been achieved in *S. cerevisiae*by expression of heterologous xylose catabolizing pathways employing either xylose reductase (XR) and xylitol dehydrogenase (XDH) or xylose isomerase (XI) [4,5,6]. A major limitation of xylose fermentation by recombinant *S. cerevisiae*is the low ethanol productivity compared to glucose fermentation. Most importantly, however, the activity of the heterologous xylose catabolizing enzymes, XR/XDH or XI, is low, and expression from strong promoters or multicopy plasmids is required for efficient xylose utilization [7, 8, 9]. In addition, the cofactor usage in the XR/XDH pathway is unbalanced, with XR preferring NADPH on NADH and XDH that strict to NAD usage. This imbalance leads to by-product formation in terms of xylitol and reduced ethanol yield and productivity [10, 11]. To improve the cofactor balance and the flux through the xylose pathway, the NADH/NADPH specificity of XR and/or XDH has previously been engineered by site-directed mutagenesis [12, 13, 14]. The cofactor binding site of XR has been aligned to aldose reductases from different organisms) and amino acid residues responsible for discriminating between NADH and NADPH binding in XR have been engineered by site-directed mutagenesis to increase the specificity for NADH [15, 16]. *S. cerevisiae*strains harboring mutant XRs with enhanced NADH specificity have thus been demonstrated to exhibit increased ethanol yield and productivity [17, 18, 19]. In this study a new and natural xylose reductase preferring NADH to NADPH from *S. passalidarum* was isolated and overexpressed in *S. cerevisiae*.

## Material and methods

### Strains, media, and growth conditions.

*E. coli* 10 G Chemically Competent Cells (Lucigen, USA) were used for plasmid construction and propagation. *E. coli* was grown in LB medium (5 g/liter yeast extract, 10 g/liter tryptone, 10 g/liter NaCl, pH 7.0) at 37°C and ampicilin(100 µg/ml) was added with shaking at 200 rpm. The *S. cerevisiae*strain used in this study was CEN.PK2-1D. Yeast strains were grown in yeast extract-peptone-dextrose (YPD) medium (10 g/liter yeast extract, 20 g/liter peptone, and 20 g/liter glucose) or defined mineral medium (YSCD) containing 6.7 g/liter yeast nitrogen base without amino acids and supplemented with the appropriate auxotrophic requirements and 20 g/liter glucose at 30°C with shaking at 200 rpm. The pure cultures were stored at -80 with the addition of 15% glycerol.

### DNA manipulation, plasmid, and strain construction.

All the procedures were done according to [20] unless specified. The plasmid pSN303, used for the overexpression of XR driven by the *TDH3* promoter and terminator from *S. cerevisiae*was constructed as follow: *S. passalidarum XYL1* gene sequence was taken from JGI (Joint Genome Institute-www.jgi. doe .gov) which have an open reading frame of 954 nucleotides and it codes for 317 amino acids as shown in figure 1. A pair of specific primers

(CCCCGCGGAAAATGTCTTTTAAATTATC TTCAGGTTATGAAATGCCAAAAAT), Y1F and (CCGCGGCCGCCTTAAACAAAGATTG GAATATG) Y1R, (Integrated DNA technologies, USA) was designed using Lasergene 9.0. It was amplified using PCR (MJ thermal cycler, Biorad, USA), in a 50 µl reaction mixture containing 0.5 µM of each primer, 200 µM dNTPs, 1U Phusion DNA polymerase, and 100 ng of genomic DNA. Amplification was done with the following conditions: initial denaturation at 94° C for 10 min, 25 cycles of ( denaturation at 94° C for 15 sec, annealing at 55° C for 30 sec, and extension at 72°C for 30 sec), and final extension at 72° C for 5 min. The amplified DNA fragment was double digested with SacII and NotI. The pSN303 was digested with the same enzyme pair and dephosphorylated with the Antratic phosphatase. Both digested plasmid and PCR product were ligated with T4DNA ligase(New England Biolabs,USA)

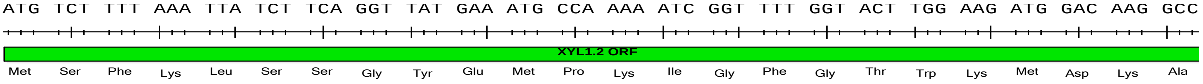

**Figure 1**- Nucleotide sequence of the *XYL1* gene and its amino acids

resulting in pYIM1 plasmid which was transformed into *E. coli* [21]. The transformed cells were grown on LB broth containing ampicilin (100µg/ml) for selection and were screened for the plasmid containing the *XYL1* gene by isolation of the plasmid (Gene JET plasmid Miniprep Kit-Fermentas, USA) and then digestion with the enzyme pair mentioned above. From these, one of them was selected randomly for sequencing (PCR Bid-Dye sequencing kit, Applied Biosystem, USA) with the DNA Analyzer (Applied Biosystem, USA). The pYIM1 plasmid was transformed into *S. cerevisiae*with the lithium acetate method [22]. SCD-URA plates were used for the selection of the *URA3* gene. The correct strain was verified by PCR using primer pair Y1F and Y1R and designated as YJTY1.

## Preparation of cell extract and measurement of enzyme activity.

*In vitro* enzymatic activity assay of XDH was done according to [23] with a slight modification. *S. passalidarum* and *S. cerevisiae* were grown overnight at 30° C, 200 rpm. Cells were harvested from 10-ml culture volume by centrifugation at 4,500 rpm (Cooled centrifuge, Eppendorff, Germany) for 10 min at 4 °C. The cell pellet was washed twice with 50 mM potassium phosphate buffer (pH 7.0), and the pellet was resuspended in 1 ml of the same buffer with the addition of 1 g acid-washed glass beads (particle size: 425–600 µm, Sigma, USA). The suspension was transferred into 13x100 mm glass tube. Cell disruption was conducted by using a vortex (Fischer Scientific, USA) for 1.5 minutes (in 30 seconds bursts) with an alternative cooling of cell homogenate on ice. The final homogenate was centrifuged at 11,000 rpm (Microcentrifuge, Eppendorff, Germany) for 20 min at 4° C. The supernatants were used for enzymatic activity assay. Protein concentrations in the cell-free extract were determined by “Bradford Protein Assay Kit” (Bio-Rad, USA) following the manufacture’s instruction. Enzymatic activity was determined spectrophotometrically by following the reduction of the coenzymes at 340 nm. One unit of the enzyme is defined as the amount of enzyme necessary to convert 1 µmol of substrate per minute at 25° C. Dynamic measurement of A340 nm was carried out by (UV/VIS spectrophotometer, Agilent, USA), with an interval time of 10 seconds for recording and a total measuring time of 2.5 min for each reaction. A quartz cuvette was used because it is the most accurate one with this wavelength. Enzyme activity was measured according to this equation: Specific enzyme activity (U/mg) = [(Δ A340.min$^{-1}$* Total volume)/ (6.22* Protein concentration mg.ml$^{-1}$* Volume of homogenate used)]. Readings were done with both blank (without xylose) and samples (with xylose) to determine the Δ A340.min$^{-1}$.The constant (6.22) in this equation represents the absorbance of 1 µM solution of NAD(P)H at 340 nm. The reaction mixture contained 50 mM potassium phosphate buffer pH 7.0, 0.4 mM NAD(P)H, 50 mM xylose ,cell-free extract, and distilled water in a total volume of 1.2 ml [23].

## Results and discussion

In this study, isolation, cloning, and characterization of the enzyme (Xylose Reductase-XR) from *S. passalidarum*[24] were done. The genomic DNA of *S. passalidarum*was isolated and purified to be the template for *XYL1* gene amplification. Specific primers (Y1F, Y1R) containing SacII and NotI restriction sites respectively were used to amplify this gene by PCR. Discrete bands were obtained with an approximate molecular weight of 950 bp as shown in figure 2. Both the plasmid pSN303 and the PCR products were double digested SacII and NotI. The recommended reaction was done with the same buffer but the two enzymes have activities of 50% and 75% respectively.Therefore, increased amounts of the two enzymeswith a prolonged period of time (2-3 hr) were used. This was done to ensure complete digestion of both the gene and the plasmid. The plasmid is then dephosphorylated with Antratic phosphatase to decrease the plasmid self re-ligation, resulted in false positive colonies within the transformants plate, during the ligation process.Ligation of both the plasmid and the PCR product was done using T4 DNA ligase and transformed into *E. coli.* The transformed bacteria were incubated overnight at 37° C. Two plates were done, the blank which contains only the plasmid whereas the other one containing the ligation mixture of plasmid and the PCR product. The control plate contains about 35 colonies whereas the plate for transformants contains too many colonies to count which gives an indication that the transformation process was successful as seen in figure 3. The colonies obtained within the control plate due to some uncut plasmids or they were not dephosphorylated. The screening process was done as follows five colonies were picked up from the plate and incubated overnight at 37° C on LB medium containing 100 µg/ml ampicillin. The resulting plasmid pYIM1 was isolated and purified from transformants. The plasmid was then digested with the same two restriction enzymes as seen in figure 4. In this figure we can see only 1 of 5 are false positive that contains only the plasmid pSN303 (≈7.2Kb) without *XYL1* gene. Four colonies contain the right construct (the plasmid and the gene). By digestion of the construct with the two restriction enzymes, two bands with the expected molecular weight (7.2 and 0.95 kb) are seen.One strain was selected for sequencing to confirm that the open reading frame is correct and to ensure amino acids sequence to give the functional enzyme. The sequence of this clone was completely correct. The DNA polymerase enzyme used for amplification of gene from genomic DNA of *S. passalidarum* was (Phusion). It is a novel *Pyrococcus*-like enzyme with a processivity enhancing domain which generates PCR products with accuracy (Error rate is 4.4 X $10^{-7}$ in Phusion HF buffer) and speed even on most difficult templates (http://www.neb.com/ ) .The plasmid was re-isolated from sequenced strain and transformed into *S. cerevisiae* using lithium acetate method. After three days of incubation at 30° C, the transformants were appeared in the SCD-URA plates as seen in figure 5. Good transformation efficiency rate was obtained indicating that this method can be used to transform *S. cerevisiae* and other yeast like *Schizosaccharomyces pombe*, *Candida albicans*, and *Pichiapastoris* with high efficiency transformation rate [25].

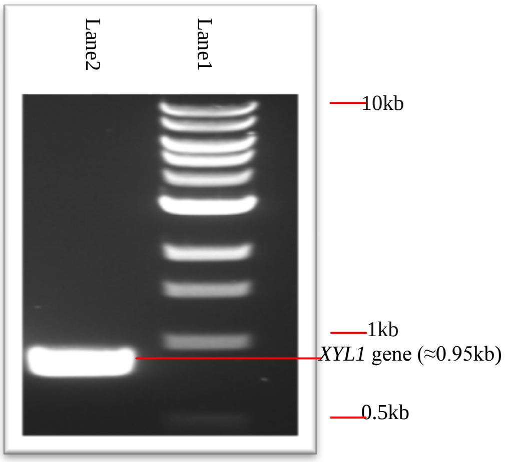

**Figure.2-** PCRamplification of *XYL1* gene with Y1F, Y1R primer pair. Electrophoresis was done on 1% agarose for 1 hr. Lane 2, *XYL1* band with a molecular weight of about 0.95 Kb are seen. Lane 1, 1Kb DNA ladder.

These transformants were screened for plasmid identification. The plasmid was isolated and purified using plasmid purification kit and then amplified using the originally primers for gene amplification. DNA bands within the expected molecular weight were found in some strain that designated as YJTY1 as seen in figure 6.

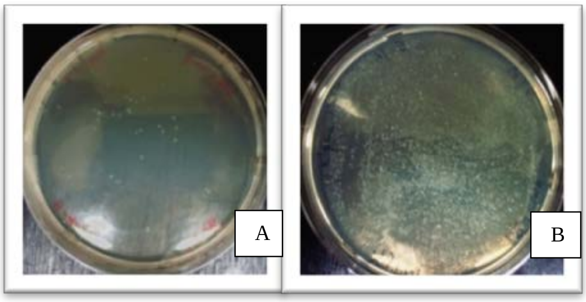

**Figure 3 -**Transformation of the plasmid pYIM1 containing *XYL1* gene into *E. coli.* (A): Control plate containing *E. coli* colonies carrying only the pSN303 plasmid without *XYL1* gene. (B) Plate that contains some positive *E. coli* colonies carrying the plasmid pYIM1**.**

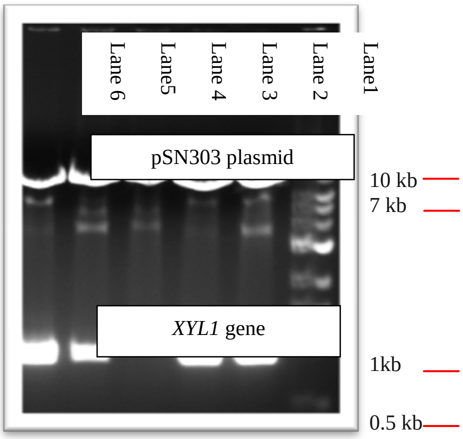

**Figure 4-** Screening for *XYL1* gene in *E. coli*transformants by digestion of pYIM1 plasmid with SacII and NotI restriction enzymes. Lane4 is false positive containing only the plasmid. Lanes 2, 3, 5, and 6 are true positives containing the plasmid pSN303 and the gene *XYL1*. Lane 1, 1Kb DNA ladder**.**

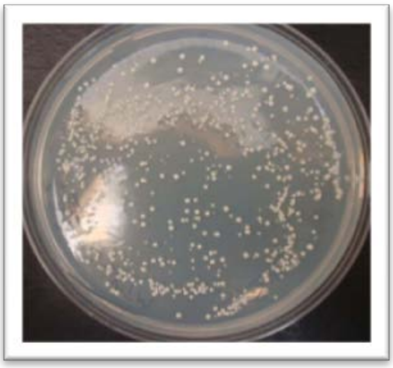

**Figure 5-** *S. cerevisiae* grown in SCD-URA plates after transformation with the plasmid pYIM1**.**

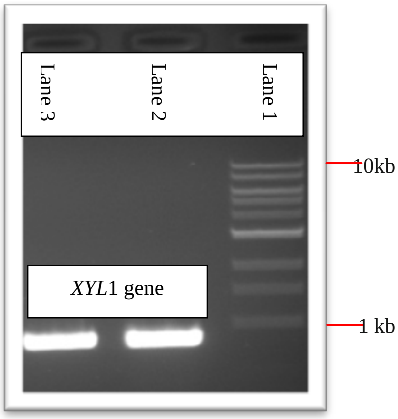

**Figure 6-** Screening for *XYL1* gene in *S. cerevisiae* transformants. The gene was amplified using PCR with the primer pairs Y1F, Y1R. Lanes 2, 3 are the gene bands with the approximate molecular weight of 0.95 kb. Lane 1, 1 kb DNA ladder.

The enzyme XR was tested for its specific activity on NADH and NADPH cofactors in *S. cerevisiae* (CENPK2-1D), and the strain *S. cerevisiae* (YJTY1) as seen in figures 7 and 8 respectively. The xylose is converted into xylitol with these two equations:

$\text{Xylose+ NADPH} \longrightarrow \text{Xylitol +NADP}^{+}$

$\text{Xylose+ NADH} \longrightarrow \text{Xylitol + NAD}^{+}$

These two equations show that the cofactors are oxidized and a curve down will be drawn as shown in figures 7,8. The specific enzymatic activities for the two strains were measured. The *S. cerevisiae*(CENPK2-1D) did not show any activity on both cofactors and this true because this strain does not have the ability to utilize xylose due to the lack of XR enzyme in its nature. The engineered strain obtained from this study, *S. cerevisiae* (YJTY1), showed a good specific activity for xylose 1.55 and 0.48 U/mg on both NADH and NADPH respectively. This overexpression obtained using a vector that contains strong promoter and terminator. In this study, the pSN303 plasmid was used that contains the TDH3 promoter and terminator (from *S. cerevisiae*) that classified as a strong one. This enzyme has a preference to NADH when compared to NADPH converting xylose into xylitol. This feature is a useful one to decrease cofactors imbalance when combined with the other enzyme (NAD$^{+}$ dependent xylitol dehydroense) that converts xylitol into xylulose. The XR enzyme was isolated from many yeasts like *Candida shehatae* (26), *Candida tenuis* (27), and *Schefferomyces stipits*(28) but it has a preference for NADPH over NADH.

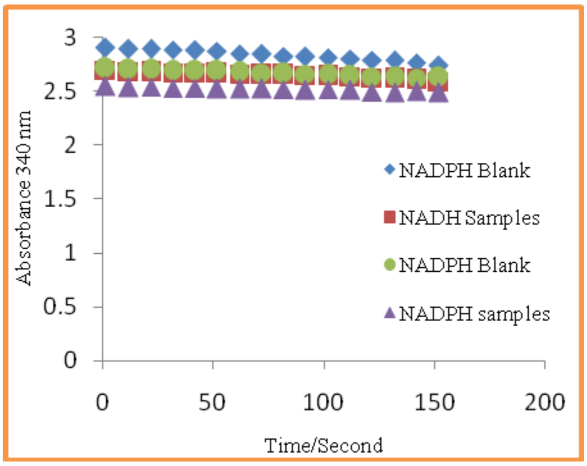

**Figure 7-** The Absorbance of NADHand NADPH oxidation at 340 nm against time without xylose (blank) or with xylose (sample) for xylose reductase for *S. cerevisiae* (CENPK2-1D).

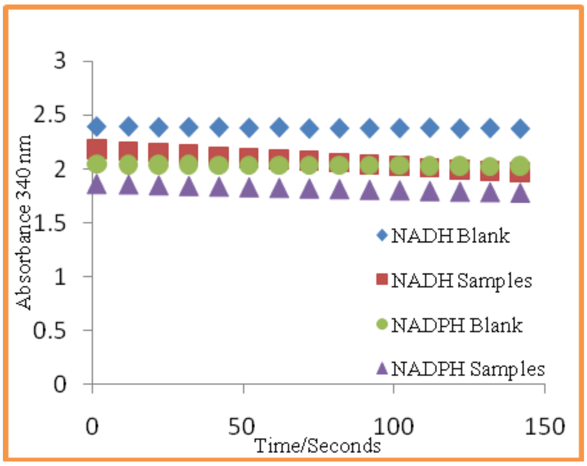

**Figure 8-** The Absorbance of NADHand NADPH oxidation at 340 nm against time without xylose (blank) or with xylose (sample) for xylose reductase for *S. cerevisiae* (YJTY1).

## Conclusion

The enzyme xylose reductase from *S. passalidarum* cloned and overexpressed in *S. cerevisiae* with high efficiency. This enzyme has a dual cofactor activity on both NADH and NADPH with a preference for NADH over NADPH that may improve the xylose conversion process. This enzyme and could be combined with xylitol dehydrogenase to complete the xylose pathway.

## Acknowledgments

The authors would like to thank the Ministry of Higher Education and Scientific Research in Iraq for its support to achieve this study. We would also like to thank Professor

Thomas W. Jeffries and his lab stuff for offering advice, materials, and lab space.

**References**

1. Galbe, M. and Zacchi,G. **2007**. Pretreatment of lignocellulosic materials for efficient bioethanol production. *Biofuels*, **108**,pp:41–65.
2. Hahn-Hägerdal, B., Wahlbom C.F., Gardonyi M., van Zyl, W.H., Cordero Otero, R.R. and Jönsson L.J. **2001**. Metabolic engineering of *Saccharomyce scerevisiae* for xylose utilization. *Adv. Biochem. Eng. Biotechnol.*,**73**,pp: 53–84.
3. Olsson, L. and Hahn-Hägerdal, B. **1993**. Fermentative performance of bacteria and yeasts in lignocellulose hydro- lysates. *Proc. Biochem.*, **28**,pp:249–257.
4. Hahn-Hägerdal, B., Karhumaa, K., Jeppsson, M. and Gorwa-Grauslund, M. F. **2007**. Metabolic engineering for pentose utilization in *Saccharomyces cerevisiae*. *Adv.Biochem Eng. Biotechnol*,**108**,pp:147–177.
5. Jeffries, T. W. **2006**. Engineering yeasts for xylose metabolism. *Curr. Opin. Biotechnol.*,**17**,pp:320–326.
6. van Maris, A. J., Winkler, A. A., Kuyper, M., de Laat, W. T., van Dijken, J. P. and Pronk J. T. **2007**. Development of efficient xylose fermentation in *Saccharomyces cerevisiae*: xylose iso- merase as a key component. *Adv. Biochem. Eng. Biotechnol.*, **108**,pp:179–204.
7. Jeppsson, M.,Träff, K., Johansson, B., Hahn-Hägerdal, B. and Gorwa- Grauslund, M. F. **2003**. Effect of enhanced xylose reductase activity on xylose consumption and product distribution in xylose-fermenting recombinant *Saccharomyces cerevisiae*. *FEMS Yeast Res.*, **3**,pp:167–175.
8. Karhumaa, K., Hahn-Hägerdal, B. and Gorwa-Grauslund, M. F. **2005**. Investigation of limiting metabolic steps in the utilization of xylose by recombinant *Saccharomyces cerevisiae* using metabolic engineering. *Yeast*, **22**,pp:359– 368.
9. Kuyper, M.,Hartog, M. M.,Toirkens, M. J.,Almering, M. J.,, Winkler, A. A., van Dijken, J. P. and Pronk, J. T. **2005**. Metabolic engineering of a xylose isomerase expressing *Saccharomyces cerevisiae*strainfor rapid anaerobic xylose fermentation. *FEMS Yeast Res.*,**5**,pp:399–409.
10. Jeppsson, M., Johansson, B., Hahn-Hägerdal, B. and Gorwa-Grauslund, M. F. **2002**. Reduced oxidative pentose phosphate pathway flux in recombinant xylose-utilizing *Saccharomyces cerevisiae* strains improves the ethanol yield from xylose. *Appl. Environ. Microbiol.*,**68**,pp:1604–1609.
11. Kotter, P. and Ciriacy, M. **1993**. Xylose fermentation by *Saccharomyces cerevisiae*. *Appl. Microbiol. Biotechnol.*, **38**,pp:776–783.
12. Bengtsson, O., Hahn-Hägerdal, B. and Gorwa-Grauslund, M. F. **2009**. Xylose reductase from *Pichia stipitis* with altered coenzyme preference improves ethanolic xylose fermentation by recombinant *Saccharomyces cerevisiae*. *Biotechnol. Biofuels*, **2**,pp:9-19.
13. Petschacher, B.,Leitgeb, S., Kavanagh, K. L., Wilson, D. K. and Nidetzky, B. **2005**. The coenzyme specificity of *Candida tenuis*xylose reductase (AKR2B5) explored by site-directed mutagenesis and X-ray crystallography. *Biochem. J.*, **385**,pp:75–83.
14. Watanabe, S., Abu Saleh, A., Pack, S. P.,Annaluru, N., Kodaki, T. and Makino, K. **2007**. Ethanol production from xylose by recombinant *Saccharo- myces cerevisiae* expressing protein-engineered NADH-preferring xylose reductase from *Pichia stipitis*. *Microbiol.*, **153**,pp:3044–3054.
15. Bohren, K. M., Page, J. L., Shankar, R., Henry, S. P. and Gabbay, K. H. **1991**. Expression of human aldose and aldehyde reductases. Site-directed mutagenesis of a critical lysine 262. *J. Biol. Chem.*, **266**,pp:24031–24037.
16. Kostrzynska, M.,Sopher, C. R. and Lee, H. **1998**. Mutational analysis of the role of the conserved lysine-270 in the *Pichiastipitis*xylose reductase. *FEMS Microbiol.Lett.*,**159**,pp:107–112.
17. Jeppsson, M., Bengtsson, O., Franke, K., Lee, H., Hahn-Hägerdal, B. and Gorwa-Grauslund, M. F. **2006**. The expression of a *Pichia stipitis* xylose reductase mutant with higher KM for NADPH increases ethanol production from xylose in recombinant *Saccharo- myces cerevisiae*. *Biotechnol., Bioeng.*,**93**,pp:665–673.

18. Petschacher, B. and Nidetzky, B. **2008**. Altering the coenzyme preference of xylose reductase to favor utilization of NADH enhances ethanol yield from xylose in a metabolically engineered strain of *Saccharomyces cerevisiae*. *Microb. Cell Fact.*,**7**,pp:9-21
19. Runquist, D., Hahn-Hägerdal, B. and Bettiga, M. **2009**. Increased expression of the oxidative pentose phosphate pathway and gluconeogenesis in anaerobically growing xylose-utilizing *Saccharomyces cerevisiae*. *Microb. Cell Fact.*, **8**,pp:49-58.
20. Sambrook, J. and Russel, D.W. **2001.** Third edition. Molecular cloning: a laboratory manual. Cold Springer harbor laboratory press, New York, USA.
21. Inoue, H., Nojima, H. and Okayama, H. **1990.** High efficiency transformation of *Escherichia coli* with plasmids. *Gene*, **96**,pp: 23-28.
22. Gietz, R.D., Schiestl, R.H., Willems, A.R. and Woods, R.A. **1995.** Studies on the transformation of intact yeast cells by the LiAc/SS-DNA/PEG procedure. *Yeast*, **11**,pp: 355-360.
23. Hou,X. **2012.** Anaerobic xylose fermentation by *Spathaspora passalidarum*. *Appl. Microbiol. Biotechnol.*,**94**,pp: 205-214.
24. Nguyen, N. H.,Suh, S., Marshall C. J. and Blackwell, M. **2006**.Morphological and ecological similarities: wood-boring beetles associated with novel xylose-fermenting yeasts, *Spathaspora passali- darum* gen. sp. nov. and *Candida jeffriesii* sp. nov. *Mycol. Res.*, **110**(10),pp: 1232-1241.
25. Kawai, S.; Hashimoto, W. and Murata, K. **2010.** Transformation of *Saccharo- myces cerevisiae* and other fungi: Methods and possible underlying mechanism. *Bioengineered Bugs*,**1**,pp:395-403.
26. Ho, N. W. Y., Lin, F. P., Huang, S., Andrews, P. C. and Tsao, G. T. **1990**. Purification, characterization, and amino terminal sequence of xylose reductase from *Candida shehatae. Enzyme Microbiol. Technol.*,**12**,pp:33–39.
27. Neuhauser, W.,Haltrich, D.,Kulbe, K. D. and Nidetzky, B. **1997**. NAD(P)H-dependent aldose reductase from the xylose-assimilating yeast *Candida tenuis*. *Biochem. J.*, **326**,pp:683–692.
28. Verduyn, C., Van Kleef, R., Frank, J., H., Schreuder, van Dijken, J. P. and Scheffers, W. A.**1985**. Properties of the NAD(P)H-dependent xylose reductase from the xylose-fermenting yeast *Pichia stipitis*. *Biochem. J.*, **226**,pp:669– 677.