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Cloning and Expression of Xylitol Dehydrogenase Enzyme from *Spathaspora passalidarum* in *Saccharomyces cerevisiae*

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Abstract

Spathaspora passalidarum is a natural xylose fermenting yeast that have the fungal pathway for converting xylose to ethanol. The second enzyme in this pathway is NAD^+- dependent Xylitol dehydrogenase which converts xylitol to xylulose. In this study, the sequence of the nucleotides for *XYL2* gene is found in JGI site. It consists of 1098 bp and code for 365 amino acids. The forward and reverse primers were designed with restriction sites on the 5` termini which are SacII and NotI restriction enzyme respectively using Lasergene 9.0 program. Genomic DNA was isolated and purified from *S .passalidarum* and amplified using PCR and it cloned into pSN303 resulting of the pYIM2 plasmid. Then it is transformed into *Escherichia coli*. This plasmid was isolated from *E. coli* and retransformed into *S. cerevisiae* and transformant is called YJTY2. Results showed that enzyme specific activities with NAD^+ as cofactors were 2.32 and 0.0 U/mg for *S. cerevisiae* YJTY2 and *S. cerevisiae* (CENPK2.1D) respectively. The enzyme did not show any activity with $NADP^+$ as a cofactor. This enzyme is NAD^+ dependent and can be used in combination with xylose reductase in *S. cerevisiae* to be able to ferment xylose.

Keywords: *Spathaspora passalidarum, Saccharomyces cerevisiae,* xylitol dehydrogenase, xylitol, xylose.

Introduction

Bioethanol production from lignocellulosic material has received considerable attention recently. It is considered as a clean liquid fuel and is potentially a replacement for fossil fuel that give rise to reduce global warming and demand for petroleum. Bioethanol has been produced by sugar fermentation from crops such as corn and sugarcane. These crops are used for both human and animal feed resulting in a competition between using them as a food or a fuel production [1]. *Saccharomyces cerevisiae* is the most promising candidate for lignocellulosic ethanol production due to its excellent glucose fermentation capability, high ethanol tolerance, and resistance to inhibitors presented in lignocellulosic hydrolysates [2]. However, native *S. cerevisiae* strains cannot utilize xylose for either growth or ethanol production, leaving a significant fraction of lignocellulosic hydrolysates (20–30%) unusable [3, 4]. A lot of research has been done during the last 20 years on the yeast conversion of xylose to ethanol, with major efforts focused on the functional expression of bacterial and fungal xylose utilizing genes and manipulating the pentose phosphate pathway (PPP) to enhance xylose utilization and fermentation in *S. cerevisiae* [5, 6, 7, 8]. There have been two common metabolic pathways for xylose utilization in fungi and bacteria. In most fungi and xylose-fermenting yeasts, such as *Scheffersomyces stipitis* [9], *Candida shehatae*, and *Pachysolen tannophilus*, xylose first is reduced to xylitol by NAD(P)H-dependent xylose reductase (XR), which is encoded by *XYL1*, and then xylitol is oxidized to xylulose by NAD^+-dependent xylitol dehydrogenase (XDH), which is encoded by *XYL2* [10]. Finally, xylulose is phosphorylated into xylulose-5-phosphate, which is further metabolized through the PPP [11]. An alternative pathway is the non-cofactor-requiring xylose isomerase (XI) pathway from bacteria or fungi, which can isomerize xylose to xylulose [12, 13]. Introducing the *S. stipitis* XR-XDH system into *S. cerevisiae* has successfully allowed xylose to be fermented to ethanol. However, a major drawback of the XR-XDH system is cofactor imbalance, because XR strongly prefers NADPH over NADH [14] and XDH uses NAD^+ [4] as a cofactor. This cofactor imbalance leads to the excess accumulation of

xylitol and reduced final ethanol yield. The surplus NADH cannot be reoxidized sufficiently through respiration under oxygen-limited conditions during bioethanol fermentation and thus causes the formation of by-product glycerol which further affects ethanol yield. To overcome this problem, the XI pathway has been introduced into *S. cerevisiae*, resulting in higher ethanol yield but lower growth rate and ethanol productivity during xylose fermentation [15, 16, 17]. Several metabolic engineering approaches have been implemented previously to balance the cofactors in *S. cerevisiae*: the overexpression of the *Kluyveromyces lactis GDP1* gene, which encodes an $NADP^+$-dependent glyceraldehyde-3- phosphate dehydrogenase [18]. Another promising approach for reducing xylitol production and enhancing ethanol yield using recombinant *S. cerevisiae* involves alterations in the coenzyme specificity of XDH (with the coenzyme preference shifted from NAD^+ to $NADP^+$ [19, 20] by protein engineering. In this study a new XDH from *S. passalidarum* was cloned and overexpressed in *S. cerevisiae* and its enzyme activity was measured. This enzyme could be used along with XR to complete the xylose pathway metabolism in *S. cerevisiae.*

Materials and methods

Strains, media, and growth conditions.

Escherichia 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 was 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 [21] unless specified. The plasmid pSN303, used for the overexpression of XDH driven by the promoter and terminator for TDH gene from *S. cerevisiae*, was constructed as follow: *S. passalidarum XYL2* gene sequence was taken from JGI (Joint Genome Institute-www. jgi.doe.gov) which contains 1098 bp (Fig(1)) and code for 365 amino acids (Fig.(2)). A pair of specific primers "Y2F (CCCCGCGGAAAAATGTCTGTTGCTAACCCATCAT), and Y2R, (CCGCGG CCGCCTTAATGGACCATCA) (Integrated DNA technologies, USA) was designed using Lasergene program 9.0. The genomic DNA from *S. passalidarum* was isolated using Master Pure Yeast DNA Purification Kit (Epicentre, USA). It was amplified using PCR (MJ thermal cycler, Biorad, USA), High fidelity DNA polymerase (Phusion) was used, 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 T4 DNA ligase (New England Biolabs, USA) resulting in pYIM2 plasmid. This plasmid was transformed into *E. coli* [22]. The transformed cells were grown on LB plates containing ampicilin (100µg/ml) for selection and were screened for the plasmid containing the *XYL2* 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 pYIM2 plasmid was transformed into *S. cerevisiae* with the lithium acetate method [23]. SCD-URA plates were used for the selection of the *URA3* gene. The correct strain was verified by PCR using primer pair Y2F and Y2R and designated as YJTY2.

ATGTCTGTTGCTAACCCATCATTAGTTC
TTAACAAGATTGACGACATCACCTTCG
AAACCTACGAAGCCCCAGAAATTGTCG
AACCAACCGACGTTATTGTCGAAGTTA
AAAAGACTGGTATCTGTGGTTCTGATA
TCCACTACTATGCCCACGGTAAGATTG

GTAACTTTATCTTGACCAAGCCAATGGT
TTTGGGTCACGAATCTGCCGGTGTTGTT
TCCCAAGTTGGTAAGGGTGTCAAGCAC
TTGAAGGTTGGTGACAGAGTTGCCATT
GAACCAGGTATTCCATCCAGATTATCC
GACGCTTACAAGTCTGGTCACTACAAC
TTGTGTCCTCACATGTGTTTTGCTGCCA
CTCCAAACTCCACTGAAGGTGAACCAA
ACCCACCAGGTACCTTGTGTAAATATTT
CAAGTCCCCAGAAGATTTCTTGGTTAA
GTTGCCAGAACACGTCTCCTTGGAATT
GGGTGCCATGGTTGAACCATTGTCTGTC
GGTGTCCACGCCTCCAAGTTAGGTAAG
GTTACTTTCGGTGACAATGTTGCCGTTT
TCGGTGCTGGTCCAGTTGGTTTATTGGC
TGCTGCCACCGCCAAGACCTTTGGTGCT
GCCAGAGTCATTGTCATTGATATCTTTG
ACAACAAGTTACAAATGGCCAAGGACA
TTGGTGCTGCCACTCACACCTTCAACTC
CAAGACTGGTGGTGATTACAAGGACTT
GATTGCTGCCTTTGACGGTGTTGAACCA
AATGTTATTTTGGAATGTACCGGTGCTG
AACCATGTATTGCCATGGGTGTCCAAA
TTGCTGCTCCAGGTGGTAGATTTGTCCA
AGTTGGTAATGCTGGTGCCGCTGTCAA
GTTCCCAATTACTGAATTTGCTACTAAG
GAATTGACCTTATTCGGTTCTTTCAGAT
ATGGTTACGGTGACTACCAAACTGCCG
TTAACATTTTCGATGCCAACTACAAGA
ATGGTAAGGACAAGGCTCCAATTGACT
TTGAACAATTGATTACCCACAGATTCA
AGTTTGACGATGCCATCAAGGCTTACG
ACTTGGTTAGAGCCGGTTCTGGTGCCGT
CAAGTGTTTGATTGATGGTCCATTATAA
.

Fig. (1): The nucleotide sequence of the gene *XYL2* which is 1098 bp.

MSVANPSLVLNKIDDITFETYEAPEIVEPT
DVIVEVKKTGICGSDIHYYAHGKIGNFILT
KPMVLGHESAGVVSQVGKGVKHLKVGD
RVAIEPGIPSRLSDAYKSGHYNLCPHMCF
AATPNSTEGEPNPPGTLCKYFKSPEDFLV
KLPEHVSLELGAMVEPLSVGVHASKLGK
VTFGDNVAVFGAGPVGLLAAATAKTFG
AARVIVIDIFDNKLQMAKDIGAATHTFNS
KTGGDYKDLIAAFDGVEPNVILECTGAEP
CIAMGVQIAAPGGRFVQVGNAGAAVKFP
ITEFATKELTLFGSFRYGYGDYQTAVNIF
DANYKNGKDKAPIDFEQLITHRFKFDDAI
KAYDLVRAGSGAVKCLIDGPL

Fig. (2): The amino acids sequence of the xylitol dehydrogenase.

Preparation of cell extract and measurement of enzyme activity.

In vitro enzymatic activity assay of XDH was done according to [24] with a slight modification. *S. passalidarum* and *S. cerevisiae* were grown overnight at 30° C with shaking at 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 5 seconds for recording and a total measuring time of 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 xylitol) and samples (with xylitol) to determine the Δ A340.min^{-1}.The constant (6.22) in this equation represents the absorbance of 1 µM solution of NAD(P)$^{+}$ at 340 nm. The reaction mixture contained 35 mM glycine buffer pH 9.0, 0.4 mM

$NAD(P)^+$,20 mM xylitol,cell-free extract, and distilled water in a total volume of 1.2 ml.

Results and Discussion

S. passalidarum is able to use xylose as the sole carbon source for biomass growth and ethanol. In this study, isolation, cloning, and characterization of the enzyme (xylitol dehydrogenase-XDH) from this organism were done. The genomic DNA of *S. passalidarum* was isolated and purified to be the template for *XYL2* gene amplification.It was amplified using PCR with specific primers (Y2F, Y2R) containing SacII and NotI restriction sites respectively. These restriction sites can be amplified along with the PCR product, easily digested with the specified restriction enzymes, and loaded onto the plasmid. Discrete bands were obtained with an approximate molecular weight of 1100 bp as shown in Fig.(3). This is agreeing with the theoretical 1098 bp of *XYL2* gene found in the JGI site. 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 enzymes with 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. Both the plasmid and the PCR product were ligated to each other using T4 DNA ligase and transformed into *E. coli.* After overnight incubation at 37° C, two plates were done, the blank which contains only about 3 to 4 colonies where as the plate for transformants contain more than 40 colonies which gives an indication that the transformation process was successful as seen in Fig.(4). The colonies obtained within the blank plate due to some uncut plasmid or it was not dephosphorylated.
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* is (Phusion). It is a novel *Pyrococcus*-like enzyme with a processivity enhancing domain which generates PCR products with accuracy (Error rate is 4.4×10^{-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 Fig.(6). 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 *Pichia pastoris* with high efficiency transformation rate [25]. These transformants were screened for plasmid identification. The plasmid was isolated and purified using plasmid purification kit and then amplified using the primers used originally for gene amplification. DNA bands within the expected molecular weight were found in two screened colonies. One of them is selected randomly and designated as YJTY2 as seen in Fig.(7). The enzyme XDH was tested for its activity on NAD^+ and $NADP^+$ cofactors in *S. cerevisiae* CENPK2-1D, and the strain *S. cerevisiae* YJTY2 as seen in Figs. (8 and 9) respectively. The xylitol is converted into xylulose with this equation: NAD^+ + xylitol xylulose + NADH. This equation shows that the cofactor is reduced and an up curve will be formed as shown in Fig.(8, 9). The *S. cerevisiae* CENPK2-1D did not show any activity on both cofactor and this true because this strain does not have the ability to utilize xylitol because it has not the XDH enzyme in its nature. On the other hand, The engineered strain obtained from this study, *S. cerevisiae* YJTY2, showed a good specific enzyme activity which was 2.32U/mg and this due to that this enzyme overexpressed form engineered *S. cerevisiae* because its driven by strong promoter and terminator for *TDH3* gene. This strain also did not show any enzymatic activity on $NADP^{+.}$ This enzyme has the same cofactors dependence isolated from many organisms like *Aspergillus niger* [26], and *Pantoea ananatis* [27].

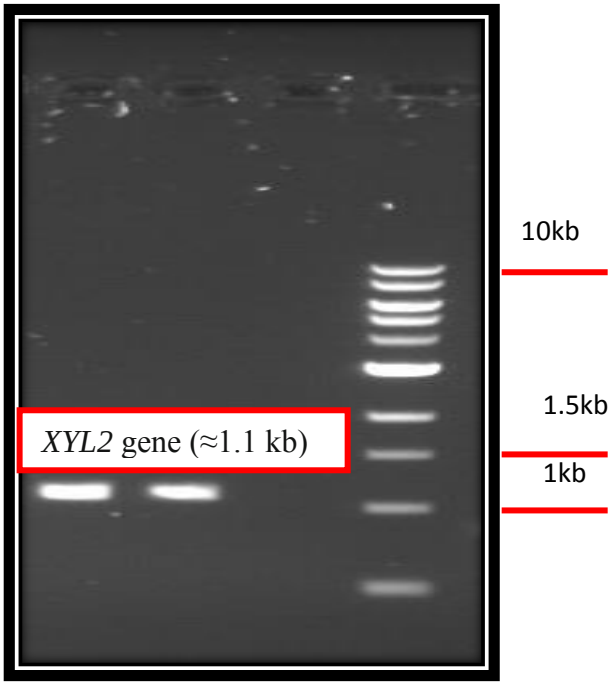


Fig. (3) PCR amplification of XYL2 gene with Y2F, Y2R primer pair. Electrophoresis was performed on 1% agarose gel and run with 5 volt/cm for one hour. Lanes 2 and 3, XYL2 bands with a molecular weight of about 1.1 Kb, are seen. Lane 1, 1Kb DNA ladder.

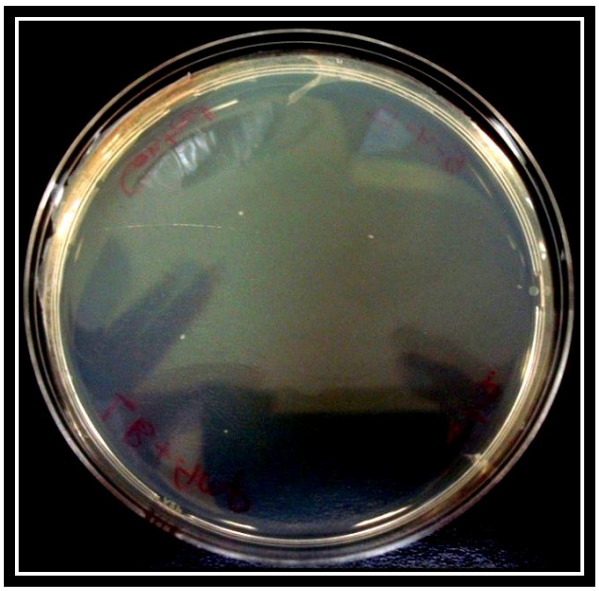


Fig. (4-A) Transformation of the plasmid pYIM2 containing XYL2 gene into E. coli. Control plate containing four E. coli colonies carrying only the pSN303 plasmid without XYL2 gene.

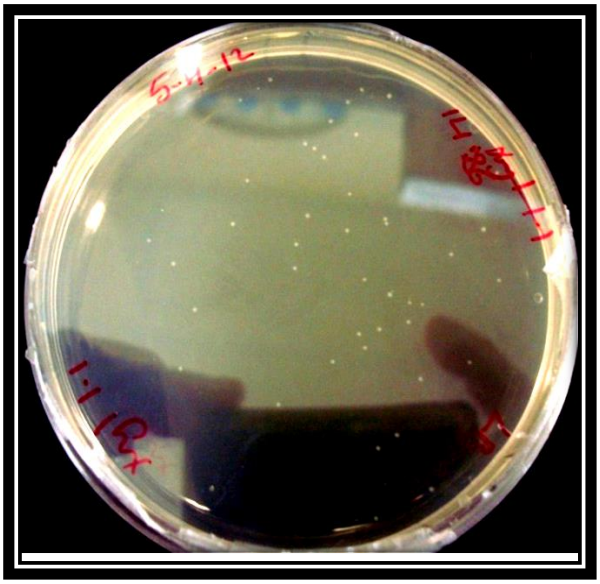


Fig. (4-B) Transformation of the plasmid pYIM2 containing XYL2 gene into E. coli. Plate which contains some positive E. coli colonies carrying the plasmid pYIM 2.

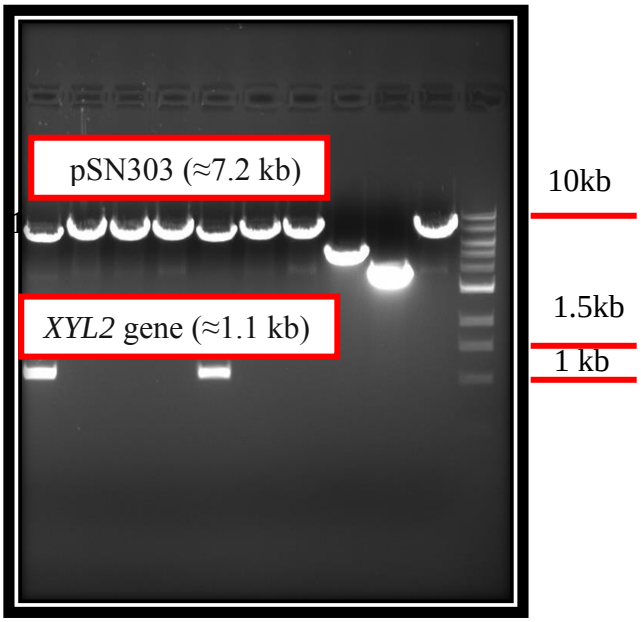


Fig. (5) Screening for XYL2 gene in E. coli transformants by digestion of pYIM2 plasmid with SacII and NotI restriction enzymes. Electrophoresis was performed on 1% agarose gel and run with 5 volt/cm for one hour. Lanes 2, 3, 4, 5, 6, 8, 9, and 10 are false positive. Lanes 7 and 11 are true positives because they contain the plasmid pSN303 and the gene XYL2. Lane 1, 1Kb DNA ladder.

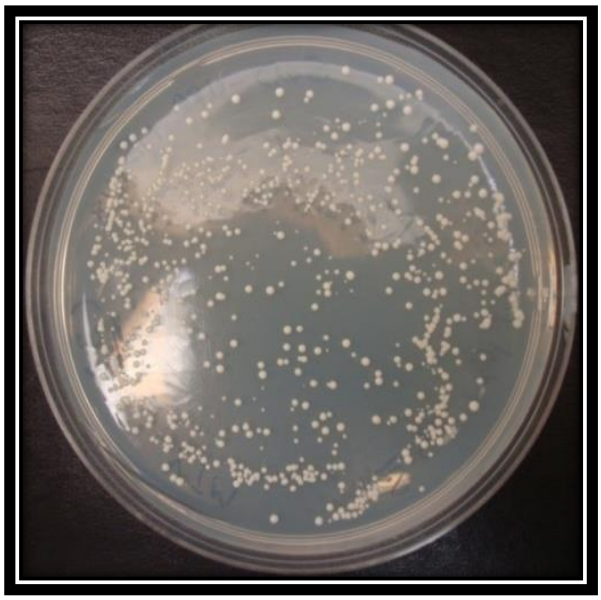


Fig. (6) S. cerevisiae grown in SCD-URA plates after transformation with the plasmid pYIM2.

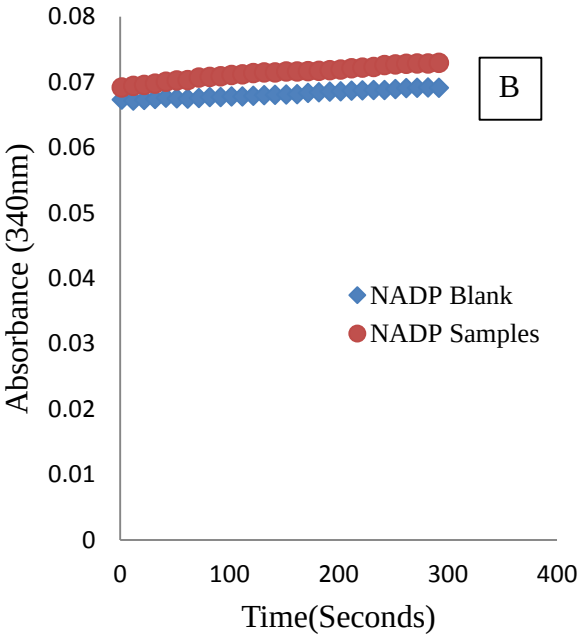
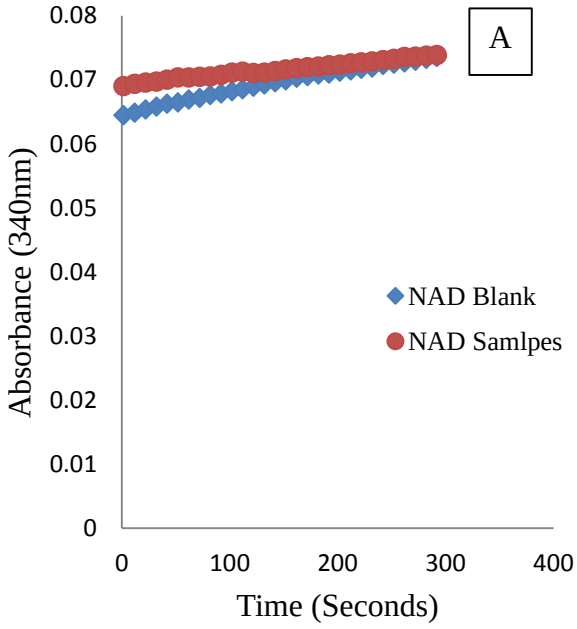
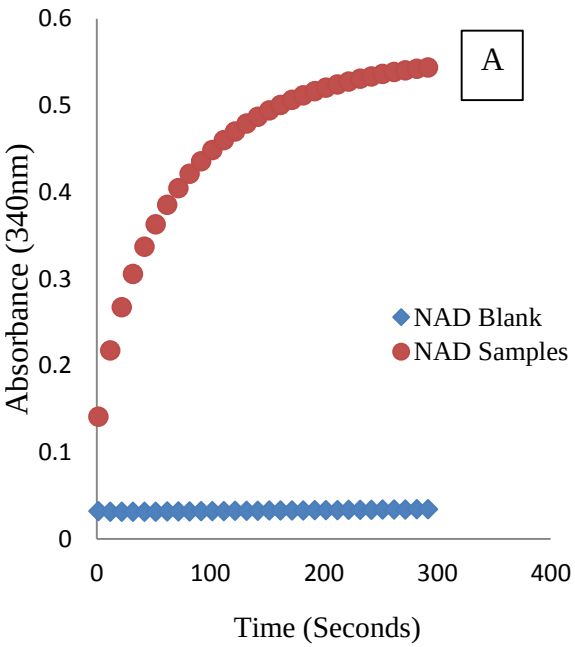


Fig. (7) A, The Absorbance of NAD^+ reduction at 340 nm against time without xylitol (blank) or with xylitol (sample) for xylitol dehyrogenase from S. cerevisiae CENPK2-1D. B, The absorbance of $NADP^+$ reduction at 340 nm against time without xylitol (blank) or with xylitol (sample) for xylitol dehyrogenase from S. cerevisiae CENPK2-1D.





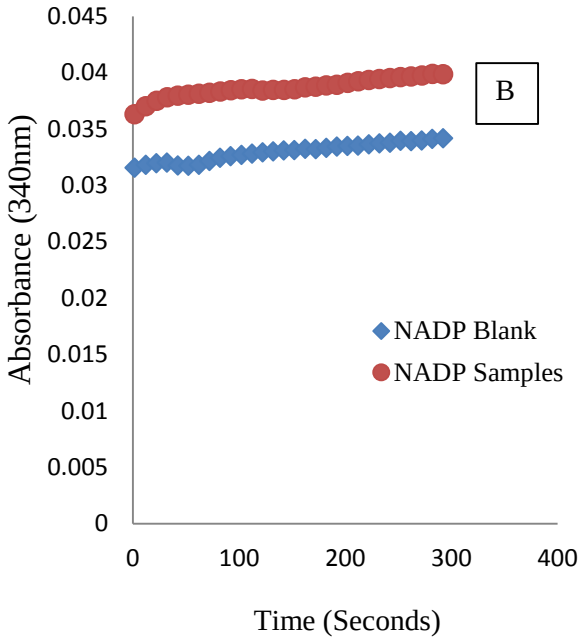


Fig. (8) A, The Absorbance of NAD$^+$ reduction at 340 nm against time without xylitol (blank) or with xylitol (sample) for xylitol dehyrogenase from S. cerevisiae YJTY2. B, The Absorbance of NADP$^+$ reduction at 340 nm against time without xylitol (blank) or with xylitol (sample) for xylitol dehyrogenase from S. cerevisiae YJTY2.

Conclusion

The enzyme xylitol dehydrogenase from *S. passalidarum* cloned and overexpressed in *S. cerevisiae* with high efficiency. This enzyme is NAD+ dependent enzyme and could be combined with xylose reductase to complete the xylose pathway.

Acknowledgments

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الخلاصة

S. passalidarum هي خميرة تخمر الزايلوز طبيعيا ولها مسار الفطريات في تحويل الزايلوز الى ايثانول. الانزيم الثاني في هذا المسار هو NAD^+ - dependent xylitol dehydrogenase الذي يحول الزايلتوز الى زايللوز والذي يمكن استغلاله بواسطة مسار Pentose phosphate pathway ليعطي الناتج النهائي وهو الايثانول. في هذه الدراسة، التعاقب النيوكليوتيدي لهذا الجين *XYL2* وجد في موقع JGI وهو مكون ١٠٩٨ زوج قاعدي ويشفر لـ ٣٦٥ حامض اميني. صمم البادئ الامامي والعكسي مع مواقع القطع في النهايات من الطرف ٥ للانزيمين SacII, NotI على التوالي بواسطة البرنامج Lasergen 9.0. عزل الدنا الجينومي من *S. passalidarum*. ضخم الجين بواسطة الPCR و كلون في البلازميد pSN303 لينتج البلازميد pYIM2. ثم حول في *Escherichia coli*. عزل هذا البلازميد وحول مرة اخرى الى *S. cerevisiae* سميت هذه المتحولة بـ YJTY2. اظهرت النتائج بان الفعالية الانزيمية الخاصة مع NAD^+ كعامل مساعد كانت ٢،٢٣ و ٠،٠ وحدة/ملغم لـ *S. cerevisiae* YJTY2 و *S. cerevisiae* CEN.PK-1D على التوالي. لم يظهر الانزيم اي فعالية مع $NADP^+$ كعامل مساعد. من هذه النتائج نستخلص بان هذا الانزيم معتمد على NAD^+ ويمكن استخدامه مع xylose reductase في *S. cerevisiae* لكي تستطيع ان تخمر الزايلوز.