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MOLECULAR EXPRESSION OF THERMOSTABLE XYLOSE ISOMERASE (XYLA) ISOLATED FROM BREVIBACILLUS FORMOSUS NF2 AND ITS BIOPHYSICAL CHARACTERISTICS

Musadak Iqbal[1], Hamid Mukhtar[1],Rashid Mir[2],Ruqaiah I Bedaiwi[2], Aziz Dhaher Albalawi[4], Ahmed Khaled J Alatawi[4], Sultan khalid Aljohani[5] ,Fares Falah Alblwi[5] and Bushra Muneer[1*]

[1-]Dr.Ikram-ul-Haq Institute of Industrial Biotechnology, Government College University, Lahore, Pakistan.
Email: musadiq81@gmail.com, hamidmukhtar@gcu.edu.pk, dr.bushramuneer@gcu.edu.pk

[2-]Division of Biomedical and Life Sciences, Lancaster University. Lancaster, United Kingdom. Email: muhammad.munir@lancaster.ac.uk

[3-] Department of Medical Laboratory Technology Faculty of Applied Medical Sciences, Prince Fahad Bin Sultan Chair for Biomedical Research, University of Tabuk, 71491, Tabuk, Saudi Arabia. Email: rashid@ut.edu.sa, rbedaiwi@ut.edu.sa,

[4-] Medical Laboratory ,King Khalid Hospital,Tabuk, Saudi Arabia . Email: azizda@moh.gov. sa,akalatwi@moh.gov.sa , Skaljohani@moh.gov.sa

[5-]Prince Abdulmohsen Hospital, AlUla, Saudi Arabia . fares.0252@hotmail.com

*Corresponding Author: Dr. Bushra Muneer . dr.bushramuneer@gcu.edu.pk

Abstract

The study aims to produce catalytic active xylose isomerase enzyme for the production of high fructose corn syrup as an alternative source of health hazardous inverted and non-calorific sweeteners. Xylose isomerase gene of Brevibacillus formosus NF2 was cloned in pTZ57R/T vector and expressed in Escherichia coli using pET28a(+) vector. The over-expressed xylose isomerase was purified to homogeneity using UNOsphere Q anion exchange column chromatography and Resource ISO pre-packed hydrophobic interaction column chromatography followed by 70% ammonium sulphate precipitation. Using this optimized approaches, we obtained 39.03% yield and enzyme was 8.13 folds purified. Size of xylose isomerase enzyme was estimated to be ~38 kDa using SDS-PAGE. We further characterised that purified xylose isomerase enzyme showed optimal activity at 55 °C and pH 7.0. Xylose isomerase retained 100% activity at 45 °C and 55 °C for 56 h, retained 90-95% of its activity at pH range of 6.5-7.5 for 40 min, exhibited maximum activity in the presence of 5 mM Mg^{2+} and strictly inhibited by Ca^{2+}. The enzyme was capable to produce 54.67% of D-fructose from conversion of D-glucose. The kinetic parameters K_m, V_{max}, K_{cat} and K_{cat}/K_m were determined to be 3.73 mM, 65.36 µmol/min, 19395 sec^{-1} and 5200 sec^{-1}/mM for D-glucose and 2.12 mM, 243.9 µmol/min, 72374 sec^{-1} and 34139 sec^{-1}/mM for D-xylose. Taken together, our finding provide foundational work on the exploitation of xylose isomerase enzyme from Brevibacillus formosus NF2 potential candidate for the production of high fructose corn syrup (HFCS) at industrial scale.

Keywords: Characterization; Cloning; Over-expression; Purification; high fructose corn syrup; Brevibacillus formosus NF2; xylose isomerase.

1. INTRODUCTION

Owing to increasing awareness of the harmful effects of sucrose and other invert sugars on human health, there is a deep desire to explore suitable sucrose substitutes. This is further compounded by the high production cost. Many non-calorific and non-carbohydrate artificial sweeteners have been proposed including saccharine, cyclamate, acesulfame K, aspartame, and thaumatin. However, majority of these substitutes are discouraged due to health concerns or other drawbacks. High fructose corn syrup (HFCS) is an equilibrium blend of glucose and fructose (with a 1:1 ratio) and is 1.3 times sweeter than sucrose and 1.7 times sweeter than glucose [1]. Sweetening capacity of glucose is 70 to 75% of that of sucrose, while fructose is twice as sweet as sucrose [1]. Taken together, HFCS is a sweetener and can be used as an alternative for sucrose or invert sugar in the food and beverage industries [2].

During last 100 years, chemical conversion of glucose to fructose has been comprehensively studied. This chemical reaction consists of a group of reactions collectively referred to as the "Lobry de Bruyn Alberda van Ekenstein" transformation and conducted at high pH and temperature [3]. The reaction does not exhibit specificity and results in the production of non-metabolizable sugars [4]. However, achieving a fructose concentration exceeding 40% is challenging

using this method. Furthermore, chemically generated fructose possesses off-flavours and reduced sweetness that cannot be easily rectified. Thus, this chemical method is unsuitable for commercial use. Conversely, for conversion of glucose to fructose enzymatic method offers multiple advantages, including (i) the reaction's specificity, (ii) the ability to operate under ambient pH and temperature conditions, and (iii) the absence of side product formation. Consequently, enzymatic conversion is the preferred approach over chemical isomerization of glucose to fructose. Currently, the process involving xylose isomerase enzyme (XI) has experienced significant growth in the industrial market [1].

Presently, industrial biotechnology has huge influence on diverse industries for the production of genetically engineered products. Imparting the physical change in a molecule without change in original molecular formula are the results of isomerase enzymes. D-Glucose/xylose isomerase (XI) is also known as glucose isomerase (GI) and D-xylose ketol isomerase; E.C.# 5.3.1.5 holds the third position among the mostly used industrial enzymes and the other two are protease and amylase [4]. It reversibly isomerizes D-glucose to D-fructose and D-xylose to xylulose and vice versa. The interconversion of xylose to xylulose fulfils a nutritional need for saprophytic bacteria thriving on decaying plant material and it also plays a vital role in the bioconversion of hemicellulose to bioethanol. Commercially, the isomerization of glucose to fructose holds significant importance in the production of HFCS. Recent reports from Bektas [5] and Colak et al. [5] have reported potential industrially important thermostable enzymes producing strains, among them was Brevibacillus species. Bektas [6] reported that Brevibacillus species are the potential reservoir for industrially important enzymes. In this study, XI gene Brevibacillus Formosus NF2 was cloned and overexpressed in E. coli. The fermentation conditions were optimized to obtain maximum expression of biologically active XI. Over-expressed XI was then purified and characterized. The aim of this study is to produce biologically active and thermostable XI that can be used for the production of HFCS which can be used as an alternative source of health hazardous inverted and non-calorific sweeteners.

2. MATERIALS AND METHODS

2.1 Culture media and primary reagents

Escherichia coli strain DH5a and BL21 (DE3) were used as host for cloning and expression studies and grown in LB medium at 37°C. Taq DNA polymerase (Cat # 10342053), dNTP Mix (Cat # R0192), agarose I (Cat # 17850), restriction endonucleases [Nco-I (Cat# ER0571) and Not-I (Cat# ER0595)], T4 DNA ligase (Cat# EL0014), InsTA- clone PCR Cloning Kit (Cat# K1214 were purchased from Fermentas. A purification kit for PCR products and DNA restriction fragments was obtained using QIAquick Gel Extraction kit (Cat# 28704) was purchased from Qiagen. The substrates and other chemicals used in experimental work were analytically purified and were purchased from Carl ROTH and Sigma (Germany).

2.2 Genomic DNA and plasmids

Genomic DNA of Brevibacillus formosus NF2 strain was kind granted by Dr M. Javed Iqbal, University of the Punjab, Lahore and was used as the source of xylA. The cloning vector (pTZ57R/T) and expression vector (pET28a(+)) were utilized (write source)

2.3 Cloning of xylose isomerase

2.3.1 PCR amplification of xylA

Xylose isomerase gene (xylA) was amplified using gene specific primers designed using OligoCalc tool (forward primer: 5`-**GCCCATGGAGCTTGGCGTATTTA**-3` and reverse primer: 5`-**GCGCGGCCGCCTAAACCCACCATA-3**`) bearing Nco-I and Not-I restriction site respectively and genomic DNA of Brevibacillus formosus NF2 was used as template. PCR reaction comprised 30 cycles each, with preliminary denaturation for 5 min at 94 °C, cyclic denaturation for 1 min at 94 °C, cyclic annealing for 1 min at 48 °C, cyclic extension for 80 sec for 72 °C and final extension for 20 min at 72 °C. The PCR product was purified by QIAquick Gel Extraction kit and then cloned using InsT/Aclone cloning kit. Standard protocol initially proposed by Sambrook and Russel [7] was used for transformation of E. coli DH5α competent cells using recombinant vector. Blue and white screening was used for the selection of positive clone. White colonies indicate positively transformed colonies. Positive clone was also confirmed using PCR, restriction analysis and Sanger's sequencing.

2.3.2 Sequence analysis

Nucleotide sequence obtained was subjected to Expasy translate tool to determine amino acid sequence and was then analysed for determination of homology among different species belonging to family Paenibacillaceae. CLUSTALW alignment tool was used for multiple alignment and FastTree tool of CLUSTALW was used for construction of phylogenetic tree.

2.4 Cloning of xylA in pET28a(+)

Recombinant pTZ57R/T:: xylA was isolated using standard alkaline lysis protocol [8]. Both Recombinant cloning plasmid and pET28a(+) vector were digested with Nco-I and Not-I restriction enzymes. Two Digestion mixtures (each 25 µl) were prepared which contained 5 µl of pET28a(+) expression plasmid and recombinant pTZ57R/T:: xylA plasmid each in separate tube. 20 µl of master mix (2.5 µl of 10X buffer O, 1 µl of Nco-I (10 U), 1 µl of Not-I (10 U) and 15.5 µl injection water) was added to each tube. The contents were short spun and placed in incubator at 37 ℃ for 3 h. The double restricted expression vector and xylA gene were purified using QIAquick Gel Extraction kit and were ligated by using T4 DNA ligase enzyme. DH5α E. coli competent cells were transformed using recombinant expression plasmid (pET28a(+) :: xylA) and plated on LB agar plates supplemented with kanamycin (50 µg/ml). Positive transformants were screened using colony PCR. After isolation of positive recombinant expression plasmid, E. coli BL21 (DE3) competent cells were transformed. The transformed cells were incubated at 37 ℃ for overnight on LB agar supplemented with kanamycin (50 µg/ml).

2.5 Xylose isomerase assay

Bradford assay was used to estimate protein concentration using bovine serum albumin as standard [9]. Enzyme solution (250 µg/ml) was incubated in a glass tube with 200 µl of test solution (100 mM MOPS/NaOH pH 7.0, 100 mM D-glucose, 5 mM Mg^{2+}) at 55 ℃ for 10 min. For xylose isomerase activity, a total of 1500 µl of a freshly prepared 1:1 mixture (v/v) of solution A (0.05% resorcinol in ethanol) and solution B (0.216 g $FeNH_4(SO_4)_2$.$12H_2O$ in 1 L conc. HCl) were added. For colour development, the tube was incubated at 70 ℃ for 40 min. The absorption was measured at 490 nm and 630 nm for D-fructose and D-xylulose, respectively. One unit of isomerase activity is defined as the amount of enzyme that produced 1 µmol of product per min under specific conditions [10].

2.6 Over-expression of XI and optimization of fermentation conditions for recombinant E. coli BL21 (DE3)

For over-expression of recombinant XI, BL21 (DE3) harbouring pET28a(+):: xylA was inoculated in LB-kanamycin (50 µg/ml) for overnight. Culture (1 ml) was transferred to fresh LB- medium (100 ml) supplemented with kanamycin (50 µg/ml) and incubated at 37 ℃ till OD_{600} reached 0.5. For over-expression, 0.5 mM of Isopropyl β-D-thiogalactopyranoside (IPTG) was added and culture was incubated at 37 ℃ for 16 h and cell culture was centrifuged. The cell pellet was re-suspended in three buffers of different pH values: 100 mM sodium acetate buffers (pH 4.0-5.5) pH adjusted with acetic acid, 100 mM 3-(N-morpholino) propanesulfonic acid (MOPS) buffers (pH 6.0-7.5) adjusted with NaOH and 100 mM 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES) Buffers (pH 8.0-9.0) adjusted with NaOH separately supplemented with 5 mM $MgCl_2$ to determine the optimum buffer for recombinant XI.

The cells were lysed using sonication and cell lysate was used to determine the expression of XI. The fermentation conditions for the cultivation of BL21 (DE3) harbouring pET28a (+):: xylA were optimized gradually separately at every step of cultivation focusing to obtain maximum expression of XI. These includes: LB medium pH (5.5, 6.0, 6.5, 7, 7.5 8.0), Optimum culture density (0.4, 0.5, 0.6, 0.7, 0.8, 0.9), effect of heat shock at 42℃ for 1 h along with comparison of effect of heat shock before IPTG (0.5mM) induction (BI), and after IPTG (0.5mM) induction (AI) and control (without heat shock), concentration of IPTG (0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 mM) comparing with control (un-induced), temperature of incubation after induction (15, 20, 25, 30, 35, 40, 45 ℃), time of incubation after induction (6, 12, 18, 24, 30, 36, 42, 48 h), speed of agitation during incubation (50, 100, 150, 200, 250 RPM). XI activity was determined using standard enzyme assay method at each step of optimization.

2.7 Purification of xylose isomerase

Purification of recombinant XI enzyme was performed using various chromatographic techniques. After ammonium sulphate precipitation, the sample were applied on pre-equilibrated UNOsphere Q anion exchange column using 100mM MOPS buffer (pH 7.0) for anion exchange chromtography. The unbound proteins were washed away. The bound XI protein was eluted with Gradient NaCl in same buffer. Further XI was purified using Resource ISO pre-packed hydrophobic interaction column using same buffer. After binding to the column desalting was used for elution of XI enzyme. Purity of xylose isomerase was analysed by 12% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE).

2.8 Characterization of xylose isomerase enzyme

The purified xylose isomerase enzyme was characterized to determine its optimum properties are mentioned below.

2.8.1 Optimum temperature determination

In order to determine the effect of temperature on activity of XI, the enzyme (2.5 mg/µl) was incubated with 100 mM (MOPS/NaOH) buffer (pH-7.0) containing Mg^{2+} (5 mM) and glucose (0.4 M) at the temperatures of interest (30-80 °C) for 10 min. The amount of D-fructose formed was quantified by standard enzyme assay.

2.8.2 Thermostability determination

The time course of irreversible thermo-inactivation was measured by incubating the apoenzymes (2.5 mg/µl) in 100 mM (MOPS/NaOH) buffer (pH-7.0) containing Mg^{2+} (5 mM) for various time periods at 45-65 ℃ in a heated water bath for 56 h, in the absence of substrate. Thermo-inactivation was stopped by incubating the tubes on ice. Residual XI activity was measured at 55 ℃.

2.8.3 Optimum pH determination

The effect of pH on XI activity was measured by using the standard enzyme assay protocol at 55 ℃, under different pH conditions using noninteracting biological buffer i.e., 100 mM sodium acetate (acetate/acetic acid) buffer (pH: 4.0-5.5), 100 mM (MOPS/NaOH) buffer (pH: 6.0-7.5) and 100 mM (HEPES/NaOH) buffer (pH: 8.0-9.5) containing Mg^{2+} (5 mM).

2.8.4 pH stability determination

The effect of pH on enzyme stability was measured by incubating the enzyme (2.5 mg/µl) at 55 ℃ for 40 min in 100 mM sodium acetate (acetate/acetic acid) buffer (pH: 4.0-5.5), 100 mM (MOPS/NaOH) buffer (pH: 6.0-7.5) and 100 mM (HEPES/NaOH) buffer (pH: 8.0-9.5) in the presence of Mg^{2+} (5 mM). The residual XI activity was measured at 55 °C and pH-7.0 by using the standard enzyme assay protocol.

2.8.5 Effect of divalent cations on activity of xylose isomerase

To determine the effect of divalent metals ions on XI activity, the apo-enzyme (2.5 mg/µl) was pre-equilibrated with Co^{2+}, Mn^{2+}, Mg^{2+} and Ca^{2+} in 100 mM (MOPS/NaOH) buffer (pH-7.0) containing 0.5, 2.0, 5.0, 8.0, 10 mM individually and in combination of Mn^{2+}/Mg^{2+}, Co^{2+}/Mn^{2+}, Mg^{2+}/Co^{2+} containing 5/0.5 mM of each metal pair for 30 min at 30 ℃. Residual activities were determined at 55 °C, pH-7.0 and presented as relative residual activity with respect to control or apo-enzyme (without metal).

2.8.6 Determination of time course isomerization of D-glucose to D-fructose

The conversion reaction containing enzyme (2.5 mg/µl), D-glucose (0.4 M), $MgCl_2$ (5 mM) in 100 mM MOPS buffer (pH-7.0) proceeded for 28 h at 55°C. Samples were taken at different time intervals for the analysis of D-fructose formation. The conversion rate was the ratio between D-fructose formed and the initial D-glucose.

2.9 Kinetics of Glucose/xylose isomerase

At temperature of 55 °C, the kinetic parameters of XI were investigated using D-xylose and D-glucose as substrates. A Michaelis-Menten plot, representing the velocity as a function of substrate concentration [S], was constructed. The line on the plot was generated using a non-linear least-squares fit that was based on the Michaelis-Menten Model Equation 1 to analyze the data.

$$v = V_{\max}[S]_o / K_m + [S]_o \quad \text{.....(1)}$$

In Lineweaver-Burk Plots, the linear regression was applied to fit double reciprocal data and standard equation 2 of straight line was obtained (given below).

$$y = mx + b \quad \text{.....(2)}$$

Where, m represents slope and b is the y intercept. The kinetic constants, K_m, V_{max}, k_{cat}, k_{cat}/K_m and V_{max}/K_m were determined using the double reciprocal Lineweaver Burk plots Lineweaver and Burk [11] of 1/V versus 1/[S]. Standard enzyme assays were performed in MOPS buffer (100 mM, pH 7.0) containing Mg^{2+} (5 mM) and various concentrations of D-glucose and D-xylose (1 to 10 mM) at 55 °C for 10 min.

Docking studies of XI - D-glucose

The minimized energy model structure of XI predicted by PEP FOLD 4.0 tool was used for Docking studies by AutoDock vina. Substrate (D-glucose) was used to determine the possible details of enzyme substrate actions and used as ligand that docked with XI. Docking result was visualized by PyMOL.

3 RESULTS AND DISCUSSION

3.1 Characteristics of xylose isomerase gene

Target xylA gene was amplified through PCR using gene specific primers and genomic DNA as template. After completion of PCR, the amplicons were analyzed. Amplicon size of xylA gene was determined to be ~980 bp on 1% agarose gel by comparing with 1 Kb DNA Ladder marker (Fermentas, Catalog # SM0311) (**Fig. 1A**). Sequence obtained after sequencing showed the size of xylA is 969 bp (**Fig. 1B**) that was deposited in NCBI and was assigned accession number PZ315830.

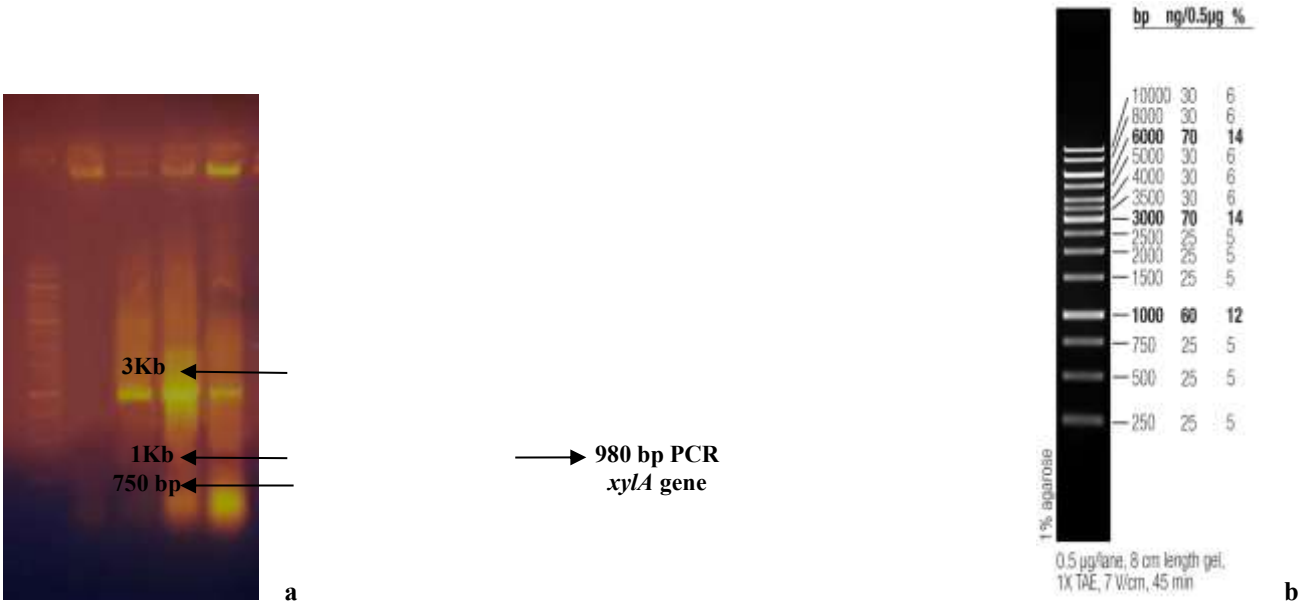


Fig. 1A. a) Amplification of xylA gene analyzed on 1% agarose gel. Lane M is 1Kb DNA Ladder Marker (Fermentas, Catalog # SM0311)), Lane 1 is non-template control and Lanes 2 to 4 represent amplified xylA gene sized ~980bp. **b)** 1Kb DNA Ladder Marker (Fermentas, Catalog # SM0311). The gel was cropped for clarity. The original full length raw gel is provided in Supplementary Fig 1

ATGAAGCTTGGCGTATTTACTGTTCTTTTTAGCGAAAAGTCTTTCGAAGAAATGCTGGATCA
TGTGAAGGCAGCAGGCTTAGAAGCGGTGGAAATCGGCACGGGCTGCTATCCGGGCAATTC
GCACTGCAATCTGGATGAGCTGTTGGAAAGTCCGGAAAAAAGACGAGCGTACAAAAAAG
CAGTAGAGGATCGGGGATTGATAATCAGTGCGCTTAGTTGTCACGGCAATCCGTTGACACC
AGAGAAAAGCTTTGCGCAGCAATCCCACGATACGTTTGTGAAAACGGTACAGCTAGCCGA
GATGCTGGAGGTGCCTGTGGTCAATTGCTTTTCTGGCACGGCAGGCGACCACGAAGGAGC
GAAGTACCCGAGCTGGCCCGTTGCTCCTTGGCCAAATGAGTATCGCGATGTGCTCCATTGG
CAATGGAACGAAAAGCTCATCCCGTATTGGCGCGAATGGGCTGCTTATGCGACTGAGCATC
ATGTCAAGGTTGCGTTGGAACTGCATGGCGGCTTTCTCGTCCACACACCAGGGACACTTCT
GAAACTGCGGGAACAGGTAGGCGAGGGAATCGGGGCAAACTTCGATCCCAGTCATATGTG
GTGGCAAGGAATTGATCCAGTAGCTGCAATCAAAATCTTGGGCAAAGAAAAAGCCATTTAT
CATTTCCATGCCAAAGACACGTATATTGATCAGGAAAAAGTGAATATGTACGGGCTGACGG
ATATGAATTCCTACGCCAATCTACATGAACGTGCGTGGTATTTCCGAACCGTAGGCTATGGG
CATTCGCAGCAGACGTGGGCGGATATGATGAGTGCACTGCGCATGAATGGATACGATTATG
TCGTGAGCATCGAGCACGAGGATGCCATCATGTCGATTGAGGAAGGCTTTCAGCGAGCTGT
TCAAAATCTGCAGCAAGTCATCTTGCGAGAACCAGTCCAGAATCTATGGTGGGTTTAG

Fig. 1B: Nucleotide sequence of xylA.

Amino acid sequence of xylA obtained from expasy translate tool showed homology among species belonging to the family Paenibacillaceae determined by multiple sequence alignment (Fig. 2A). This showed that amino acid sequence of XI is highly conserved among genus Brevibacillus. The analysis of the phylogenetic tree (Fig. 2B) showed that amino acid sequence closely resembled with Brevibacillus formosus (WP_088907468.1).

Fig. 2A. Multiple sequence alignment of amino acid sequence of xylA gene among the genus Brevibacillus; Brevibacillus formosus (WP_088907468.1), Brevibacillus brevis (WP_188068224.1), Brevibacillus porteri (WP_376893108.1), Brevibacillus sp. AG (WP_069845115.1) and Brevibacillus antibioticus (WP_137029093.1) having homology E value 0 showed that amino acid sequence of XI is high conserved among bacterial species belonging to genus Brevibacillus.

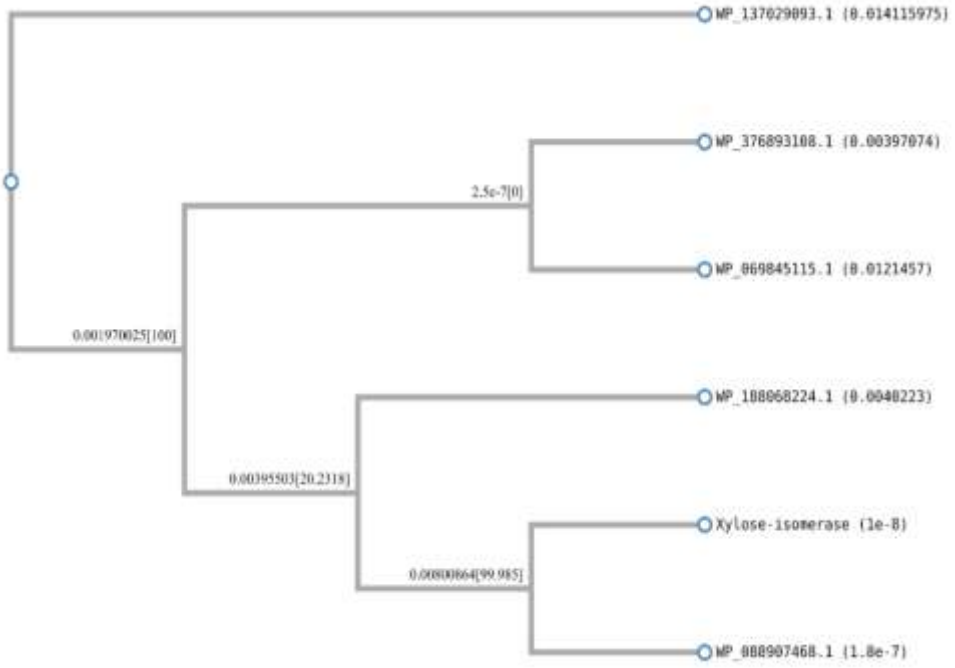
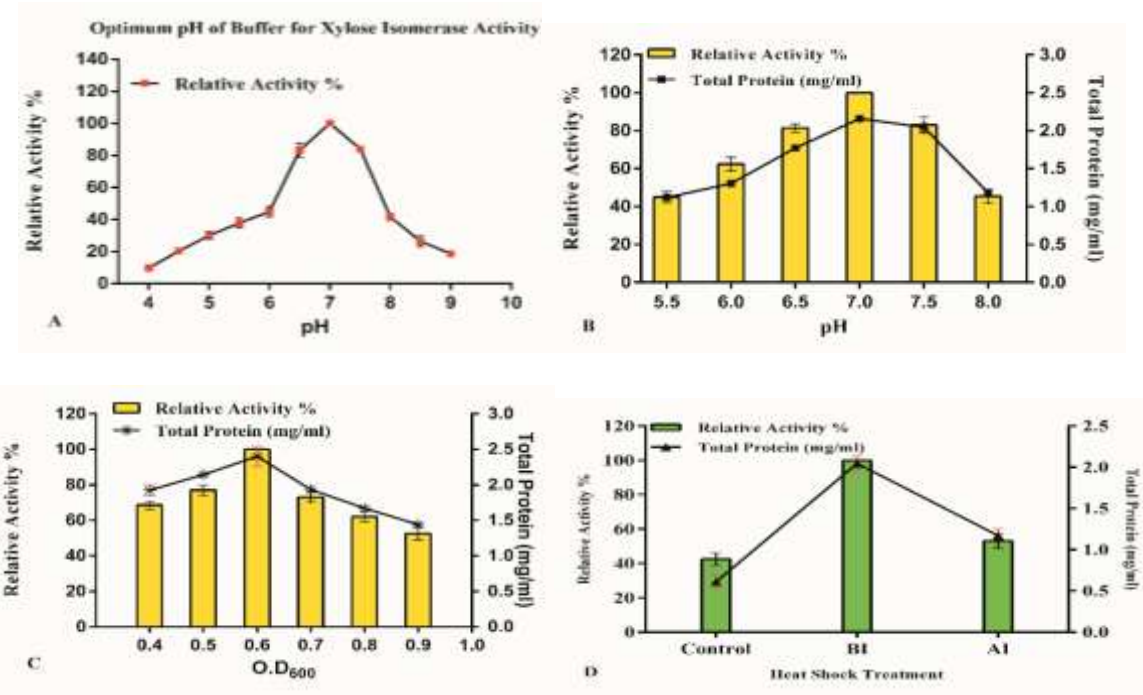


Fig. 2B. Phylogenetic analysis of amino acid sequence of XI showing that the sequence obtained has close resemblance with Brevibacillus fromosus NF2.

3.2 Optimum conditions for the overexpression of xylose isomerase

Over-expressed xylose isomerase showed maximum activity at pH 7.0 buffer. At other pH values (4.0-6.0 and 8.0-9.0), a significantly less enzyme activity was observed as compared to pH 7.0 (Fig. 3A). At pH 7.0 of medium, maximum XI activity was observed. Optimization of pH has significant importance as it is involved in the optimal growth of the recombinant bacterial strain and protein folding. The XI activity was affected significantly at either side of pHs (Fig. 3B). The optimal enzyme activity was observed when recombinant bacterial culture was induced at 0.6 optical density ($O.D_{600}$) of E. coli cells (Fig. 3C). This shows that host cell density has influence on the expression of XI enzyme. Batt et al. [12] have induced the expression of XI in E. coli JM105 at cell density of $O.D_{600}$ 0.5. There was comparatively low enzyme activity in the culture that was treated with heat shock after induction (AI) as compared to that culture which was treated with heat shock before induction (BI) (Fig. 3D).

Molecular chaperons (heat shock proteins) are produced in the bacterial cells as the result of heat shock treatment. These molecular chaperons are involved in adequate protein folding also controlling and limiting the formation of inclusion bodies thus supporting the appropriate expression of XI [13, 14]. The optimum over-expression of xylA gene was obtained with 0.5 mM IPTG induction. Maximum XI activity was observed along with the cell density and total protein content. Furthermore, with increase in IPTG concentration there was relatively decrease in XI activity as well as decrease in total protein content (Fig. 3E). Thus, using the low concentration of IPTG is effective approach for the expression of XI and making the fermentation process economical. Batt et al. [12] have induced the expression of XI in E. coli JM105 using IPTG of 2 mM concentration. Dekker et al. [15] reported the expression of thermostable XI from Thermus thermophilus in E. coli using 0.4 mM concentration of IPTG. Lajoie et al. [16] used IPTG (0.218 g/L) for the expression of recombinant XI from marine bacterium Fulvimarina pelagi in Rosetta-gami 2 (DE3) E. coli cells. At 30 °C maximal protein content along with the XI activity was determined (Fig. 3F). Low induction temperature is effective method for adequate protein folding thus obtaining maximum soluble protein expression and inhibiting the production of inclusion bodies. Brat et al. [17] expressed bacterial XI in Saccharomyces cerevisiae by incubating the culture system at low temperature (30 °C). Ata at al. [18] have produced XI in Pichia pastoris at 30 °C. The recombinant XI enzyme activity was increased up to 30 h after IPTG induction. After 30 h there was no significant increase in cell culture as well in XI activity instead xylose isomerase activity was dropped by up to about 15% (Fig. 3G). XI was maximally produced at the agitation speed of 200 RPM along with maximal total protein content (Fig. 3H).



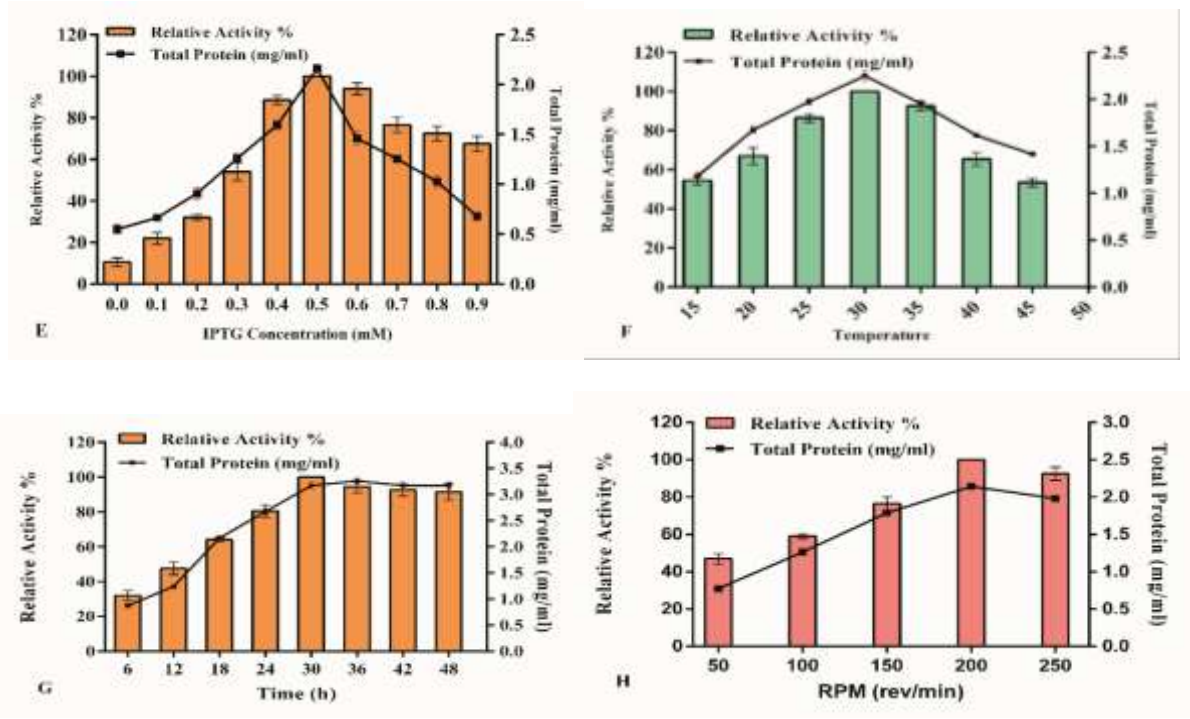
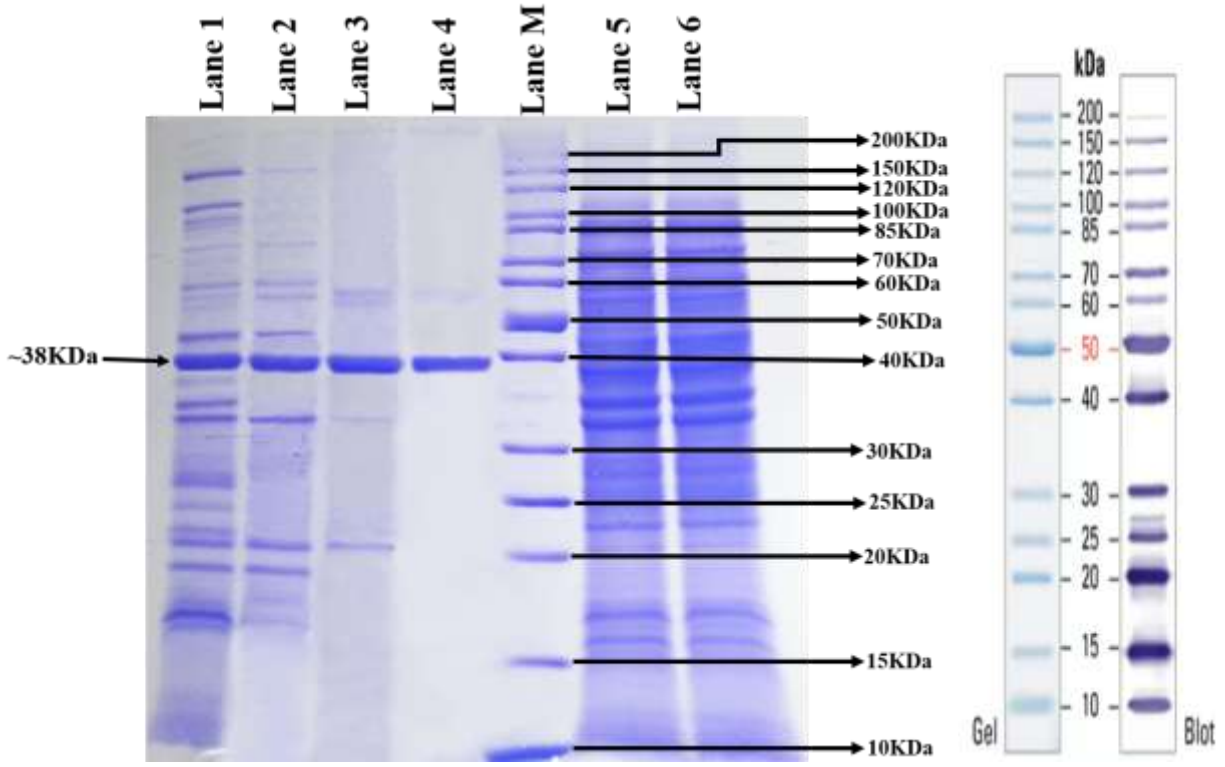


Fig. 3. Optimization of different parameters of E. coli BL21 (DE3) for over-expression of xylA cloned in pET28a(+). Enzyme activity was determined at 55℃ in the presence of 5 mM Mg^{2+} using standard enzyme assay protocol. Highest activity obtained is defined as 100% relative activity. All experiment were carried out in triplicates. (A) Optimum buffer and pH determination, 100 mM sodium acetate buffer (pH 4.0-5.5), 100 mM MOPS buffer (pH 6.0-7.5) and 100 mM HEPES buffer (pH 8.0-9.0) were used. (B) Effect of pH of growth medium. 5.5 to 8.0 pH range was used and maximal activity was measured at pH 7.0. (C) Effect of Pre-induction optimal cell density of cloned bacterial cells. At different optical density (0.4-0.9), IPTG induction was carried out and enzyme activity assays showed that maximum enzyme was produced with IPTG induction at 0.6 $O.D_{600}$. (D) Effect of heat shock, Heat shock was carried out both before induction (BI) and after induction (AI) and was compared with control (without heat shock) at 42 ℃ for 1 h. Significant increased enzyme activity was measured in BI as compared to control and AI. (E) Effect of different IPTG concentration. At 0.5 mM IPTG concentration maximum xylose isomerase enzyme was expressed (F) Effect of different induction temperatures. At 30 ℃, maximum XI was produced. (G) Effect of incubation time. At 30 h incubation at 30 ℃ after 0.5 mM IPTG induction maximum xylose isomerase was produced. (H) Effect of agitation speed. At 200 RPM, maximum enzyme was produced.

3.3 Purification of xylose isomerase enzyme

XI was partially purified with overall yield of 57.34% having specific activity (980.61 U/mg) using 70% ammonium sulphate precipitation and enzyme was purified by using UNOsphere Q anion exchange column and Resource ISO pre-packed hydrophobic interaction column with purity level of 8.13 folds having specific activity 5450.83 U/mg (Table 1). Purity of XI was confirmed by 12% SDS-PAGE (Fig. 4). Size of purified xylose isomerase was estimated to be ~38 kDa (Fig. 4). Kawai et al. [19] have partially purified Bifidobacterium adolescentis xylose isomerase using up to 65% ammonium sulphate precipitation. Karaoglu et al. [20] partially purified novel glucose isomerase from Anoxybacillus gonensis $G2^T$ enzyme using 70% ammonium sulphate precipitation. Fatima and javed [21] partially purified XI enzyme from E. coli BL21 using 70% ammonium sulphate concentration. Kluskens et al. [22] purified XI from e thermophilic bacterium Fervidobacterium gondwanense using Q-Sepharose anion exchange column and Superdex 200 gel filtration column. Yanmis et al. [23] purified Anoxybacillus gonensis $G2^T$ XI using DEAE-Sepharose and Phenyl-Sepharose 6 Fast Flow having purification yield of 73.14% and was 10.98 folds purified.



a) **b)**

Fig. 4. a) Purification of XI enzyme. Lane M: Protein ladder marker (Fermentas, Cat# 26614), Lane 1: Crude protein from E. coli harboring pET28a(+) :: xylA after 0.5 mM IPTG induction. Lane 2: protein from E. coli harboring pET28a(+) :: xylA after ammonium sulphate precipitation, Lane 3: protein from E. coli harboring pET28a(+) :: xylA anion exchange chromatography, Lane 4: Purified XI enzyme from E. coli harboring pET28a(+) :: xylA, Lane5: Negative control E. coli harboring pET28a(+) without insert and Lane 6: Negative control E. coli harboring pET28a(+) :: xylA without IPTG induction. **b)** Protein ladder marker (Fermentas, Cat# 26614). The gel was cropped for clarity. The original full length raw gel is provided in Supplementary Fig.2.

TABLE 1: Purification of XI enzyme from Brevibacillus formosus NF2.*

Steps	Total Protein (mg)	Total Enzyme Activity (U)	Specific Activity (U/mg) ⁋	Yield (%) ⁋⁋	Purification (Fold) ⁋⁋⁋
Crude Extract	25±1.8	16760±120	670.4	100.00	1.00
Ammonium Sulphate Precipitation(70°C)	9.8±1.3	9610±103	980.61	57.34	1.46
Anion Exchange Chromatography	3.2±0.4	7880±65	2462.5	47.01	3.67
Hydrophobic interaction chromatography	1.2±0.2	6541±73	5450.83	39.03	8.13

⁋ Specific activity= Total enzyme activity / total protein
⁋⁋ Yield = Total enzyme activity of fraction / Total enzyme activity of crude enzyme
⁋⁋⁋ Purification Fold = Specific Activity of fraction / Specific activity off crude enzyme.
* All experiments were performed in triplicates.

3.4 Effect of temperature on XI

XI enzyme optimally acts at 55 ℃ (Fig. 5A). It also showed significant activity (>80%) at temperature range of 50 to 60 ℃. There was significant decrease in activity (>80%) was observed below 50 ℃ and above 60 ℃. After incubation of apo-enzyme (2.5 µg/µl) at different temperatures (45 to 65 ℃) for various time periods (2-56 h). XI enzyme retained 100 of its activity at 45 ℃ and 50 ℃ for 56 h. At 55 ℃, XI retained 100% activity for 52 h and later retained >90% of its activity. At 60 ℃, XI retained 100% of its activity for 20 h and gradual decline in activity was observed after 2 h. There was significant decline in XI activity at 66 ℃ after 12 h of incubation. (Fig 5B). Thermostabilities vary widely in class I xylose isomerases but found to be identical in case of class II xylose isomerases this is because of presence different salt bridges among class I and II xylose isomerases [24]. XI from Vibrio sp. was optimally active around at 60 ℃ [25]. Konak et al. [26] reported that XI from Geobacillus thermodenitrificans TH2 was optimally active at 85 ℃ and enzyme remained highly for 4 days at 50 °C. Park et al. [27] reported XI from Anoxybacillus kamchatkensis G10 was optimally active at 80 ℃ and heat stability tests resulted that 55% of residual activity was maintained after 2 h of incubation at 60 ℃. Piromyces sp. E2 exhibited optimum activity at 65 ℃ and retained full activity for 48 h at 30 ℃ and 12 h at 60 ℃ [28].

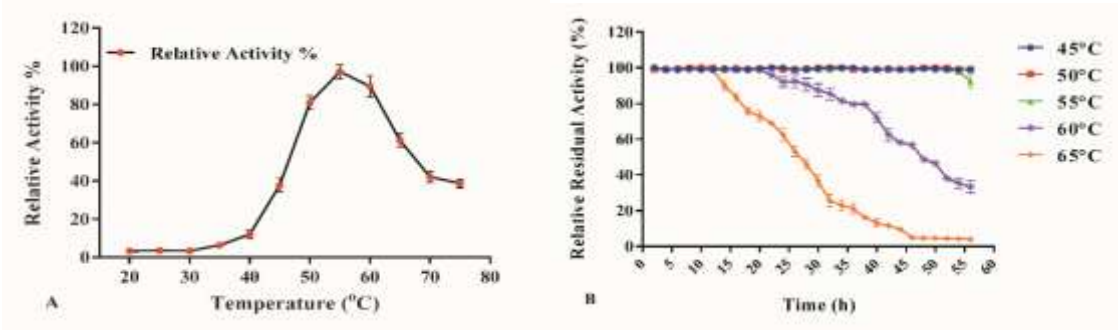


Fig. 5. (A) Optimum temperature was determined to be 55 ℃ when XI enzyme was incubated at different temperature ranges (20-75 ℃) at pH 7.0 in the presence of D-glucose (0.4 M) and Mg^{2+} (5 mM) (B) Thermostability for the activity of Brevibacillus formosus NF2 XI enzyme. It as determined by incubating the Apo-XI enzyme for 720 min at different temperature ranges (45-65 ℃). XI enzyme retained 100% and >80% of its activity at 45 ℃ and 50 ℃ respectively for 720 min. All experiments were carried at in triplicates and maximum XI activity was termed as 100% relative activity.

3.5 pH and pH stability

XI was optimally active at pH-7.0 (Fig. 6A). There is sharp decline in activity of XI on either side of optimal pH. Vieille and Zeikus [29] reported that most of the thermostable xylose isomerases have optimal pH range between 7.0-7.6. The specific activity of XI was significantly decreased at pH below. This is due to the presence of imidazole nitrogen of the active site histidine (His-101) that transforms to positively charged as His-101 provides electrostatic bonds to stabilize the substrate [30]. XI retained 90-95% of its activity at pH 6.5 to 7.5 for 40 min (Fig. 6B). Tolerance of XI at slightly acidic to neutral pH have significance in industries for the production of HFCS at high temperature. According to the Vieille et al. [31], brown byproducts are formed at alkaline pH and high temperature during non-enzymatic reactions. This process is also known as Millard reaction in which enzyme get deactivated [32]. In the absence of substrate XI retained 100% activity at pH 7.0. XI tolerated the pH change and showed >80% of activity in the absence of substrate at pH range of 6.0 to 8.0 after incubation for 40 min at 55 ℃ (Fig. 6B). Chanitnun and pinphanichakarn [33] reported that XI from Streptomyces sp. CH7 remained stable within a pH range of 5.5 to 8.5. Yanmis et al. [23] reported optimum pH of XI from Anoxybacillus gonensis $G2^T$ is 6.5. Dong et al. [34] reported that XI from Caldicellulosiruptor acetigenus and Thermoanaerobacter thermocopriae were optimally active at pH 8.0.

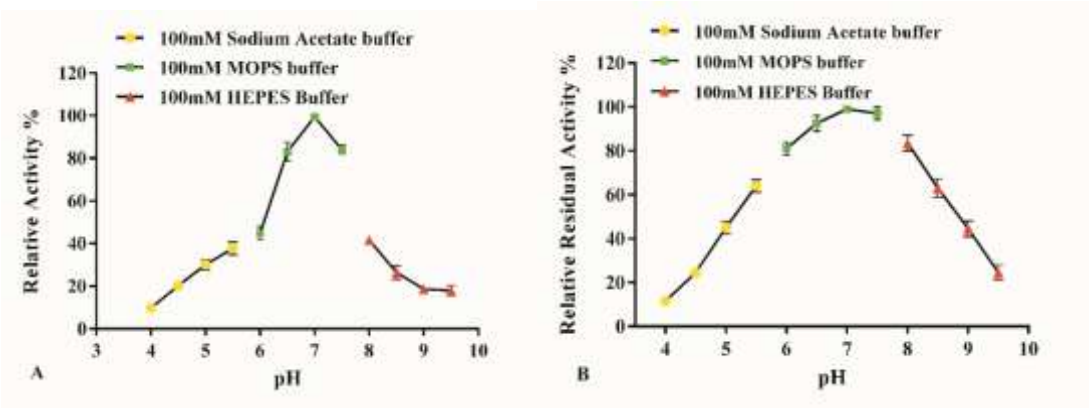


Fig. 6. (A) Optimum pH; XI enzyme was incubated at 55 ℃ for 10 min in the presence of D-glucose (0.4 M), Mg^{2+} (5 mM) and 100 mM sodium acetate buffer (pH 4.0-5.5), 100 mM MOPS buffer (pH 6.0-7.5) and 100 mM HEPES buffer (pH 8.0-9.0) and. Enzyme exhibited maximum xylose isomerase activity in the presence of 100 mM MOPS buffer (pH: 7.0). (B) pH stability; apo-enzyme was incubated in the presence of 100 mM sodium acetate buffer (pH 4.0-5.5), 100 mM MOPS buffer (pH 6.0-7.5) and 100 mM HEPES buffer (pH 8.0-9.0) in the absence of substrate and cofactor for 40 min. XI enzyme retained 100% at pH 7.0 and >90% activity at pH 6.5 and 7.5. All experiments were carried out in triplicates.

3.6 Effect of Divalent metal ions determination

XI was found to be most active in the presence of 5 mM Mg^{2+} and also exhibited peak activity in the presence of 6 and 7 mM Mg^{2+}. The activity was found to be increased by 385% as compared to that of apo-enzyme in MOPs Buffer (pH-7.0) at 55 ℃ (Fig. 7). In the presence of Co^{2+} and Mn^{2+}, the enzyme activity was found to be increase by 165% and 32% with concentration of 8 mM for both metal ions. In pairwise combination of Mn^{2+}/Mg^{2+} and Co^{2+}/Mg^{2+} and Mn^{2+}/Co^{2+}, XI was found to be most active in the combine state of Co^{2+}/Mg^{2+}. However, the activity in all pairs metal concentration is found to be less as compared to Mg^{2+} (alone). The enzyme activity was found to be decreased significantly in the presence of Ca^{2+} as compared to that of apo-enzyme concluding that Mg^{2+} is best activator and Ca^{2+} is inhibitor of XI (Fig. 7). Khire et al. [35] reported the highest activity of XI from Chainia sp. (NCIM 2960) in the presence of 5 mM Mg^{2+}. Smith et al. [36] reported the maximum activity of XI from Arthrobacter strain N.R.R.L. B3728 in the presence of Mg^{2+} and inhibited in the presence of Ca^{2+}. Sukumar et al. [37] reported the maximal activity of XI from Bacillus sp. in the presence of 5 mM Mg^{2+}. Park et al. [27] reported the inhibition of XI from Anoxybacillus kamchatkensis G10 in the presence of Ca^{2+}. Class I XI was best activated in the presence of Mg^{2+} and glucose as substrate followed by Co^{2+} and Mn^{2+}. While Class II XI was found to be best activated in the presence of Co^{2+} then in Mg^{2+} and then Mn^{2+} [38].

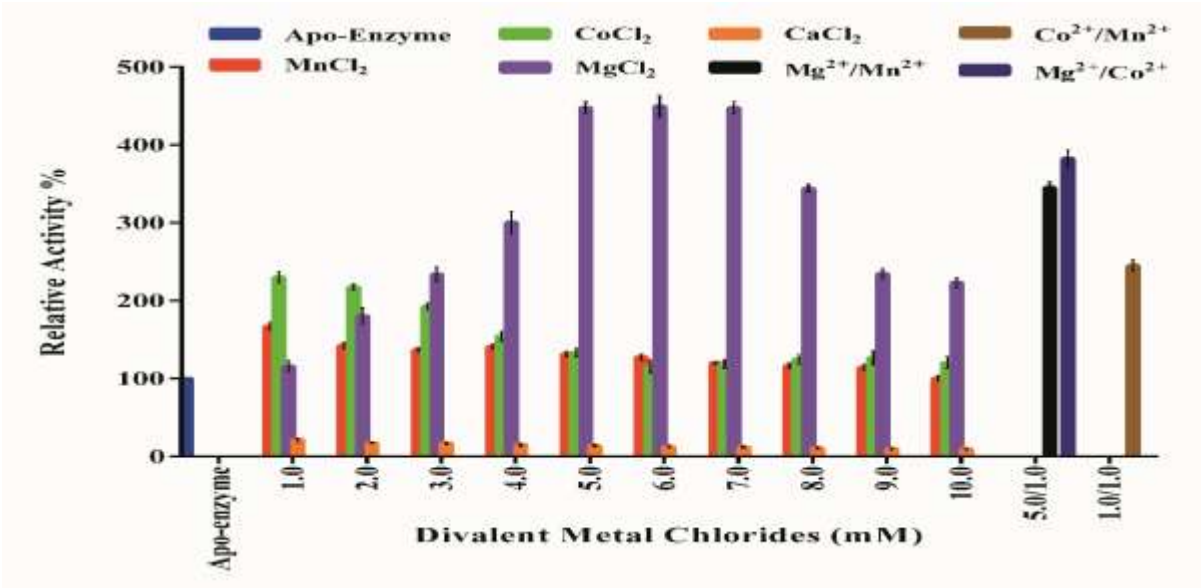


Fig. 7. Effect of divalent metal ions on XI. Apo-enzyme was incubated in the presence of different concentrations of divalent metal ions (Mn^{2+}, Co^{2+}, Mg^{2+}, Ca^{2+} and combination of two divalent metal ions). Xylose isomerase assay was carried out in the presence of these metal ions, at 55 ℃ in the presence of 100 mM MOPS buffer pH 7.0. The activity of apo-enzyme obtained was termed at 100% relative activity. The increase or decrease in enzyme activity was represented in relative activity. All experiments were carried out in triplicates. XI activity was maximally increased in the presence of 5 mM Mg^{2+} and decreased in activity was determined in the presence of Ca^{2+}.

3.7 Time Course isomerization of D-glucose to D-fructose by xylose isomerase

After 10 h of incubation at 55 ℃, maximum D-fructose of concentration of 54.67% was produced from conversion of D-glucose. (Fig. 8). About 48.33% and 41.67% D-fructose was produced from D-glucose isomerization after 5 h and 3 h of incubation. D-fructose of 48% concentration was achieved by Staudigl et al. [39]. The thermophilicity of XI from Brevibacillus formosus NF2 permits the production of HFCS at industrial scale. Moreover, it also eliminates further chromatographic steps for purification and enrichment of HFCS.

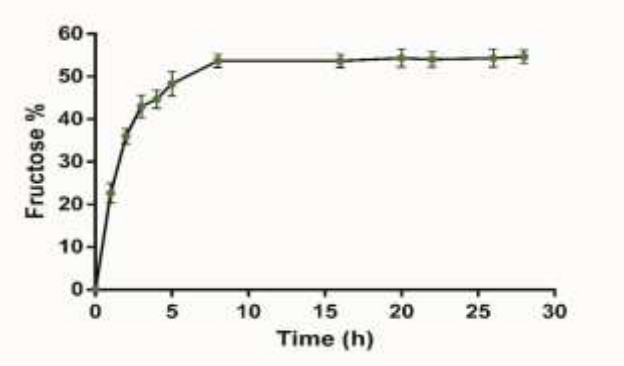


Fig. 8. Time course isomerization of D-glucose to D-fructose XI. XI enzyme was incubated at 55 ℃ in the presence of 100 mM MOPS buffer pH 7.0, 0.4 M D-glucose and 5 mM Mg^{2+}. At different time intervals, the percentage of D-fructose produced was determined and after 10 h the 54.67% of D-fructose was produced. All experiments were carried out in triplicates.

3.8 Kinetic Studies

Michaelis-Menten plot resulting in a hyperbolic curve that illustrated that rate of reaction increased with increase in concentration of D-xylose (Fig. 9A) and D-glucose (Fig. 9B). Kinetic parameters (K_m, V_{max}, K_{cat}, V_{max}/K_m and K_{cat}/K_m) are known as Michealis Menton constants were determined using Lineweaver-Burk Plots (Fig. 10A and 10B) and are given in Table 2. XI have lower K_m value for xylose (2.12 mM) as compared to glucose (3.73 mM). Similar pattern was observed in term of turnover rate (K_{cat}) showing that the efficiency (K_{cat}/K_m) of XI for natural substrate xylose as compared to glucose. Karaoglu et al. [20] reported K_m, K_{cat} and K_{cat}/K_m for D-glucose 146.08 mM, 2188.2 min^{-1} and 15 $min^{-1}.Mm^{-1}$ respectively of XI from Anoxybacillus gonensis $G2^T$. Rengasamy et al. [40] reported K_m value for D-glucose of XI from S. lividans RSU26 to be 29.4 mM. The XI from Brevibacillus formosus NF2 has greater affinity for D-glucose as compared to the reported ones and thought to be have more forward rate of reaction for the production of HFCS.

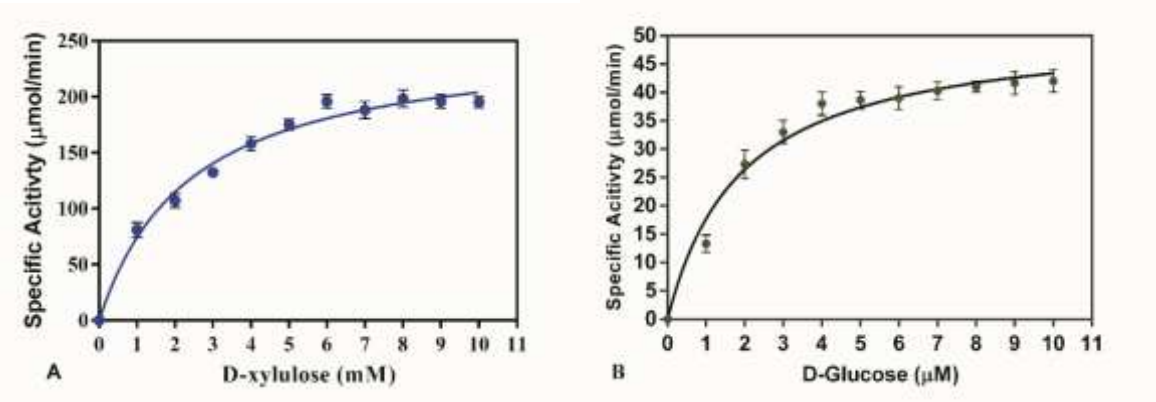


Fig. 9. Michaelis–Menten model of **(A)** D-Xylose, **(B)** D-Glucose for isomerization by XI enzyme. All experiments were carried out in triplicates.

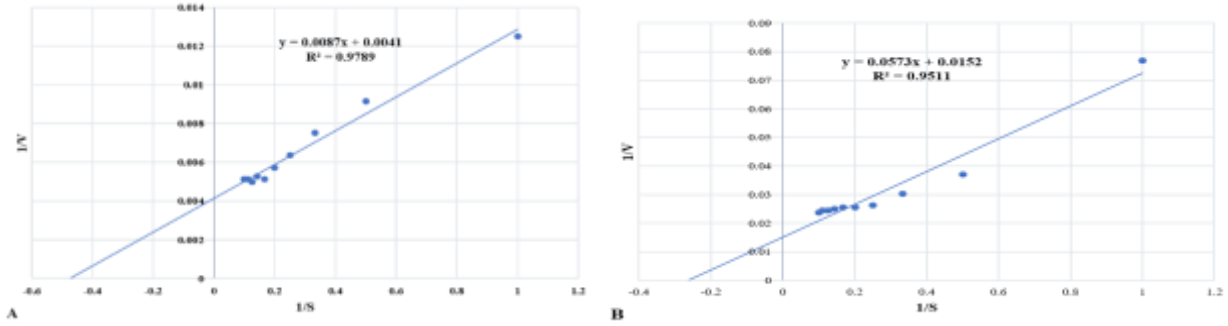


Fig. 10. Lineweaver-Burk plot for the determination of Michaelis kinetic constants for **(A)** D-xylose, **(B)** D-Glucose isomerization by XI enzyme.

Table 2: Kinetic parameters of XI from Brevibacillus formosus NF2.

Substrate	K_m (mM)*	V_{max} (µmol/min)*	K_{cat} (sec^{-1})**	K_{cat}/K_m (sec^{-1}/mM)
D-Glucose	3.73	65.36	19395	5200
D-Xylose	2.12	243.9	72374	34139

* Michaelis kinetic constants were determined at 55 °C in 100 mM MOPS buffer bearing pH of 7.0 supplemented with 5 mM $MgCl_2$.
** The K_{cat} is the turnover number: the number of substrate molecules reacted per active site per min, was calculated using the relation: $K_{cat} = V_{max}$ /[E] where, [E] is molar concentration of enzyme.

DOCKING STUDIES

Xylose isomerases are metalloproteins and activated in the presence of divalent metal ions (Mg^{2+}, Mn^{2+} and Co^{2+}). In large homotetramer, each monomer comprises two metal binding sites (M1 and M2). M1 comprises four carboxylates (Glu-190, Glu-218, Asp-228 and Asp-232). M1 is in tetrahedral and octahedral coordination in the absence and in the presence of substrate, respectively. The distance between M1 and M2 is less then 5Å and both share common residue (Glu-219) [41]. In this study, the distance is calculated to be 4.199Å. Inactivation of enzyme occurs, if mutation occur in either metal binding site [42]..

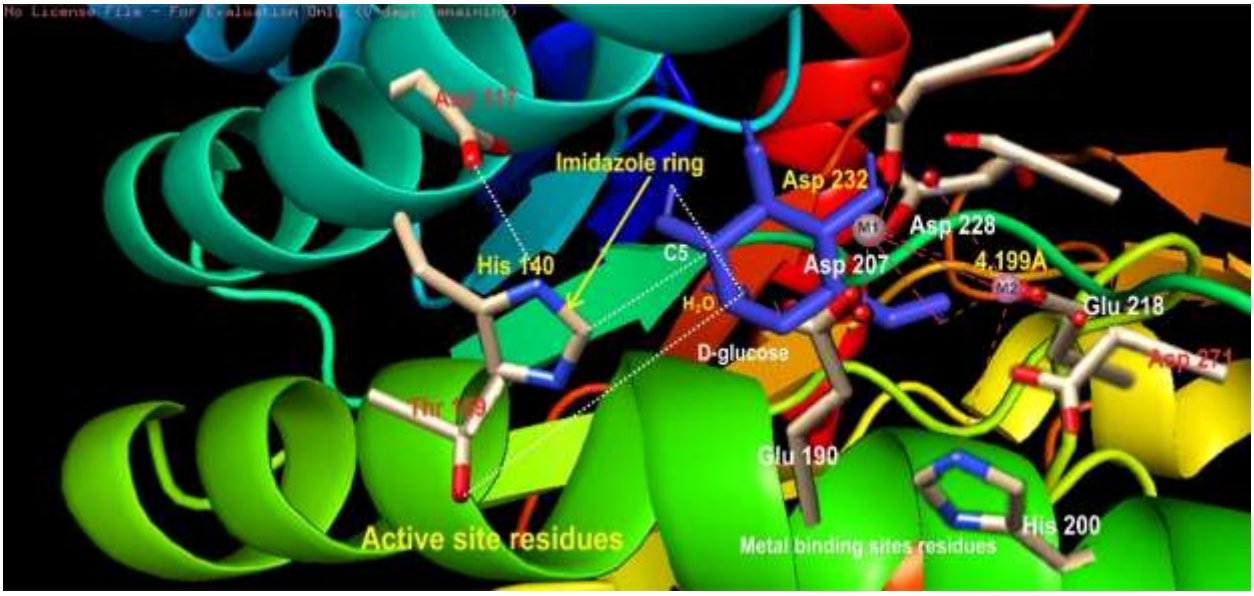


Fig. 11. Molecular docking of D-glucose with xylose isomerase showing metal binding sites and catalytic site residues interacting with D-glucose for isomerization process (Predicted by AutoDock vina and visualized by PyMOL version: 3.1.5.1)

First of all, formation of activated complex occurs by opening of pyranose ring of aldo sugars either pentoses or hexoses by formation of hydrogen bond between imidazole ring of His-140 and hydroxyl group of C5. Then translocation of the C1 and C2 atoms of the open-ring intermediate occurs towards the metal ions. While Asp-117, His-140 and Thr-159 maintains the structural orientation of the linear sugar intermediate by formation of H-bond with C5 of the linear sugar (Fig. 11). Then, metal ion induced hydride shift occur from C2 to C1 (isomerization from aldo to keto sugar). Finally closing of the ring occur bt hemiketal formation

4. CONCLUSION

In this study, xylose isomerase enzyme was purified with high protein content and activity. The enzyme was stable at wide temperature range and pH values. The kinetic parameters showed its affinity for D-glucose for the production of HFCS. The D-fructose percentage produced from D-glucose showed that xylose isomerase enzyme cloned in this study from Brevibacillus formosus NF2 can be used as industrial scaled potential candidate for the production of HFCS. More-over in addition its affinity for D-xylose to produce D-xylulose this enzyme can also be used at industrial level for the production of bioethanol (also requires further lab studies).

Abbreviations

Ethics approval and consent to participate: This study did not contain human or animal data. Therefore, ethical approval and consent to participate were not required.

Consent for publication: Not Applicable

Availability of data and materials: The datasets generated and/or analyzed during the current study are available in the [NCBI database] repository, under the accession number [PZ315830].

Competing Interests: Every contributing author affirms that they have no competing interests.

Funding: Not applicable.

Acknowledgements: Not applicable.

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