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Cloning, expression and characterization of xylose isomerase from thermophilic *Geobacillus caldoxylosilyticus* TK4 strain

[*Geobacillus caldoxylosilyticus* TK4 termofilik bakterisinden ksiloz izomerazın klonlanması, ekspresyonu ve karakterizasyonu]

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ABSTRACT

Aim: To clone the gene of Xylose isomerase (XyI) from thermophilic *Geobacillus caldoxylosilyticus* TK4 strain and to express and characterize this enzyme.

Methods: XyI gene of *Geobacillus caldoxylolyticus* was cloned into pET28a(+) vector and expressed in *Escherichia coli*. The recombinant protein was purified by using nickel affinity chromatography and characterization of the purified enzyme was done.

Results: The recombinant enzyme had 1326 bp and optimum pH and temperature of the XyI were found to be 6.5 and 80°C, respectively. At 4.0-9.0 pH range and 4°C, after 15 days incubation, the enzyme was quite stable. The enzyme retained nearly 80% of its original activity after 3 hours incubation at 60°C and 70°C. In the presence of glucose as substrate, K_m and V_{max} values of the XyI were determined as 20.58 mM and 0.67 U/mg protein, respectively.

Conclusion: A XyI gene from *Geobacillus caldoxylosilyticus* TK4 was cloned and expressed in *E. coli*. The XyI shared common characteristics with known XyIs in terms of conserved amino acid residues, electrophoretic behaviour and kinetic parameters. The recombinant XyI may be used in industrial applications need high temperatures and low pHs

Key Words: Xylose isomerase, gene expression, *Geobacillus caldoxylosilyticus,* thermophilic, cloning, characterization

ÖZET

Amaç: Termofilik *Geobacillus caldoxylosilyticus* TK4 bakterisinden elde edilen ksiloz izomerazı (XyI) kodlayan genin klonlanması, ekspresyonu ve bu enzimin karakterizasyonudur.

Yöntem: *Geobacillus caldoxylosilyticus* TK4 termofilik bakterisinin XyI kodlayan geni pET28a(+) vektörüne klonlanıp *Escherichia coli*'de eksprese edildi. Rekombinant protein, nikel afinite kromatografisiyle saflaştırıldı ve enzimin karakterizasyonu gerçekleştirildi.

Bulgular: XyI kodlayan gen 1326 baz çiftinden oluşmuştur ve rekombinant enzimin optimum pH ve sıcaklığı sırasıyla 6.5 ve 80°C'dir. pH 4.0-9.0 aralığında 4°C'de 15 gün bekletildikten sonra rekombinant XyI'ın, oldukça kararlı olduğu gözlendi. Enzim 3 saat süreyle, 60°C ve 70°C'de bekletildiğinde, aktivitesini %80'nin üzerinde koruduğu gözlendi. Glukoz substratı varlığında, rekombinant Xyl'ın K_m ve V_{maks} değerleri, sırasıyla, 20.58 mM ve 0.67 U/mg protein olarak belirlendi.

Sonuçlar: *Geobacillus caldoxylosilyticus* TK4'ten bir XyI geni klonlandı ve *E. coli*'de eksprese edildi. Rekombinant enzimin korunmuş amino asit birimleri, elektroforetik davranışı ve kinetik parametreleri bakımından diğer XyI'larla benzer karakteristik özelliklere sahip olduğu belirlendi. Rekombinant XyI, yüksek sıcaklık ve düşük pH gerektiren endüstriyel uygulamalarda kullanılabilecektir.

Anahtar Kelimeler: Ksiloz izomeraz, gen ekspresyonu, *Geobacillus caldoxylosilyticus*, termofilik, klonlama, karakterizasyon

Introduction

Xylose isomerase (EC 5.3.1.5), widely known as glucose isomerase, is an intracellular enzyme catalyzing the reversible isomerization of D-xylose to D-xylulose *in vivo* and D-glucose to D-fructose *in vitro* [1]. Glucose isomerization ability of the enzyme is of commercial importance in the production of high-fructose corn syrup (HFCS) [2] used as sweetener in soft drinks and other food products where it replaces beet and/or cane sugar. At equal sweetener level, fructose is cheaper than sucrose and is less caloric because of lower resorption. Glucose and fructose have better solubility compared to sucrose and therefore lesser tendency to cristalize in a wide range of food product as ice-cream, canned products, baking, pickes, sauces, meal product etc. [3].

At high temperature, the rate of the isomerization reaction increases and allows the shift of equilibrium between glucose and fructose toward the latter [4]. In industry, this is useful in developing a one-step process for the conversion of starch to HFCS [5]. Although low pHs are desired in this industrial process, the majority of isolated and studied enzymes have a high optimum pH. However, it is a major drawback on industrial scale that the isomerization at high temperature and alkaline pH leads to the formation of unwanted side reactions generating undesirable bitter (browning) sub-products such as mannose, psicose, and other acidic compounds in the resulting fructose solution [6]. Hence, the new challenge consists of identifying new xylose isomerases acting at higher temperature and low pH.

The presence of metal ions as activator in the reaction mixture is also sometimes necessary for enzyme activity. For xylose isomerase (XyI) stability and activity, Co^{2+}, Mg^{2+}, and Mn^{2+} metal cations are essential [7]. Xylose isomerases are best activated with one of these metal ions or, in some cases, by a combination of two of them [8, 9]. Metal specificity also depends on the nature of the substrate [10].

The isolation of the XyI gene from *E. coli* was the first report on this subject [11]. After that, this gene has been cloned from many (thermophilic or not) organisms such as *Clostridium thermosulfurogenes* [12], *Thermus thermophilus* [13], *Bacillus subtilis* [14]*, thermoanaerobacterium* strain jw/sl-ys 489 [15], *Streptomyces griseofuscus* S-41 [16] and expressed in different hosts. It was also reported that XyI purified from *Streptomyces griseofuscus* S-41 was used in industry. After the understanding of the molecular structure of the enzyme related to its catalytic properties, the studies were focused on cloning XyI gene and this could help the improvement of the industrial applications of the enzyme [16].

Here, we reported the cloning, expression, purification and biochemical characterization of the xylose isomerase from a thermophilic bacterium *Geobacillus caldoxylosilyticus* TK4 (obtained from Kestanbol thermal springs in Çanakkale-Turkey) growing at 40-75 °C and utilizing xylose. The enzyme was active at high temperatures and slightly acidic pHs which are remarkable properties for the use of this enzyme in food industry.

Materials and Methods

Materials

All reagents used were of analytical grade and purchased from Sigma Chemical Co. (St. Louis, MO). The plasmids used for genomic DNA library construction, PCR product cloning and gene expression were pUC18 (Promega, Madison, USA), pGEM-T Easy (Promega, Madison, USA) and pET-28a(+) (Novagen, Madison, WI, USA), respectively. *E. coli* JM101 and *E. coli* BL21(DE3) pLysS were used as the host for cloning and gene expression, respectively. Amino acid alignments were performed by using the online BLAST search engine at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/). Genomic and plasmid DNAs were isolated by using Wizard Genomic DNA Purification Kit (Promega, Madison, USA) and Wizard Plus SV Minipreps DNA Purification System (Promega, Madison, USA), respectively. Restriction enzymes were purchased from Promega (Madison, USA) and Fermentas (Vilnius, Lithuania). PCR products were purified by using Wizard Plus SV Minipreps DNA Purification Systems (Promega, Madison; USA). DNA sequence analyses were performed by Macrogen Inc. (Seoul, Korea). MagneHis Protein Purification System (Promega, Madison, USA) was used for purification of the recombinant protein.

Construction of Genomic DNA library

Geobacillus caldoxylosilyticus TK4 (*G. caldoxylosilyticus* TK4) was grown in Luria-Bertani (LB) medium at 60°C and *G. caldoxylosilyticus* TK4 genomic DNA was isolated by Wizard Genomic DNA Purification Kit following the manual instruction. To construct the genomic DNA library, the genomic DNA of *G. caldoxylosilyticus* TK4 was digested with *Eco*R I and resulting DNA fragments were inserted into pUC18, previously digested with *Eco*R I. Recombinant vectors were transformed into *E. coli* JM101 strain [17]. The resulting transformants were plated on Lauria-Bertani (LB) agar plates including 50 mg/mL ampicillin. The ampicillin-resistant clones were selected by screened for blue/white test and isolated. Recombinant vectors were sequenced by Macrogen Inc. (Seoul, Korea). Sequence alignments were performed by using the online blast search engine at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/).

Inverse PCR

Based on the already known DNA sequence of *G. caldoxylosilyticus* TK4 Xyl gene, a set of primer (forward; XyIF1 5'-TTGAACATGAACTGCGAGTAGC-3'and reverse XyIR1 5'-TCATAGGATATGGCGTTGATGG-3')

was designed and inverse PCR was performed to find the missing DNA sequence of the gene in the 3' end.

To find out the missing part of *G. caldoxylosilyticus* TK4 XyI gene ,the genomic DNA was digested with *Hinf* I and self-ligated overnight at 22°C by using 3 µl (3 U/µl) T4 DNA ligase in a total volume of 500 µl. After the ligation, DNA was absolutely precipitated with ethanol, washed with 70% ethanol and airdried. The pellet was resuspended in nuclease free water. The suspension was used as template DNA for PCR amplification. PCR was amplified by 36 cycles for each primer set with the following protocol: denaturation, 94°C for 1 min; annealing, 50°C for 1 min; extension, 72°C for 2 min and additional extension, 72°C for 5 min. The inverse PCR products were purified and inserted into the pGEM-T easy vector. After the isolation of plasmid, they were sequenced.

Cloning and expression of XyI gene from G. caldoxylosilyticus TK4

To find out the whole DNA sequence of the gene, two specific primers (forward; XyIF2 5'-GGCTAGCATGTCTTATTTCAACACCATCAACG-3' (*Nhe* I site underlined)and reverse; XyIR2 5'-GCTTGAAGTTTGTGCAACCCGTTAAGTGACAAGCTTG-3' (Hind III site underlined) were used. The reverse primer contains an additional stop codon immediately upstream of the gene stop codon (TAA).

PCR was amplified with *G. caldoxylosilyticus* TK4 genomic DNA as a template, using following PCR procedures: denaturation, 94°C for 1 min; annealing, 59°C for 1 min; and extension, 72°C for 2 min and additional extension for 5 min. The PCR product was digested with *Nhe* I and *Hind* III and cloned between *Nhe* I and *Hind* III region of pET-28a(+) expression vector, previously digested with the same enzymes, containing the IPTG (isopropyl β-D-1-thiogalactopyranoside)-inducible *trc* promoter.

The resulting recombinant plasmid, pET28a-XyI, was transformed into *E. coli* BL21(DE3)pLysS. The recombinant *E. coli* was cultured in LB medium containing 50 µg/ml kanamycin at 37°C overnight and inoculated into new media at ratio of 1:100. The bacteria were grown until the optical density of medium reached to 0.6-0.8 (OD_{600nm}) and expression of XyI was induced by the addition of 1 mM IPTG. After induction for 3 h at 37 °C, the cells were centrifugated at 10 000 rpm for 10 min at 4°C.

For the purification of enzyme, the recombinant-induced-cells were centrifugated (10 000 rpm, 4°C and 10 min), resuspended in cold 50 mM MOPS (3-Morpholinopropanesulfonic acid) (pH 6.0) containing 0.5 mg/mL lysozyme and incubated at 37°C for 30 min followed by sonication in an ice bath. The sonicate was removed by centrifugation (10 000 rpm, 4°C, and 20 min) and supernatant was incubated at 70°C for 10 min. For partially removing *E. coli* proteins, the mixture was centrifugated again. The recombinant enzymes contained 6xHis-tagged at its N-terminal, loaded on MagneHis Protein Purification System containing paramagnetic precharged nickel particles by using manual protein purification system protocol.

Protein concentrations were determined by the Lowry method using bovine serum albumin as the standard [18].

Enzyme assay

The XyI activity was assayed in the presence of glucose as a substrate and the amount of fructose produced in the enzymatic reaction was measured. The enzyme was incubated in 50 mM, pH 6.5, MOPS buffer containing 10 mM $MgCl_2$, 1mM $CoCl_2$ and 100 mM glucose (total volume of this mixture was adjusted to 100 µL) at 80ºC for 30 min. After the incubation, enzymatic reaction was stopped with 0.5 M perchloric acid addition. The amount of fructose formed was estimated by the cysteine–carbazole–sulfuric acid method [19]. One unit of enzyme activity was defined as 1 µmol fructose formed per minute under the assay conditions.

SDS-polyacrylamide gel electrophoresis

Denaturing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed by Laemmli [20] using 12% polyacrylamide gel. Coomassie brilliant blue R-250 was used for staining and the estimation of molecular weight of the polypeptide was performed by using a calibration curve of the protein standards.

Influence of pH and temperature

To assess the effect of pH on the enzyme activity, XyI activity was measured at various pH values ranging from 4.0 to 9.0. 50 mM of the following buffers were used at the indicated pH; sodium acetate buffer (pH 4.0 to 5.5), MOPS buffer (pH 6.0 to 7.5) and Tris-HCl buffer (pH 8.0 to 9.0). The optimum temperature was determined according to standard assay at different temperatures ranging from 40 to 100ºC at intervals of 10ºC. *The results for optimum pH and temperature studies were presented as percentage of the XyI activity.* The optimum pH and temperature values were used for further characterization studies.

pH and thermal stability

Thermal stability of the enzyme was investigated by incubating the enzyme at 60-90°C with 10°C increments for 1-3 h. Samples were withdrawn at indicated incubation times, cooled to room temperature on an ice bath and assayed for the residual enzyme activity under standard assay conditions at optimum pH and temperature. The percentage residual enzyme activity was calculated by comparison with the activity of the enzyme which was not incubated [21].

The pH stability of the *G. caldoxylosilyticus* TK4 XyI was determined by incubating the purified enzyme for 1, 5, 10 and 15 days at 4°C in several buffer solutions (indicated above) ranging from pH 5.0 to 9.0 *(in ratio*

of 1:1). At the end of the incubation period, XyI activity was determined under the standard assay conditions. The percentage residual XyI activity was calculated by comparison with the activity of the enzyme which was not incubated [21].

Effects of metal ions on the enzyme activity

Effects of various metal ions (at 5 mM final concentration of Ni^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, Ca^{2+}, Co^{2+}, Sn^{2+}, Cu^{2+}*) on the recombinant XyI activity were tested by the same procedure described above. To define the effect of these investigated metal ions on XyI activity, MOPS buffer containing any* $MgCl_2$ *and* $CoCl_2$ *was used in the reaction mixture for standard activity assay.* Enzyme activity determined in the presence of any metal ions was defined as 100%. *The XyI activity was expressed as relative percentage activity.*

Results and Discussion

Molecular cloning and sequence analysis of the XyI gene

The genomic DNA library of *Geobacillus caldoxylosilyticus* TK4 (*G. caldoxylosilyticus* TK4) strain was constructed using pUC18 plasmid in *E. coli* JM101. Genomic library was constructed in order to be able to clone the genes coding for industrially important enzymes of the organism. Recombinant pUC18 plasmids containing gene fragments larger than 2 kb were sequenced and the DNA sequences were converted to the amino acid sequences. These sequences were compared with known sequences using the online BLAST program. According to alignment results, a DNA fragment containing 927 bp was obtained after overlapping two clones and it shared approximately 96% similarity to XyI from *Geobacillus stearothermophilus*. In order to determine the missing part of the gene, encoding C-terminus of the component, genomic DNA was digested with *Hinf I*, circularized by ligation and subjected to inverse PCR with XyI F1 and XyI R1 primers designed from the known sequence of the gene. The inverse PCR product, approximately 1250 bp, was cloned into pGEM-T Easy vector and sequenced. Subsequently, the total length of missing part encoding C-terminus of the component including stop codon was determined to be 399 bp. The whole XyI gene was amplified by using XyI F2 and XyI R2 primers. PCR product was cloned into pET-28a(+) expression vector and transformed into *E. coli* BL21(DE3)pLysS for expression. The nucleotide sequence of XyI gene was submitted to GenBank (under accession number ACO55082.1). The open reading frame (ORF) of XyI consisting of 1326 bp encoded 441 amino acids residues with a calculated molecular mass of 50.18 kDa. The molecular mass of the 6xHis-tagged XyI was calculated to be 52.64 kDa *(calculated by ProtParam, http://www.expasy.org).*

Comparison of the deduced amino acid sequence of the XyI with those of proteins in the GenBank database indicated that the greatest similarity was with the XyI of *Geobacillus stearothermophilus*, 97% identity and 98% positive (GenBank accession no. CAA66715). Many of the bacterial XyI registered in the GenBank database also showed high homology to the XyI of *G. caldoxylosilyticus* TK4 (*Geobacillus thermodenitrificans* NG80-2, GenBank accession no. YP_001125866; *Geobacillus kaustophilus* HTA426, GenBank accession no. YP_l47728; *Bacillus halodurans* C-125, GenBank accession no. NP_243623).

It can be seen in the ClustalW results that the catalytic residues (His99, Asp102 and Asp337), substrate binding pocket (Trp48, Phe59, Thr139, Phe143, Thr144, Trp186, Glu229 and Lys231) and metal binding residues (Glu229, Glu266, His269, Asp294, Asp337) are entirely conserved (Figure 1). Similar results were previously reported by Hartley et. al. (2000) [7].

Expression and purification of recombinant XyI

Expression of the XyI was successfully achieved in *E. coli* BL21(DE3)pLysS, with the induction by isopropyl β-D-1-thiogalactopyranoside (IPTG) at 37°*C*, under the control of T7 RNA polymerase promoter with 6xHis-tagged in the N-terminal of the protein. The expressed recombinant protein was efficiently purified with one-step purification procedure using Ni^{2+} affinity chromatography. Paramagnetic precharged nickel particles were used to isolate recombinant protein directly from the crude cell lysate using a manual procedure according to the manufacturer.

Electrophoresis analysis

A single band with a molecular mass of approximately 52.12 kDa was monitored in the electropherogram (Figure 2) obtained from SDS-PAGE for the purified XyI containing 6xHis-tagged in the N-terminal. It was in a good agreement with the molecular mass calculated from predicted amino acid sequence (52.64 kDa). Known XyI proteins are geneally homodimeric or homotetrameric with subunit molecular mass in the range of 45–55 kDa [15,22,23]. Subunit molecular weight of trimeric recombinant XyI from a novel thermophilic bacterium *Bifidobacterium adolescentis* was reported to be 53.00 kDa on SDS-PAGE analysis [23]. A tetrameric recombinant XyI from *Thermoanaerobacterium* JW/SL-YS 489 and a trimeric XyI purified from the thermophilic *Bacillus* sp. strain have been reported to have 50.00 kDa subunit molecular weight for each of them on SDS-PAGE analysis [15,22].

Effect of pH and temperature on XyI activity

XyI activity assays at different pHs (4.0-9.0) in the presence of glucose as substrate revealed that the optimum pH was 6.5 (Figure 3A). The pH optima for different XyIs are generally in the alkaline range. But in industry, acidic conditions are desirable. This minimize the pro-

G. stearothermophilus -MPYFDNISTIAYEGPASKNPLAFKFYNPEEKVGDKTMEEHLRFSVAYWH 49
*G. caldoxylolyticus*_T -MSYFNTINAISYEGPTTKNPLAFKFYNPEEKVGDKTMEEHLRFSVAYWH 49
G. termodenitrifican -MAYFPNIGKIAYEGPESRNPFAFKFYNPEEKVGGKTMEEHLRFSVAYWH 49
*G. kaustophilus*_HTA42 -MAYFPNIGTIPYEGPESRNPLAFKFYNPDEKVGGKTMEEHLRFSVAYWH 49
*B. coagulans*_36D1 MMAYFPNVSKITYSGKQLKSGLSFNHYNPKELVGGKTMEEQLRFSVAFWH 50

G. stearothermophilus TFTGDGSDPFGAGNMIRPWNKYSGMDLAKARVEAAFEFFEKLNIPFFCFH 99
*G. caldoxylolyticus*_T TFTGDGSDPFGAGNMIRPWNKYSGMDLAKVRVEAAFEFFEKLNVPFFCFH 99
G. termodenitrifican TFTGDGSDPFGVGNMIRPWDKYSGMDLAKARVEAAFELFEKLNVPFFCFH 99
*G. kaustophilus*_HTA42 TFTGDGSDPFGVGNMIRPWNTYSGMDLAKARVEAAFELFEKLNVPFFCFH 99
*B. coagulans*_36D1 TFTESGTDPFGAGSKIRPWDRFTGMDLAKARVEAAFEFFEKLGNPYFCFH 100

G. stearothermophilus DVDIAPEGETLKETYKNLDIIVDMIEEYMKTSKTKLLWNTANLFTHPRFV 149
*G. caldoxylolyticus*_T DVDIAPEGETLKETYKNLDIIVDMIEEYMKTSKTKLLWNTANLFTHPRFV 149
G. termodenitrificans DVDIAPEGETLSETYKNLDEIVDMIEEYMKTSKTKLLWNTANLFSHPRFV 149
G. kaustophilus HTA42 DVDIAPEGETLSETYKNLDEIVDMIEEYMKTSKTKLLWNTANLFSHPRFV 149
B. coagulans 36D1 DRDIAPEGDTLRETNKNLDVIVAMIKDYMKTSKVKLLWNTANMFTNPRFV 150

G. stearothermophilus HGAATSCNADVFAYAAAKVKKGLEIAKRLGAENYVFWGGREGYETLLNTD 199
*G. caldoxylolyticus*_T HGAATSCNADVFAYAAAKVKKGLEIAKRLGAENYVFWGGREGYETLLNTD 199
G. termodenitrificans HGAATSCNADVFAYAAAKVKKGLEIAKRLGAENYVFWGGREGYETLLNTD 199
G. kaustophilus HTA42 HGAATSCNADVFAYAAAKVKKGLEIAKRLGAENYVFWGGREGYETLLNTD 199
B. coagulans 36D1 HGAASSCNADVFAYAAAQVKKGLEVGKELGAENYVFWGGREGYETLLNTD 200

G. stearothermophilus MKLELDNLARFLHMAVDYAKEIGFDGQFLIEPKPKEPTKHQYDFDVATRL 249
*G. caldoxylolyticus*_T MKLELDNLARFLHMAVDYAKEIGFDGQFLIEPKPKEPTKHQYDFDVATAL 249
G. termodenitrificans MKLELDNLARFFHMAVDYAKEIGFDGQFLIEPKPKEPTKHQYDFDVATAL 249
G. kaustophilus HTA42 MKLELDNLARFLHMAVDYAKEIGFDGQFLIEPKPKEPTKHQYDFDVATAL 249
B. coagulans 36D1 LKLEQDNLARFFHMAVDYAKEIGFDAQFLLEPKPKEPTKHQYDFDAATTI 250

G. stearothermophilus AFLQTYGLKDYFKFNIEANHATLAGHTFEHELRVARIHGMLGSVDANQGD 299
*G. caldoxylolyticus*_T AFLQTYGLKDYFKFNIEANHATLAGHTFEHELRVARIHGMLGSVDANQGD 299
G. termodenitrificans AFLQTYGLKDYFKFNIEANHATLAGHTFEHELRVARIHGMLGSVDANQGD 299
G. kaustophilus HTA42 AFLQTYGLKDHFKFNIEANHATLAGHTFEHELRVARIHGMLGSVDANQGD 299
B. coagulans 36D1 AFLKTYDLDQHFKLNLEANHATLAGHTFEHEIRVARTHGLLGSLDANQGD 300

G. stearothermophilus MLLGWDTDEFPTDLYSTTLAMYEILKNGGLGRGGLNFDAKVRRGSFEPED 349
*G. caldoxylolyticus*_T TLLGWDTDEFPTDLYSTTLAMYEILKNGGLGRGGLNFDAKVRRGSFEPED 349
G. termodenitrificans MLLGWDTDEFPTDLYATTLAMYEILQNGGLGRGGLNFDAKVRRGSFEPED 349
G. kaustophilus HTA42 TLLGWDTDEFPTDLYTTTLAMYEILQNGGLGRGGLNFDAKVRRGSFEPED 349
B. coagulans 36D1 PLLGWDTDEFPTDLYSTTLAMYEVLKNGGLGRGGLNFDAKTRRASFTDED 350

G. stearothermophilus LFYAHIAGMDSFAVGLKVAHRLIEDRVFDEFIEERYKSYTEGIGREIVEG 399
*G. caldoxylolyticus*_T LFYAHIAGMDSFAVGLKVAHRLIEDRVFDEFIEERYKSYTEGIGREIVEG 399
G. termodenitrificans LFYAHIAGMDSFAIGLKVAHRLLEDRVFEQFIEERYKSYTEGIGREIVEG 399
G. kaustophilus HTA42 LFYAHIAGMDSFAIGLKVAHRLLEDRVFEQFIEERYKSYTEGIGREIVEG 399
B. coagulans 36D1 LFYAHIAGMDSFALGLKVANRLIEDRVFDAFIEERYSSYKEGIGADIVSG 400

G. stearothermophilus TADFHKLEAHALQLGEIQNQSGRQERLKTLLNQYLLEVCAAR---- 441
*G. caldoxylolyticus*_T TADFHKLEAHALQLGEIQNQSGRQERLKTLLNQYLLEVCATR---- 441
G. termodenitrificans TADFHKLEQYALQLGEIRNTSGRLERLKTLLNQYLLEVSVPSKARL 445
G. kaustophilus HTA42 TADFKKLEEYALQLGDIRNTSGRLERLKTLLNQYLLEVSAPSGSRS 445
B. coagulans 36D1 KADFKSLENYILDKKEIINQSGRLEQLKNTLNHYIVQEAYQSVNA- 445

Figure 1. Alignment of the amino acid sequence of G. caldoxylosilyticus TK4 GI with different Xyls from Geobacillus stearothermophilus, Geobacillus thermodenitrificans, Geobacillus kaustophilus HTA426, Bacillus halodurans C-125. Catalytic residues are showed as ○, residues forming the substrate binding pocket are showed as ●, metal binding sites residues are showed as #.

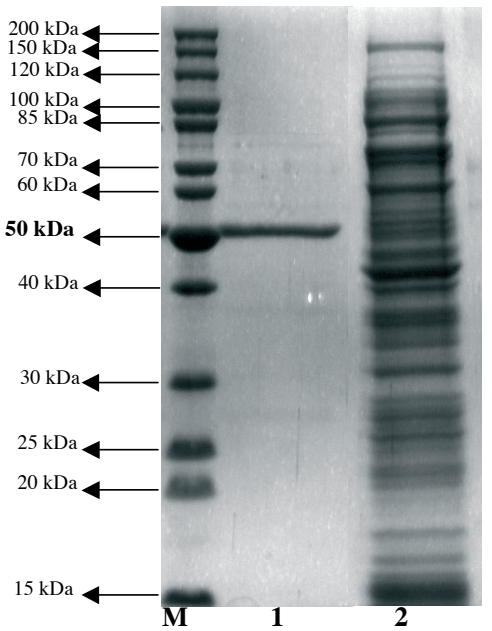

Figure 2. SDS-PAGE showing purified recombinant G. caldoxylosilyticus TK4 Xyl. Lane M: The protein molecular weight markers, Lane 1: The purified recombinant enzyme containing 6xHis-tagged, Lane 2: Intracellular component of the recombinant E. coli BL21(DE3)pLysS induced with IPTG for 3 hours.

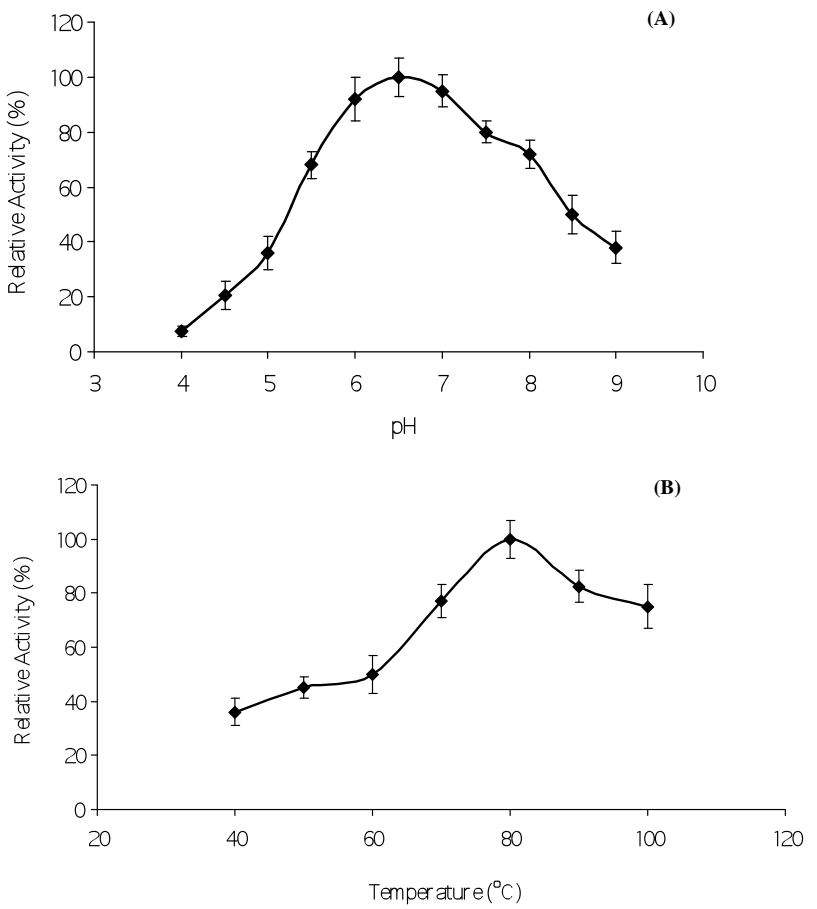

Figure 3. Effect of pH on the G. caldoxylosilyticus TK4 Xyl activity. Assays were performed in 50 mM of different buffer systems at indicated pH; sodium acetate buffer (pH 4.0 to 5.5), MOPS buffer (pH 6.0 to 7.5) and Tris-HCl buffer (pH 8.0 to 9.0). (B) Effect of temperature on the G. caldoxylosilyticus TK4 Xyl activity. The activities were determined in 50 mM MOPS buffer (pH 6.5) at different temperatures from 40 to 100°C.

duction of unwanted compounds like browning products (mannose, psicose, and other acidic compounds) and ultimately make for the development of a single step process for glucose isomerisation [6,24]. Figure 3A shows that recombinant *G. caldoxylosilyticus* TK4 XyI has an approximately 95% relative activity at pH 6.0 and it can be speculated that this enzyme can be attractive for industrial applications. Optimum activities of many XyIs were observed at pHs higher than 6.5 [23,25-27].

The temperature dependence of the recombinant enzyme was investigated in the temperature range between 40 and 100ºC. The recombinant XyI showed the highest activity at 80ºC (Figure 3B). This finding is in a good agreement with XyIs isolated from *Streptomyces* sp. [26], *Bacillus* sp. [22] and *Streptomyces chibaensis* J-59 [28]. The enzyme maintained 80% and 90% of its relative activity at 90ºC and 100ºC, respectively. The availability of thermoactive biocatalist for High-Fructose Corn Syrup production raises the possibility that higher temperatures could be used to improve the potential yield of fructose. It also raises the possibility that alternative bioprocessing strategies could be used to make strategic use of high temperatures [4].

pH and thermal stabilities

The pH stability of the recombinant XyI in different buffer solutions at pH 4.0-9.0 was evaluated at 4ºC (Figure 4A). After 1, 5, 10 and 15 days incubation the enzyme activity was enhanced about 30-70% in the broad range of pH when compared with its original activity. The pH tolerance of enzymes is very important to predict their storage condition, since enzyme can lose acitivity after stored at different pHs until use. It seems that *G. caldoxylosilyticus* TK4 can be stored for a broad range of pH for a long time.

Thermal stability was also investigated by incubating the recombinant enzyme at 60-90ºC prior to enzymatic assay for different periods up to 3 h (Figure 4B). After 1 h incubation at 80ºC, the recombinant enzyme retained 60% of its original activity. At 60 and 70ºC, after 1 h incubation, the enzyme retained more than the 90% of its orginal activity, but 3 h incubation caused more than 80% activity loses. It was also seen in Figure 4B that the recombinant XyI has lower thermal stability at 90ºC when compared to other incubation temperatures. When literature was searched, XyIs having similar thermal behaviours with *G. caldoxylosilyticus* TK4 XyI were fo-

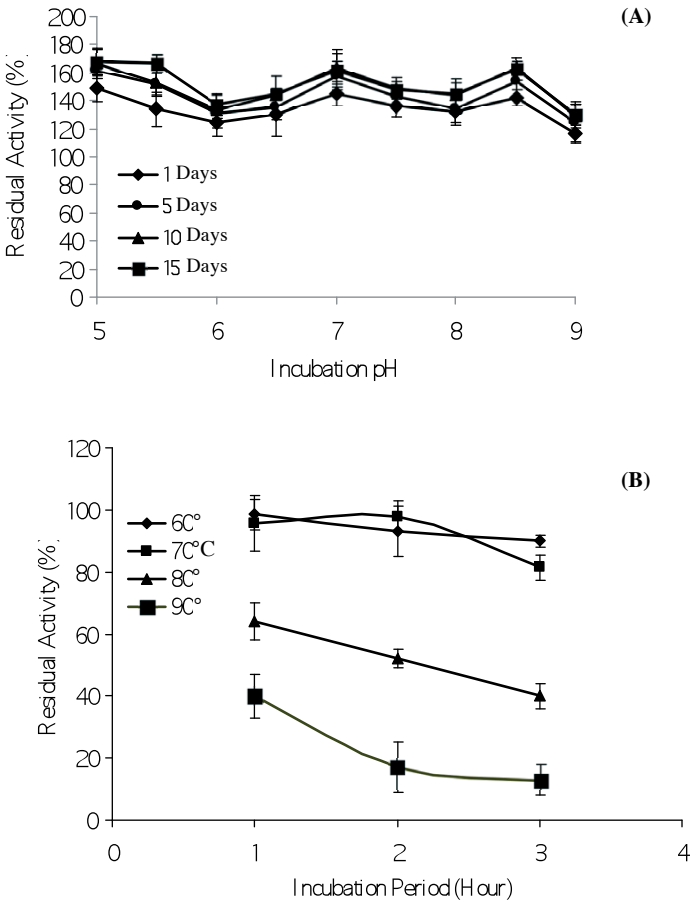

Figure 3. pH stability of recombinant G. caldoxylosilyticus TK4 Xyl. The enzyme activity was measured using the standard enzyme assay after 1, 5, 10 and 15 days incubation at different pH values. (B) Thermal stability of the G. caldoxylosilyticus TK4 Xyl. Enzyme solutions were incubated from 3 h at various temperatures between 60 and 90°C with 10°C increments and residual enzyme activities were determined by using standard assay procedure in 50 mM MOPS buffer (pH 6.5) at 80°C.

und. It was also declared that the recombinant XylI from *Streptomyces chibaensis* J-59 retained 25% of its orginal activity after 1 h incubation at 90ºC [28].

Effects of metal ions on the enzyme activity

XylIs require divalent cations (Co^{2+}, Mg^{2+} and Mn^{2+}) for their activities [1]. It was understood from the literature that their specific requirement depends on the source of enzyme and metal ions play a variety of roles in natural proteins, including nucleophilic catalysis, electron transfer and the stabilization of protein structure. When the effect of some divalent metal ions on the recombinant *G. caldoxylosilyticus* TK4 XylI activity was investigated, it was seen that efficiency in activation of enzyme activity was in the order: $Co^{2+} > Mn^{2+} > Mg^{2+}$ (Table 1). However, Ni^{2+}, Sn^{2+}, Ca^{2+}, Zn^{2+} inhibited the enzyme at different ratio. It was found that Cu^{2+} had the highest inhibitory effect for the *G. caldoxylosilyticus* TK4 XylI activity. Bhosale et al. [1] was also reported that Ag^{+}, Hg^{2+}, Cu^{2+} , Ni^{2+}, Ca^{2+} and Zn^{2+} ions inhibited XylIs activites. Strong inhibition in the presence of Cu^{2+} was reported for the *Thermus aquaticus* XylI [29]. Metal ions may have different coordination numbers and geometries in their coordination compounds and potentials as Lewis acids, they may behave differently towards proteins as ligands. These differences may also result in metal binding to different sites, and therefore, perturb the enzyme structure in different ways [30,31].

Table 1. Effect of some divalent metal ions on XylI activity.

Metal Ion [5 mM]	Relative Activity (%)
None	100±7
Mg^{2+}	160±6
Mn^{2+}	210±12
Zn^{2+}	25±4
Ca^{2+}	75±8
Co^{2+}	300±20
Cu^{2+}	05±1
Sn^{2+}	80±5
Ni^{2+}	20±3

Enzyme kinetics

Kinetic parameters for the reaction catalyzed by recombinant *G. caldoxylosilyticus* TK4 XylI were determined in the presence of different final concentration of D-glucose (2-200 mM) in the reaction mixtures. The Lineweaver-Burk plot analysis of this enzyme showed 20.58 mM K_m and 0.67 U mg protein^{-1} V_{max} values at 80°C (Figure 5). It was easily seen in the literature that K_m values of different XylIs in the presence of glucose were found variable between 0.249 mM and 5800 mM

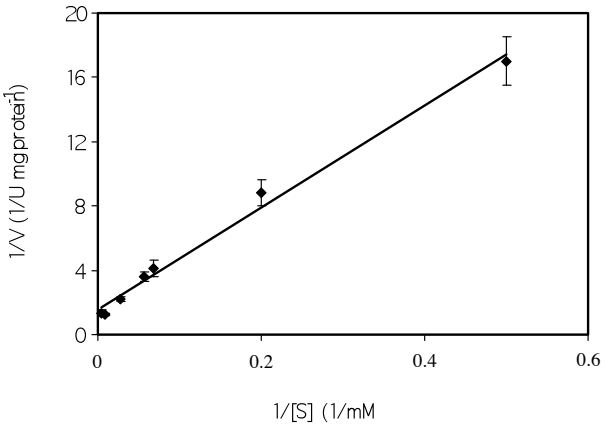

Figure 5. Lineweaver-Burk plot of G. caldoxylosilyticus TK4 Xyl for the kinetic analysis of the reaction rates, at a series of concentrations for glucose. The final glucose concentration was plotted in the range of of 2-200 mM versus XylI activity.

at different pH and temparature. The K_m value of a *Bacillus* sp. was reported as 142 mM at 80 °C [22]. The K_m values for *Actinoplanes missouriensis* [32], *Arthrobacter* sp. [33]. *Streptomyces rubiginosus* [27] XylIs were determined as 1900 mM, 250 mM, 160 mM at 60°C, respectively.

In conclusion, a XylI gene from *G. caldoxylosilyticus* TK4 was successfully cloned with 6xHis tagged and expressed in *E. coli*. The recombinant enyzme shared common characteristics of XylIs in terms of conserved amino acid residues, electrophoretic behaviour of the enzyme and kinetic parameters. Besides, this recombinant enzyme can be separated from many others in terms of its pH and temperature optima. Therefore, it is seen that the XylI has advantages in industrial applications where high temperatures and low pHs are desired.

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