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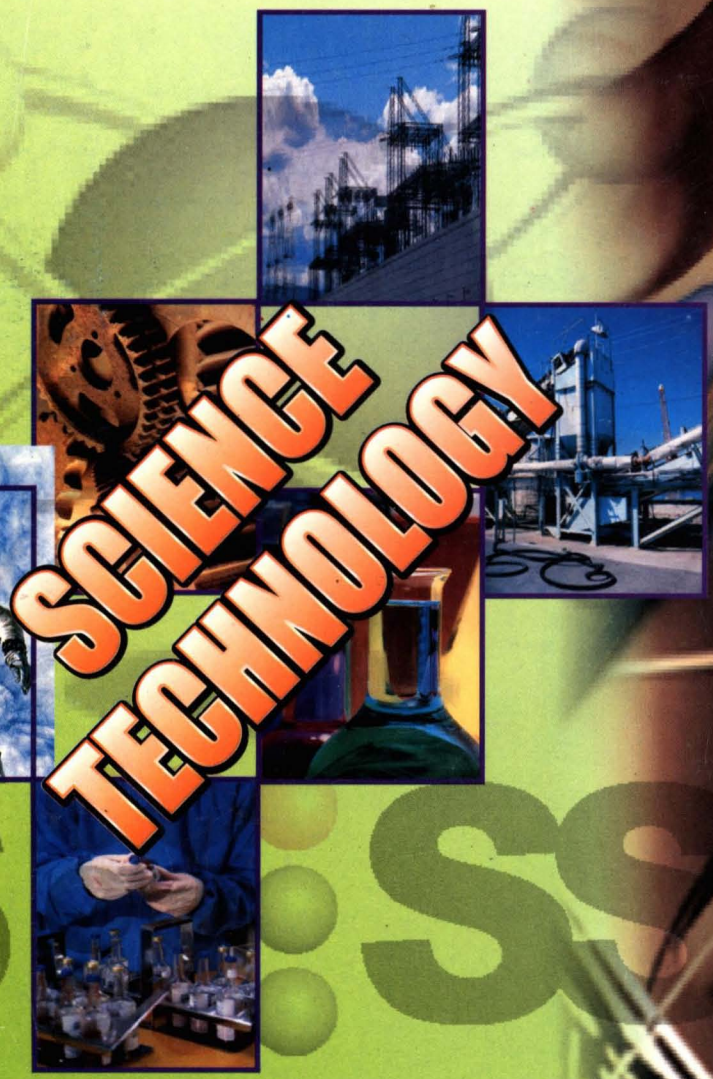

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The Cloning of The Xylose Isomerase Gene (*Xyla*) from *Bacillus subtilis* through Oligonucleotide Primers Directed PCR Amplification

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ABSTRACT

Xylose isomerase is also referred to as glucose isomerase. It reversibly catalyses the isomerisation reaction between D-xylose and D-xylulose, as well as D-glucose and D-fructose. Nucleotide sequences encoding the isomerases from various organisms are known. In our study, an isolate from soil sample that was pre-determined as Bacillus subtilis was used. Its genomic DNA was obtained through phenol chloroform extraction and alcohol precipitation. Purified genomic DNA was used as template in xylose isomerase gene (xylA) amplification by polymerase chain reaction. Several sets of oligonucleotide primers were designed with reference to published nucleotide sequences and synthesised for the use in gene amplification. The study results show successful xylA gene amplification after several attempts onto the optimisation of PCR protocols. Analysis by agarose gel electropheresis estimated the PCR amplified product to be ~1.3 kb, is in agreement to xylA published sequence, 1338 bp (NCBI GeneID: 939558). The product was further purified and endonucleases restricted by BamHI and KpnI that both recognition sites were included in the forward and reverse primers respectively. Treated PCR product was subsequently ligated into pGEM®-3Z cloning vector that has been BamHI/KpnI restricted. DNA sequencing analysis of the cloned gene shows 72% homology in comparison to the published xylA gene, implies a variant of xylose isomerase gene has been cloned.

Keywords: *xylose isomerase, Bacillus subtillis, PCR amplification*

Introduction

Xylose isomerase (D-xylose ketol isomerase, EC 5.3.1.5) is commonly referred to as glucose isomerase (GI). It catalyses the isomerisation reversibly between D-xylose and D-xylulose in the first step of xylose metabolism following the pentose phosphate cycle; as well as D-glucose and D-fructose. Wiseman (1975) predicted GI would be the most important of all industrial enzymes of the future. It is one of the largest volume commercial enzymes used, and it is used for industrial production of high-fructose corn sweetener (Crueger & Crueger 1982). Since, cloning and sequencing work on various sources of GI were extensively done. Lawlis et al. (1984) cloned a 4.2 kb fragment of *Escherichia coli* chromosome which contains the xylose isomerase gene. Sarthy *et al.* (1987) expressed the *Escherichia coli* GI in *Saccharomyces cerevisiae*. Dekker et al. (1991) cloned the GI gene from the thermophile *Thermus thermophilus*. Indeed, GI has gained attention due to its commercial importance in industrial application (Bhosale et al. 1996).

Recently, we have isolated amylase producing *Bacillus* from soil sample (Kan et al. 2003). The presence of GI was uncertain due to undetectable enzyme activity by plate assay method described by Lee at al. (1990a). Therefore, we undertook studies aimed at amplifying gene fragment that could code for GI. Here we report on our approaches identifying the possible GI gene through PCR amplification and cloning the gene fragment.

Materials and Methods

Isolate determination

Bacterial isolates were picked and tested by using API50CHB Medium and API50CH strips according to manufacturer's recommendation (bioMérieux). Reading of the results were taken after 24h, 48h and 96h of incubations.

Glucose isomerase activity plat assay

Fructose (2%), $MgSO_4$ (5mM), $CoCl_2$ (0.5mM), glucose oxidase (20U/ml), peroxidase (4 U/ml) and benzidine (0.4 mg/ml) in 100 mM MOPS buffer (pH 7.0) were mixed with top agar (0.7%) at 50°C and poured on colonies grown overnight on Luria broth agar plates with 1% xylose. After soft agar had solidified, the plates were incubated overnight at 37°C. Positive clones will show a dark brown halo around the light colonies.

DNA preparation and quantification

Total DNA from each bacterial isolate was extracted using genomic DNA purification method (Sambrook *et al.* 1989). The amount of genomic DNA was then quantified by spectrophotometry (Bio-Rad – SmartSpec Plus).

PCR amplification

Primer set contains a forward primer XylA3 (30-mer) and reverse primer XylA4 (30-mer). They are designed based on the known sequence (Wilhelm & Hollenberg 1985). The PCR profile was optimised to 94°C for 1 minute, 45°C for 1 minute and 50°C for 2 minutes. Taq polymerase from Fermentas' 2X PCR Master Mix was used in all the amplifications. The amplified products were analysed through agarose gel (1% w/v) electrophoresis (10V/cm).

Cloning and sequencing

Prior to cloning, PCR amplicons were subjected to purification using Qiagen MinElute® PCR Purification Kit followed by BamHI/KpnI restriction. Subsequently the amplicons were cloned into Promega's pGEM®-3Z cloning vector that has been BamHI/KpnI restricted. *E. coli* JM109 was made competent for transformation. Blue-White selection method on amplicil in LB agar chooses the possible clones. The recombinant construct of each clone was isolated through plasmid extraction method (Sambrook *et al.* 1989). Sequencing was bi-directional using M13/pUC sequencing primers sets.

Results and Discussion

The success of our PCR amplification relies heavily on the primer designs. Nucleotide sequence of GI gene published by Wilhelm and Hollenberg (1985) was used as template in primer designing. The primers set XylA3: 5'-gttaac**ggatcc**atggctcaatctcattctagttca-3'consists of a BamHI site (bolded) and XylA4: 5'-cgctct**ggtacc**ttattggttcaatatagttttaa-3' consists of a KpnI site (bolded) used in the PCR amplification yielded a DNA fragment that has a molecular weight between 1.0kb to 1.5kb (Fig. 1). Gel analysis on size by migration distance with reference to the DNA ladder markers estimated the amplified DNA to be 1.3kb. This is very close in agreement to what Wilhelm and Hollenberg (1985) had reported where the structural gene consists of 1320bp, and Kunst et al. (1997) published the xylose isomerase gene, *XylA* as 1338bp. The PCR amplicon was extracted and purified from agarose gel. It was then followed by BamHI and KpnI endonuclease restriction before being cloned into pGEM-3Z, a 2743bp cloning vector (Fig 3a). The recombinant plasmid pWHK7 (Fig. 3b) would have a size of total molecular weight at about 4.0 kb. Endonuclease restriction analysis digested pWHK7 with BamHI or KpnI confirmed its molecular weight (Fig. 2). When pWHK7 was restricted by both mentioned enzymes above, the 1.3 kb insert was recovered (Fig. 2). A specific enzyme activity assay that detects the conversion of fructose to glucose on agar plates (Lee et al., 1990a) was carried out on clones harbouring pWHK7. However the GI activity was not observed on this method where positive clones will show a dark brown halo around the colonies that appeared as dark halo against the light colonies. A bi-directional sequencing on pWHK7 using universal M13 forward and reverse sequencing primers resulted a 72% homology compare to nucleotide sequence of Wilhelm and Hollenberg (1985) analysed with the CLUSTALX global alignment. This implies a possible variant of GI gene has been cloned. We could not conclude exclusively due to the lack of enzyme activity. A simple explanation would be that the GI gene has not been cloned in frame. Currently, we are sub-cloning the gene for further expression studies. Another possibility would be that *Bacillus subtilis* produces thermolabile GI (Lee et al. 1990b) where the activity was lost during the assays.

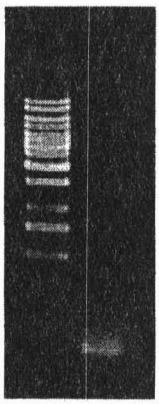


Fig. 1: Glucose isomerase gene amplification. Lane M: DNA ladder marker. Lane 1: 10ml PCR reaction after amplification. A DNA band (~1.3 kb) is observed indicated by the arrow.

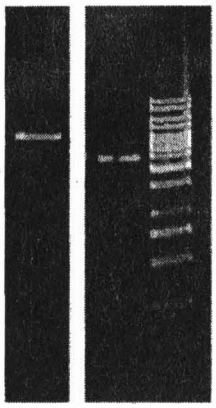
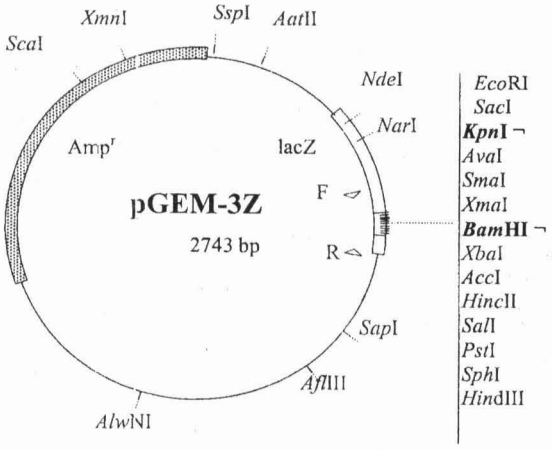


Fig. 2: Electrophoresis of endonuclease digestions of pWHK7. Lane 1: pWHK7 was single digested with BamHI. Linearised plasmid shows its molecular weight to be ~4 kb. Lane 2: pWHK7 was double digested with BamHI-KpnI. Lane M: DNA ladder marker. Numbers indicate the molecular weights.



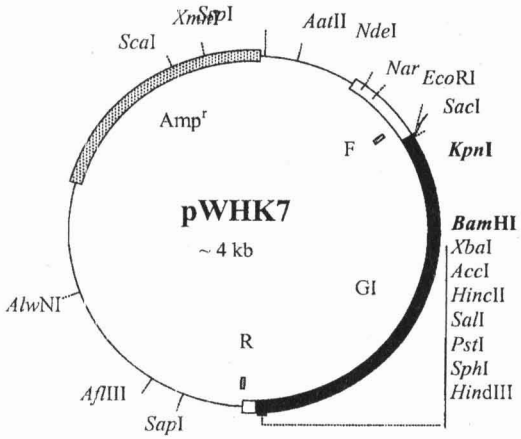


Fig. 3: (a) Cloning vector pGEM-3Z. It was double digested at its multiple cloning site using BamHI and KpnI prior to cloning. (b) Recombinant plasmid pWHK7 where BamHI-KpnI treated amplified GI gene was cloned into pGEM-3Z. F: M13 forward sequencing primer site. R: M13 reverse sequencing primer site.

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