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Original Article

Molecular cloning of *gluconobacter oxydans* DSM 2003 xylitol dehydrogenase gene

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Abstract

Due to the widespread applications of xylitol dehydrogenase, an enzyme used for the production of xylitol, the present study was designed for the cloning of xylitol dehydrogenase gene from *Glcunobacter oxydans* DSM 2003. After extraction of genomic DNA from this bacterium, xylitol dehydrogenase gene was replicated using polymerase chain reaction (PCR). The amplified product was entered into pTZ57R cloning vector by T/A cloning method and transformation was performed by heat shocking of the *E. coli* XL1-blue competent cells. Following plasmid preparation, the cloned gene was digested out and ligated into the expression vector pET-22b(+). Electrophoresis of PCR product showed a 789 bp band. Recombinant plasmid (rpTZ57R) was then constructed. This plasmid was double digested with *XhoI* and *EcoRI* resulting in 800 bp and 2900 bp bands. The obtained insert was ligated into pET-22b(+) vector and its orientation was confirmed with *Xho*I and *Bam*HI restriction enzymes. In conclusion, in the present study the recombinant expression vector containing xylitol dehydrogenase gene has been constructed and can be used for the production of this enzyme in high quantities.

Keywords: Xylitol dehydrogenase, *Gluconobacter* oxydans, Gene**,** Cloning

INTRODUCTION

Xylitol dehydrogenase *(xhd,* EC 1.1.1.9*)* is a key enzyme in the production of xylitol (1-3). This enzyme plays an important role in ethanol fermentation and belongs to the polyol dehydrogenase subfamily (4). Xylitol is a polyol sweetner with anticarciogenic effects (i.e. it does not cause tooth decay) (5,6). Since its metabolism is insulin independent and it has low calorie value (6), it is a suitable alternative sugar in diabetic regimens. These benefits have resulted in the use of xylitol in many industries such as pharmaceuticals, as a sweetener or excipient in syrups, tonics and vitamin formulations and food industry such as chewing gum, hard candy, chocolate coating, and bakery (7,8).

Xylitol can be produced by chemical methods in which D-xylose is reduced to xylitol. These methods need high temperatures and pressures and additional purification steps to separate the by-products therefore these are not economical (5,6). On the other hand, microbial production of *xhd* and eventually xylitol has cheaper downstream processing and could be performed without toxic catalysts (9).

Some species of bacteria and yeasts have been used to produce *xhd* (1,6,10). Aarnikunnas and coworkers have cloned *xhd* in *Pantoea ananatis* in which *xhd* reduced xylitol to L-xylulose (11). In other studies this goal has been achieved using *Candida tropicalis* (2), *Pichia angusta* (3), *Aspergylus oryzae*, *Pichia stipitis*, *Saccharomyses serevisiae* (12), *Bacillus pa1lidus* (13) and *Gluconobacter oxydans* (5,10,14).

Gluconobacter genus of acetic acid bacteria has a soluble *xhd* and is able to produce xylitol efficiently (5). *Gluconobacter oxydans* strains have higher *xhd* activity (14) and could

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convert D-arabitol to xylitol following two enzymatic reactions: initially D-arabitol converts to D-xylulose by D-arabitol dehydrogenase, and then the conversion of D-xylulose to xylitol occurs with *xhd* (10).

Complete *Gluconobacter oxidans* genome contains 2,702,173 base pairs (15) and *xhd* gene is 789 bp long coding for 262 amino acids. Since this gene has no introns, it can be PCR amplified without the need for isolation of its mRNA. Also expression of this gene results in production of xylitol enzyme with high efficiency (14). Therefore, in the present study, we constructed an expression vector containing the *xhd* gene of *Gluconobacter oxydans* DSM 2003 for its production and purification from *E. coli* cells.

MATERIALS AND METHODS

Strains

Gluconobacter oxydans DSM 2003 was purchased from DSMZ, Germany. *E. coli* XL1-blue was obtained from Cinnagen, Iran.

Enzymes, primers, chemicals and plasmids

Restriction enzymes, modifying enzymes and other molecular related reagents were purchased from Fermentas, Poland. Primers for PCR reaction were purchased from Fanavarie Kowsar, Iran.

Isolation of genomic DNA

Gluconobacter oxydans DSM 2003 was initially cultured in Luria Bertani broth (LB) (0.5% Bacto yeast extract, 1% Bacto tryptone and 1% NaCl in distilled water) at 37 °C for 24 h. Then 0.5 ml of the bacterial overnight culture was centrifuged at 3000 rpm for 5 min and the obtained pellet was resuspended in 200 µl of PBS (10 mM Na_2HPO_4, 137 mM NaCl, 2.7 mM KCl, 2 mM KH_2PO_4). Subsequently, 5 µl of lysozyme buffer was added to this suspension and incubated at 37 °C for 15 min. Genomic DNA was isolated using high pure PCR template preparation kit (Roche, Germany) according to the manufacturer's instructions. Briefly, after addition of 200 µl of the binding buffer and 40 µl of proteinase K, the mixture was incubated at 72 °C for 10 min. 100 µl of isopropanol was added to the solution and the sample was pipetted into the upper reservoir of combined filter-collection tube assembly and centrifug-ed. The flowthrough was discarded and the filter tube was combined with a new collection tube. Subsequently, 500 µl of the inhibitor removal buffer was added to the upper reservoir and then centrifuged. The filter tube was combined with a new collection tube and 500 µl of the washing buffer was added to the upper reservoir and then centrifuged. The filter tube was inserted into a clean 1.5 ml reaction tube and 300 µl of pre-warmed (70 °C) elution buffer was added to it in three steps followed by centrifugation. All centrifugation steps were performed for 1 min at 8000 rpm.

PCR

Xhd gene was amplified by PCR using the following primers designed according to the *xhd* genetic sequence: forward primer: 5'-GGAATTCCATATGTCGAAGAAGTTTAA CGGTAAAGTCGT-3' and reverse primer: 5'-CCCTCGAGAGCAATCGGCAGGTTCACC-3'. PCR was performed in a total volume of 50 µl containing 1.4 µl $MgCl_2$, 1 µl of dNTP, 1 µl of each PCR primer, 5 µl 10x-PCR buffer, 1 µl of Taq polymerase enzyme and 2 µl of DNA template. Thermal cycles included initial denaturation at 95 °C for 5 min, 35 cycle of 95 °C for 1 min, 55 °C for 2 min and 72 °C for 3 min. Afterwards, 5 µl of the PCR product was electrophoresed on a 1.0% agarose gel.

Cloning of xhd gene

The purified PCR product was ligated into pTZ57R plasmid using Ins T/A clone kit (Fermentas, Poland) and transformed into *E. coli* XL1-blue cells using heat shock method (16). Subsequently, plasmid preparation was performed from the obtained colonies using Aurum plasmid mini kit (Roche, Germany) according to the manufacturer's instructions.

Construction of the recombinant expression vector

PTZ57R/*xhd* plasmid was isolated from *E. coli* cells using alkaline lysis method (16). This plasmid and expression vector pET-22b(+) were digested with *Eco*RI and *Xho*I restriction enzymes and the obtained insert and

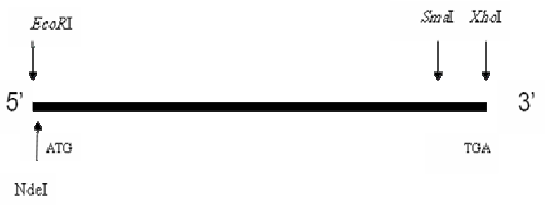

Fig. 1. Schematic presentation of *Eco*RI, *Nde*I, *Sma*I, and *Xho*I restriction sites on xylitol dehydrogenase gene. *ECoR*I site is located 10 nucleotides upstream of the start (ATG) codon.

vector were gel purified. The *xhd* gene was then ligated into pET-22b(+) in the presence of 1 µl T_4 DNA ligase and 2 µl of enzyme buffer for 24 h at 16 °C . Then this product was used for the transformation of *E. coli* BL21 cells.

Restriction digestion of DNA

In the present study the obtained DNA products were digested using restriction enzymes shown in Fig. 1. All digestions were performed for one hour at 37 ^{o}C.

RESULTS

PCR amplification of xhd gene from Gluconobacter oxydans DSM 2003

Xhd gene of *Gluconobacter Oxydans* contains 789 nucleotides encoding for 262 amino acids. Therefore, after PCR amplification of this gene, it was expected to obtain a DNA band of about 800 bp. As shown in Fig. 2, the desired product was obtained. Digestion of this DNA with *Sma*I restriction enzyme produced the expected 725 bp fragment (Fig. 3).

Insertion of xhd gene into pTZ57R plasmid

The *Gluconobacter oxydans xhd* gene was ligated into pTZ57R vector and was used for transformation of *E. coli* XL1 blue cells. The obtained recombinant plasmids were then subjected to digestion with *Bam*HI and *Xho*I restriction enzymes for determination of the presence of *xhd* gene in these plasmids.. The observed 800 bp band confirmed the presence of *xhd* gene (Fig. 4).

Construction of the recombinant expression vector

The recombinant pTZ57R plasmid was double digested with *Xho*I and *Eco*RI restriction enzymes and the obtained insert was gel purified. Additionally, pET-22b(+) plasmid was double digested with these enzymes. These were then ligated and used for the transformation of *E. coli* BL21 cells. Subsequently, the obtained plasmids were digested with *Bam*HI and *Xho*I restriction enzymes and those containing the correct orientation of the insert (showing 800 bp band) were selected (Fig. 5).

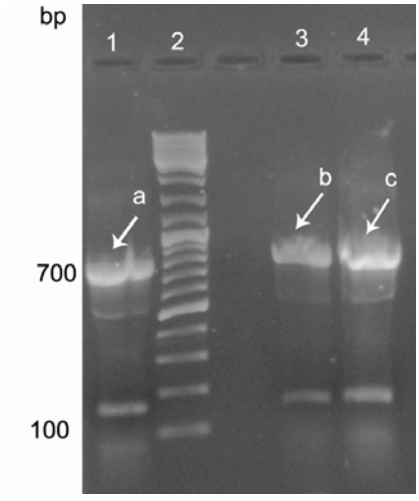

Fig. 2. PCR amplification of *Gluconobacter oxydans* DSM 2003 *xhd* gene (Lanes 1, 3, 4, a= 800 bp). Arrows show the bands of the expected PCR products. Lane 2 shows 100 bp molecular weight marker.

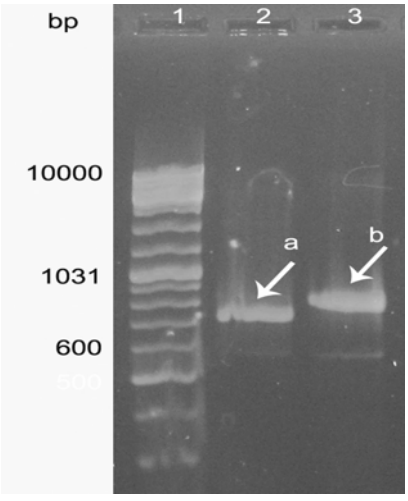

Fig. 3. Digestion of *xhd* gene with *Sma*I restriction enzyme. Lane 1 shows100 bp molecular weight marker, lane 2 digested PCR product with *Sma*I (a, 725 bp), and Lane 3 undigested PCR product (b, 800 bp).

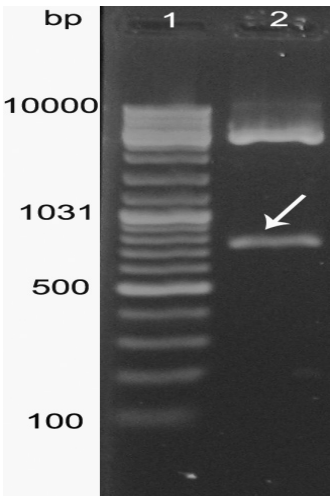

Fig. 4. Digestion of pTZ57R/*xhd* with *Bam*HI and *Xho*I restriction enzymes. Lane 1 shows molecular weight marker, Lane 2 is digested recombinant plasmid containing *xhd* gene (800 bp).

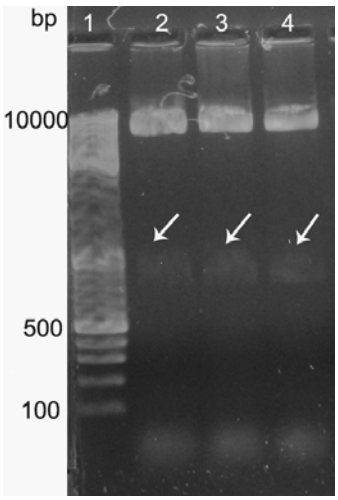

Fig. 5. Digestion of recombinant pET-22b(+) plasmid with *Bam*HI and *Xho*I restriction enzymes (Lanes 2-4). Lane 1 shows100 bp molecular weight marker.

DISCUSSION

In this study we cloned *xhd* gene of *Gluconobacter oxydans* DSM 2003 in *E. coli* XL1-blue cells and constructed an expression vector for further production of xylitol dehydrogenase enzyme. The *xhd* gene of yeasts like *Candida tropicalis* (2), *Pichia angusta* (3) and bacteria like *Pantoea ananatis* (11), *Bacillus pa1lidus* (13) and *Gluconobacter oxydans* (14) have been cloned. When the two latter bacteria were compared, *Gluconobacter oxydans* had higher *xhd* activity (14). In *Gluconobacter oxydans* and *Bacillus pa1lidus xhd* L-xylulose converted to xylitol but in *Pantoea ananatis,* D-xyloluse was converted to xylitol.

Sugiyama and coworkers have cloned *xhd* gene of *Gluconobacter oxydans* ATCC 621 in *E. coli* JM 109 cells. These investigators have used pUC18 as the vector for cloning and expression of this gene (14). In our study, we cloned *xhd* gene into pET22b(+) plasmid. This vector contains the powerful T7 promoter which is more efficient for expression of proteins as compared to the lac promoter present in pUC18 plasmids. Additionally T7 promoter system in *E. coli* BL21 (DE3) cells would provide tighter control over the expression of *xhd* gene. In our study, genomic DNA of *Gluconobacter oxydans* was extracted and amplified by PCR reaction. Annealing time was longer than usual because of the length of primers which could increase the chances of self coupling (17). T/A cloning method was used for the cloning of this gene. This method was utilized due to the fact that Taq polymerase adds an adenine nucleotide at the 3'end of the gene and pTZ57R plasmid (T-vector) has an excess thymidine nucleotide at its 3'end (18). This method of cloning was very fast and efficient and we recommend it especially when the direct digestion of PCR products fails to produce inserts suitable for ligation into a selected vector.

CONCLUSION

In this study we were able to successfully clone *Gluconobacter oxydans* DSM 2003 *xhd* gene and construct a recombinant expression vector. For production of this enzyme in high quantities, it is necessary to optimize the expression of *xhd* gene in future studies.

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