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Construction of an Expression Vector Harboring Xylonate Dehydratase Enzyme Gene and its Transformation into the Appropriate Host

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ABSTRACT

Lignocellulosic biomass such as agricultural wastes are important and available source for production of biofuels and chemicals. D-xylose, is the second important sugar in lignocellulosic hydrolysates .Nowadays, developing the microbial cell factories is important to acheive D-xylose for the production of chemicals. D-1,2,4-butanetriol (BT), an important intermediate chemical, apply in many fields, such as pharmaceuticals, polymer materials, paper and military. Bio production of BT is necessary, so a synthetic pathway (using D-xylose dehydrogenase (XD), D-xylonate dehydratase, 2-keto acid decarboxylase and aldehyde reductase) applied to produce BT from D-xylose. XD have Fe-S clusters so its belongs to the IlvD/EDD dehydratase enzymes. Crystallographic analysis demonstrated that XylD has a homotetrameric quaternary structure, and each monomer is composed of two domains. XD catalyzes conversion of D-xylonic acid to 2-keto-3-deoxy-D-xylonic acid (KDX). In this research by using restriction enzymes, Colony PCR and sequencing, the cloning process and entry of the gene *xylD* into the expression vector pET28 were confirmed. Existence of recombinant protein was verified by SDS-PAGE with the molecular weight of about 68 KDa. The expression rate of the recombinant protein was estimated to be 54% by Image J software. Therefore, the bioproduction of butanetriol requires the construction of a metabolic pathway consisting of several enzymes. The presence of *E.coli* bacteria as the target strain and using a cheap substrate such as biomass containing D-xylose and the presence of the xylonate dehydratase enzyme are necessary to produce BT at a high rate.

Key words: D-xylose, Xylonate , Butanetriol, D-xylonate dehydratase, *Escherichia coli*.

1.Introduction

D-1,2,4-butanetriol (BT) is an important chemical in plasticizers, polymers, cationic lipids, and pharmaceuticals production. It is also has an important role in 1,2,4-butanetriol trinitrate (BTTN) production, an energetic plasticizer in propellant and explosive formulations suitable for replacing the traditional nitroglycerin (Abdel-Ghany et al., 2013 ; Cao et al., 2015). Lower sensitivity to shock, higher thermal stability, and less volatile are the advantages of BTTN over nitroglycerin (Gouranlou, & Kohsary, 2010). Chemical reduction of malic acid using $NaBH_4$ leads to 1,2,4-Butanetriol producedion (Gouranlou & Kohsary, 2010; Bamba et al., 2019), Although this production process generates great amount of borate salts as a disposal by-product, but provide other catalysts such as copper chromite and rubidium for 1,2,4-butanetriol production (Niu et al., 2003), catalytic reduction needs specific condition such as high pressure and also high temperature. In addition, the inevitable production of by-products cause reduces 1,2,4-butanetriol production rate (Niu et al., 2003 ; Bamba et al., 2019).

Chemical synthesis of BT has high cost and leads to low yield, so providing its microbial bio production process seems important (Abdel-Ghany et al., 2013 ; Lu et al., 2016). Xylans is made of D-Xylose (“wood sugar”). Xylans is the majority of hemicellulose in plant cell walls and biosphere carbohydrates (Zhou et al., 2013). D-xylose, a pentose (five-carbon) sugar, forms 30–40% of the sugars recoverable from plant biomass (Jagtap et al., 2014).

The ability of microorganisms to use lignocellulosic sugars can is beneficial for the production of biofuels and chemicals like 3,4-dihydroxybutyric acid, glutaric mesaconic, and glycolic as organic acids, monoethylene glycol, 1,4-butanediol and 1,2,4-butanetriol, as alcohols (Francois et al., 2020). Several microorganisms have been studied for the conversion of xylose to different products. In general, three pathways have been described for the degradation of D-xylose in microorganisms (Moysés et al., 2016 ; Nunn et al., 2010): isomerase pathway (XIP), oxo-reductive pathway (ORP), xylose oxidative pathway (XOP), which includes Weimberg and Damms pathways (Rygus et al., 1991; Lawlis et al., 1984; Valdehuesa et al., 2018 ; Jo et al., 2017 ; Johnsen & , Schönheit., 2004 ; Dahms., 1974).

In recent years, recombinant strains, such as fungi and bacteria, have been created by genetic engineering and metabolism methods, which are able to produce various substances, including

butanetriol from xylose, using the above pathways. Concentration on bio production of BT, leads to a four steps synthetic pathway (using by D-xylose dehydrogenase, D-xylonate dehydratase, 2-keto acid decarboxylase and aldehyde reductase) to produce BT from D-xylose. Xylonate dehydratase (XD) is an important and useful enzyme that converts xylonate to 2-keto3-deoxy D-xylonate during a one-way reaction. This enzyme has different kinetic and activity characteristics in different bacterial species, which will be mentioned. This enzyme is mainly capable of dehydrating xylonate, arabonate, and galactarate. The reaction of this enzyme with a Gibbs free energy (ΔG) of about -100 KJ/mol can be done thermodynamically easily (Wang et al., 2018).

C. crescentus is a Gram-negative oligotrophic bacterium that is belongs to freshwater environments. The genome of *C. crescentus* has been demonstrated that there are several gene clusters that make certain survival during nutrient starvation or serious changes in environmental conditions (Nierman et al., 2001). Growing *C. crescentus* on D-xylose as only carbon source, revealed expression of genes coding for hydrolytic exoenzymes (xylosidases and hydrolases) and a gene cluster that was initially predicted to encode enzymes for the Weimberg pathway (Hottes et al., 2004).

The bioproduction of butanetriol requires the construction of a metabolic pathway consisting of several enzymes. Considering *E.coli* bacteria as the target strain and using xylose as a substrate, the presence of xylonate dehydratase enzyme is necessary to produce BT at a high rate. Because, as mentioned, the dehydratase enzymes in *E.coli* bacteria are not kinetically strong (YagF, YjhG). In addition, the cell needs these enzymes in its metabolic network, and the involvement of these enzymes in the extra metabolic pathway reduces the growth and activity of the host cell. Therefore, the goal of this project has been to create a metabolic pathway for rapid consumption of xylonate in *E.coli* strain, so that an inexpensive substrate such as biomass containing xylose can be used and based on the fermentation power of the host *E.coli* bacteria and the enzymes required for the metabolic pathway, BT will be produced. The best type of XylD enzyme, which has a high kinetic activity compared to other known strains, is expressed in *Caulobacter crescentus*, and due to the unavailability of this bacteria, *Caulobacter Vibrioides* strain whose *xylD* gene is 96% similar to *C.Crescentus* was used.

2. Materials and methods

2.1. Design and development of gene manipulation

In this research, in order to amplify the *xylD* gene fragment using PCR, using Snap Gene 5.2 software, and also downloading the gene sequence file of *Caulobacter vibrioides* bacteria from the NCBI site under the number NC-011916.1 and according to the available enzymes and its cleavage site in the pET28 vector, two primers with *Ndeı* and *HindIII* restriction sites were designed. Considering the overall frame of the pET28 vector and in order to place the *xylD* gene fragment and the *lacI* promoter in the opposite direction inside the vector, *xylD*-RBS-F primer with *Ndeı* restriction site with a size of 32 bases (bp) and *xylD-Hindıı* primer R, with a size of 30 bases (bp) were selected (Table 1). The structure and characteristics of these two primers were analyzed by Oligo Analyzer software. In order to extract the genome, *Caulobacter vibrioides* was cultured in the 830 R_2 culture medium, and when the optical density (OD) at 600 nm of cell culture reached around 2, the genome was extracted using the DNA extraction kit of the GTP company. Then, using designed primers and the PCR method, the xylonate dehydratase (*xylD*) gene was amplified to a length of 1794 bp from the extracted genome of *Caulobacter vibrioides* bacteria. The PCR temperature conditions is 5minutes in 95° as Pre-denaturation and then 30 seconds in 95°, 30 seconds in 53°, 2 minutes in 72° for 10 cycles and Immediately 30 seconds in 95°, 30 seconds in 57°, 2 minutes in 72° for 20 cycles and 5 minutes in 72° as final extention.

Table 1: Primers designed for *xylD* gene

Primers	Sequence (5'-3')	PCR Product length
xylD-RBS-F	5' GATATACATATGAGATCCGCCTTGTCTAACCG 3'	1794 bp
xylD- Hindıı -R	5' CTCCAAGCTTCCTTCGCATCAGTGGTTGTG 3'	

2.2. Cloning of xylD gene into pET28 vector

After amplification of the xylonate dehydratase (*xylD*) gene using PCR, *E. coli* DH5α strain containing the pET28 vector was cultured in order to extract the vector, and then the existing vector was isolated by a plasmid extraction kit (Yekta Tajhiz Azma). After extracting the pET28 vector, the vector and the xylonate dehydratase gene fragment (*xylD*) are cut using the restriction enzymes according to which the primers were sequenced, i.e., *Ndeı* and *Hindıı* enzymes. In the next step, the connection between the vector and the gene fragment should be made (Fig 1) and after that, the transformation step should be done by preparing the competent *E.coli* DH5α cell. After the transfer of the recombinant vector containing the *xylD* gene, several colonies were identified and prepared for colony PCR by the appearance of colonies on the agar plate containing kanamycin. After confirming the presence of the

xylonate dehydratase gene in the recombinant vector (pET28-*xylD*) using the colony PCR, in order to ensure the presence of the gene fragment within the recombinant vector, as well as the absence of a false positive response in the colony PCR, Enzymatic cutting was put on the agenda by restricting enzymes *Ndeı* and *Hindıı*. For this purpose, the vector was first extracted from the *E. coli* DH5α strain, and then enzymatic cleavage was performed on the extracted vector. The third step was to confirm the presence of the *xylD* gene fragment in the recombinant pET28 vector, the intended vector was sent for sequencing with *xylD*-RBS-F and *xylD- Hindıı* -R primers and the result was confirmed.

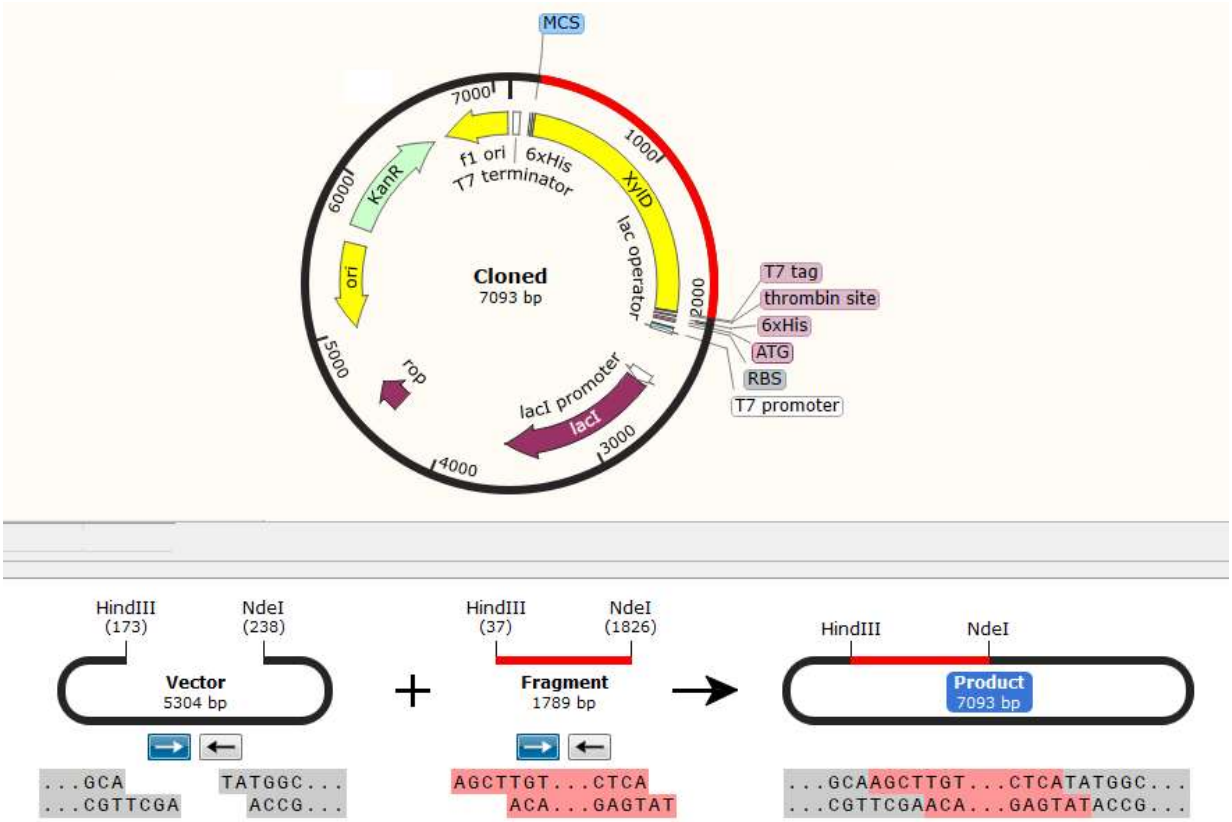

Figure 1. Design of the *xylD* gene cloning route in the pET28 vector

2.3. Protein expression

At this stage, due to the more suitable expression performance of the *E.coli* Rosetta-gami B (DE3) strain compared to the *E. coli* DH5α strain, the extracted recombinant vector was transferred after preparing the competent cell from the *E.coli* Rosetta-gami B (DE3) , and the colonies that appeared on the agar plate containing kanamycin with the recombinant vector were transferred. After inserting the pET28-*xylD* recombinant vector into the *E. coli* Rosetta-gami B (DE3) strain, in order to check the expression of the target protein, the recombinant and negative control strains (without the vector or the vector without the gene fragment) were cultured and when the optical density at 600 nm of cell culture reached around 0.6, the strains were induced under different conditions by adding 1.5 microliters of IPTG with a concentration of 1M (given that the final concentration of IPTG for the purpose of inducing the strains is equal to 0.2 mM, so after calculating the required amount a total of 1.5 microliters of IPTG with 1M concentration was used). After 4 hours of incubation at 37°C on a rotatory shaker (200 rpm), 1ml of cells were harvested by centrifugation. Furthermore, by determining the protein bands on the SDS-PAGE gel, the protein expression in the size of 68KD, was examined. After observing the expression in the recombinant strain compared to the control strain, in order to ensure the increased expression in the recombinant strain compared to the control strain, SDS-PAGE gel was analyzed using Image J software.

3. Results and Discussion

3.1. The cloning of xylD gene into pET28 vector

In this research, in order to amplify the *xylD* gene fragment, polymerase chain reaction was performed by specific primers on the extracted genome of Caulobacter vibrioides. In examining the result, a 1794 bp fragment of the xylonate dehydratase (*xylD*) gene was amplified. Then, pET28 vector was extracted from E.coli DH5α strain. Then the pET28 vector and the xylonate dehydratase (*xylD*) gene fragment were cut using Ndeı and Hindıı enzymes and then put on the gel and extracted .Then, according to the ligation method, the vector and the xylonate dehydratase enzyme gene are connected together, and after preparing the competent cell and also performing the transformation step, the colonies that appear on the agar plate have the recombinant pET28

vector containing the *xylD* gene and the presence of xylonate dehydratase enzyme gene in the recombinant vector (pET28-*xylD*) was confirmed by colony PCR. In order to fully ensure the presence of the gene fragment in the recombinant vector, enzymatic cleavage was performed by restriction enzymes Ndeı and Hindıı. 1794 bp fragment belongs to the *xylD* gene and 5304 bp fragment belongs to the cutted vector (Fig 2).

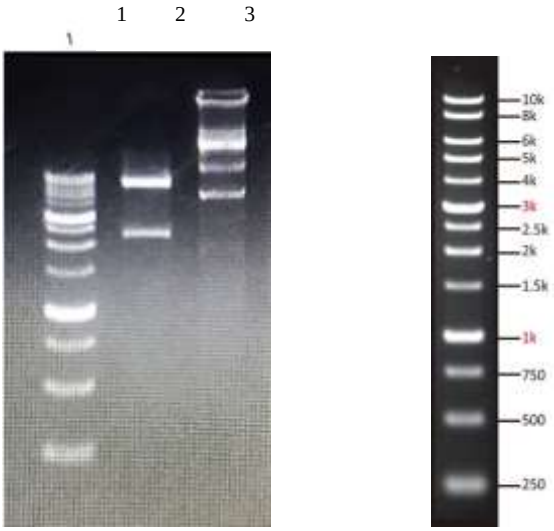

Figure 2. Confirmatory enzymatic cleavage of recombinant pET28-*xylD* vector

1) DNA Ladder 2) Digest of the pET28-*xylD* recombinant vector 3) Uncut vector

3.2 Examination of target protein expression

After confirming the pET28-*xylD* recombinant vector and extracting it, this recombinant vector was transferred to competent cells of *E.coli* Rosetta-gami B (DE3) and the colonies with the recombinant vector were grown on an agar plate containing kanamycin. Then, in order to check the expression of the target protein, after culturing the bacterial strains (recombinant and negative control) in LB medium, and when the optical density (OD) at 600 nm of cell culture reached 0.6, both strains were induced with 1.5 microliters of IPTG with 1M concentration (Fig 3). After observing the expression in the recombinant strain compared to the control strain, in order to ensure the increase in expression in the recombinant strain compared to the control strain, SDS-PAGE gel was analyzed, using Image J software and it was found that in recombinant strain, an increase in expression occurred 54 percent compared to the control strain (Table 2).

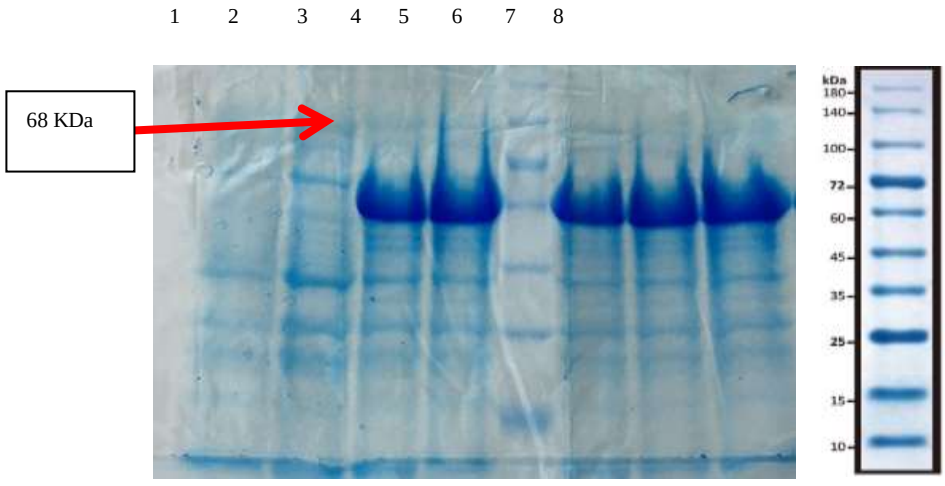

Figure 3. Induction and expression of recombinant strain pET28-*xylD* and control

1)control strain 2) t0 induced with IPTG= 0.2 mM 3,4) t4 of recombinant colony number 1 and 2 induced with 0.2 mM IPTG 5) Protein Marker 6,7,8) t4 of recombinant colony number 3, 4 and 5 induced with 0.2 mM IPTG

Table 2: Image J software graph information

	Area	Percent
1	1531.933	1.062
2	3251.095	2.254
3	4081.903	2.83
4	78331.17	54.303
5	57051.98	39.551

Sun and colleagues stated that YjhG as the main xylonate dehydratase in *E.coli* bacterium is involved in the bioproduction of BT, whose high expression increased BT production by 20% [Sun et al, 2016].

Stephens and colleagues investigated xylonate dehydratases from strains such as *Pseudomonas sp.SHC52*, *Haloferax volcanii* and *Caulobacter crescentus*, which are involved in the oxidative metabolic pathway of xylose. The degree of correspondence between the amino acid sequence of YjhG and xylonate dehydratase was very low (less than 30%). Expression of xylonate dehydratase from CcxylD and PsxylD increased BT production by 53.3% and 24.1%, respectively, compared to YjhG. While with the expression of HvxylD, BT production was not observed. Results show that XylD from *Caulobacter crescentus* is the most suitable xylonate dehydratase among the 4 enzymes mentioned for BT production in *E.coli* [Stephens et al, 2007].

Wang and colleagues considered three candidates for xylonate dehydratase: YjhG and YagF from *E.coli* and XylD from *C.crescentus*, which convert D-xylonic acid to 2-keto3-deoxy D-xylonate. To select the best dehydratase, these three enzymes were expressed and purified, and the results showed that the K_{cat} of YjhG, YagF, and XylD enzymes are 0.446 S^{-1}, 0.093 S^{-1}, and 0.519 S^{-1}, respectively. XylD has the lowest Michaelis constant and also the highest specific constant that shows the efficiency of the enzyme, so XD is more active than the enzymes in *E.coli* [Wang et al, 2017].

Therefore, according to the studies of xylonate dehydratase enzyme, *C.crescentus* strain or *C.vibrioides* NA1000 is known as the most efficient type of this enzyme.

3.3 Discussion

Considering that *E.coli* BL21(DE3) and Rosetta-gami (DE3) strains are suitable hosts for the production of foreign recombinant proteins and with the help of pET28 expression system, which easily shows the expression of all types of recombinant proteins, so this expression system was used in *E.coli* Rosetta-gami (DE3) strain. However, this bacterium for the desired route to BT production, has the gene of xylonate dehydratase, but given that the enzyme is host of its own metabolic enzyme sets and plays the role of converting xylonate to 2-keto 3-deoxyxylonic acid ،Getting this enzyme stuck in a new pathway in the cell, although it can achieve the production of the target product, but it will impose a significant metabolic rate on the cell, because the ultimate goal in establishing the BT production pathway will be a dynamic pathway with significant production. Therefore, it is necessary to consider an independent gene with coordinated expression with other genes of the BT production pathway in the cell. For this reason, it was decided to express this gene independently in the cell. After that, the selection of the suitable strain for gene expression was studied. After inducing the cells and confirming the expression of the cloned fragment in bacteria using SDS-PAGE, the expression rate was estimated to be 54% using Image J software. Because transcription and translation occur simultaneously in the cytoplasm of *E.coli*, when the protein is highly expressed, it may have difficulty in folding. In order to solve this issue, the final concentration of IPTG can be reduced, and the production of this protein can be reduced by lowering the incubation temperature after induction.

4. conclusion

The biological production of D-1, 2, 4 butanetriol from renewable materials has received much attention. For nearly 20 years, different groups in the world have designed microbial and fungal strains to produce BT, which is widely used in industry. Biological production of this product requires four enzymes named xylose dehydrogenase, xylonate dehydratase, 2-keto acid decarboxylase and aldehyde reductase. First, choosing the right strain is an important prerequisite for the successful expression of the recombinant protein and obtaining the target product at a high level. The synthetic pathway of BT in *E.coli* has been successfully established due to its easy cultivation, rapid growth, extensive knowledge of its genetics, and genetic manipulation. In *E. coli* BL21(DE3) and Rosetta-gami (DE3) strains, genes such as yjhH and yagE that catalyze competitive pathways in BT production are absent, so these strains can be a more suitable host for BT production. As mentioned, one of the enzymes of this pathway is xylonate dehydratase, which is found in various microorganisms with various kinetic activities.

Conflict of Interest

The authors declare that they have no conflict of interests.

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