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Recombinant D-tagatose 3-epimerase production and converting fructose into allulose

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Abstract

In this study, D-tagatose 3-epimerase gene was amplified from Escherichia coli JM109 by PCR and re-cloned into *E. coli* JM109 using HincII digested pUC18 cloning vector. Digestion of recombinant plasmid by HincII and PCR amplification of the gene from recombinant plasmid produced 789 bp gene band on agarose gel which indicated the gene integration. The host cell was grown in a shaking incubator at 37°C for 24 hr and then the optical density (OD) of the cells was measured in a spectrophotometer at 600 nm wavelengths. When the OD600 value reached 0.61, the protein expression was induced by the addition of 0.1 mM IPTG into the growth medium. Through His-select gel column purification, highly purified and stable DTE protein was produced. The conversion rate of D-fructose into D-allulose was determined by HPLC. The native and recombinant enzymes could effectively convert D-fructose into D-allulose with a turnover ratio of 20.76%.

Novelty impact statement: A recombinant enzyme, D-tagatose 3-epimerase, was produced and D-fructose was converted into D-allulose using this recombinant enzyme.

1 | INTRODUCTION

Rare sugars are becoming more and more popular around the world. Rare sugars are described as "monosaccharides and their derivatives which are very rare in nature" according to International Society of Rare Sugars (ISRS) (Muniruzzaman et al., 2016). One of these sugars, D-allulose (D-psicose), has been the focus of rare sugars for human beings (Hashii et al., 2015). Although rare sugars are found in very small amounts in nature, they have so many biological functions and great potential of use in the pharmaceutical, pharmacology, cosmetics, food industry, aroma industry, and many other industrial areas (Li et al., 2013; Tang, 2012). Since rare sugars are present in an extremely small amount in nature, it is hard to extract them from natural sources. Therefore, many chemical and enzymatic methods have been developed to synthesize rare sugars from common sugars (Wen et al., 2016). D-allulose, which has a very important place among rare sugars, is a calorie-free sweetener and has the ability to regulate many biological functions such as lowering blood glucose levels, improving insulin resistance, reducing body fat accumulation, and reducing fever (O'Charoen et al., 2014; Yoshihara et al., 2006; Zhang et al., 2016). D-allulose has been used as a healthy sweetener in the food industry as it has an energy of only 0.39 kcal/g and prevents many diseases that affect the quality of life (Hadipernata & Ogawa, 2016; Ushijima, 2014).

Itoh et al. (1994) isolated D-tagatose 3-epimerase enzyme from *Pseudomonas* sp. ST-24 and used this enzyme in allulose production . Using this microorganism, they produced 90 g of allulose from 500 g of D-fructose in 10 days (Li et al., 2013). However, a new method was developed by Takeshita et al. (2000) using immobilized D-tagatose 3-epimerase, which enables serial production of allulose by continuous epimerization of D-fructose, and thanks to this method rare sugar studies have started to make progress. Three enzyme groups are mainly used for the conversion of monosaccharides to each other. Two of these are keto-aldol isomerases (EC 5.3.1) and

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carbohydrate epimerases (EC 5.1.3), which belong to the group of isomerases. The third group is oxidoreductases. All of the enzymes in these three groups are used in rare sugar production, but each has its own advantages and disadvantages. Among these three groups, epimerases are potentially the most useful biocatalysts for the production of rare sugars (Beerens et al., 2012). Allulose synthesis is commonly carried out by the epimerization of D-fructose catalyzed by the D-tagatose 3-epimerase enzyme group (D-TEase, EC 5.1.3.-) at the C-3 position (Figure 1). In addition, allulose production from D-glucose can be achieved by D-xylose isomerase and D-psicose 3-epimerase enzymes together (Li et al., 2015).

The use of natural and inexpensive sugars such as D-fructose and D-glucose as the starting material is advantageous in terms of both mass production of rare sugars and high efficiency of the biochemical method applied (Izumori, 2002).

D-tagatose 3-epimerase (DTE) has been found to catalyze the epimerization of many ketoses in the C-3 position and is a very useful enzyme for rare sugar production. This enzyme is used for the synthesis of various carbohydrates in both single and multiple enzyme reactions, but has also been used to produce all possible ketohexoses on a laboratory scale (Beerens et al., 2012). There is no commercial enzyme preparation to produce D-allulose from fructose. For this reason, D-allulose production is still very limited. There are only three companies in the United States that have received GRAS approval for allulose production. These are Matsutani, SamYang, and CJ CheilJedang (FDA, 2016).

The aim of this study was to clone D-tagatose 3-epimerase (DTE) gene and convert D-fructose into D-allulose using the recombinant enzyme.

2 | MATERIALS AND METHODS

2.1 | Bacterial strains and plasmid

Escherichia coli JM109 (DSM 3423) was used as a genomic DNA source and host cell. Lyophilized *Escherichia coli* JM109 was obtained from a German microorganism and culture collection company DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH. The plasmid (pUC18) was obtained from the Molecular Genetic and Animal Biotechnology Laboratory in Çukurova University, Faculty of Agriculture, Department of Animal Science.

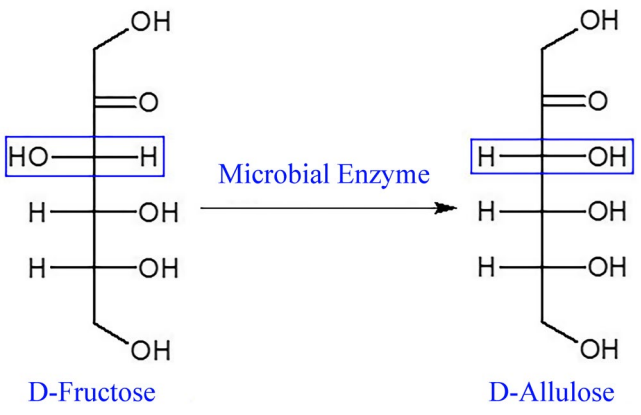


FIGURE 1 D-allulose synthesis from D-fructose

2.2 | PCR amplification and cloning

Genomic DNA was extracted from *E. coli* JM109 by Genomic DNA Purification Kit (Thermo Fisher Scientific, GeneJET Genomic DNA Purification Kit). The DTE coding region was PCR amplified from *E. coli* JM109 using primers (He et al., 2015). ECF (5′-ggcctcgagATGAAAATAGGCACACAGAATC-3′; *Xho* I restriction site underlined; non-DTE sequence in lowercase letters) and ECR (5′-ccggatccTTAGCAGGTACGCAACCAG-3′; *Bam* HI restriction site underlined; non-DTE sequence in lowercase letters).

The PCR reactions were performed using a 50 µl of reaction mixture containing 10× Pfu DNA polymerase buffer with 2 mM $MgCl_2$, 10 mM dNTPs (2.5 mM each), 20 µM primers, 2.5 U Pfu DNA polymerase (to create blunt ended gene product), 50 to 100 ng genomic DNA, and sterile distilled water. The temperature cycles used for amplification include an initial denaturation step for 30 s at 95°C; 35 cycles of denaturation at 94°C for 10 s, annealing for 20 s at 71.2 °C, and extension for 30 s at 72°C followed by a final extension step at 72°C for 10 min in a gradient thermocycler. The PCR amplified products were stored at 4°C until further use.

The blunt ended PCR product DTE gene was cloned into the *Hinc*II (Thermo Fisher Scientific, ER0491) digested pUC18 plasmid, under the control of lac promoter, using T4 DNA ligase (Thermo Fisher Scientific, EL0011).

2.3 | Transformation and expression of the DTE gene

The *E. coli* cells were inoculated in the Erlenmeyer flask containing LB broth and were incubated overnight at 37°C. The overnight grown culture was then centrifuged in Eppendorf tubes and treated with 0.1 M $MgCl_2$ and 0.1 M $CaCl_2$. The competent cells prepared were stored at 4°C. The ligated vector containing DTE insert was transformed into the competent cells by heat shock (42°C, 90 s) treatment. The transformed *E. coli* cells were screened using ampicillin (50 mg/L) and X-gal (40 mg/L) that was added to the LB agar. To confirm whether the DTE gene was successfully inserted into plasmid pUC18, the recombinant plasmid (pUC18-DTE) was digested with both *Xho* I and *Bam* HI. According to the electrophoresis, it could be determined that the DTE gene was inserted. PCR analysis that pUC18-DTE was used as template also produced a single band confirming insertion of the gene into the pUC18. The recombinant plasmid was transfected into *E. coli* JM109. Protein expression was induced by the addition of 0.1 mM isopropyl-beta-D-*thiogalactopyranoside* (IPTG).

2.4 | Purification of DTE protein

The pellets from 2000 ml of culture were resuspended in 100 ml of buffer (50 mM Tris–HCl, 0.15 M NaCl, 20% glycerol, pH 7.5). Then, the suspension was disrupted by sonication on ice and centrifuged at 8,000 rpm for 15 min. The supernatant was further subjected to ultracentrifugation (12,500 rpm, 60 min). The DTE protein was purified by Ni Sepharose 6 Fast Flow (His-GraviTrap 1 ml, GE Healthcare, USA) from the cytosolic fraction. The protein was loaded on a column that had been pre-equilibrated with 10 ml of Buffer P1 (50 mM Tris–HCl, 0.5 M NaCl, 10% glycerol, 0.1 M PMSF, 5 mM imidazole, pH 7.5). After washing four times with 5 ml of Buffer P1 (50 mM Tris–HCl, 0.5 M NaCl, 10% glycerol, 0.1 M PMSF, 5 mM imidazole, pH 7.5) and Buffer P2 (50 mM Tris–HCl, 0.5 M NaCl, 10% glycerol, 0.1 M PMSF, 20 mM imidazole, pH 7.5) successively, DTE was eluted in 1 ml of fractions by 5 ml Buffer P3 (50 mM Tris–HCl, 0.5 M NaCl, 10% glycerol, 0.1 M PMSF, 150 mM imidazole, pH 7.5) and then 5 ml of Buffer P4 (50 mM Tris–HCl, 0.5 M NaCl, 10% glycerol, 0.1 M PMSF, 500 mM imidazole, pH 7.5).

2.5 | Conversion rate of D-fructose into D-allulose

The purified DTE enzyme solution was added to 1%, 5%, and 50% fructose solution at a rate of 5% and incubated at 20°C, 40°C, 60°C, 70°C, and 80°C for 24 hr. At this stage, during the first 8 hr, the samples were analyzed by HPLC (Agilent Technologies, 1,260 series) every 2 hr and at the end of 24 hr, the amount of allulose converted from fructose was determined.

The standard substances belong to fructose and allulose were prepared by diluting with 75:25 Acetonitrile:Water and the retention times were determined by these standard substances.

The applied HPLC conditions were Injection volume (20 µl), detector (Refractive Index Detector (RID), detector temperature (35°C), column (ZORBAX Carbohydrate, 4.6 × 250 mm, 5 micron), column heater temperature (30°C), mobile phase (Acetonitrile:water (75:25 vol/vol)), mobile phase flow rate 1.4 ml/min), analysis time (15 min).

3 | RESULTS

3.1 | Cloning and identification of the *E. coli* DTE gene

The DTE gene was amplified from *E. coli* JM109 genome via PCR and re-cloned into *E. coli* JM109 competent cells. Previously, the nucleotide sequence of D-tagatose 3-epimerase enzyme gene was determined to be 789 bp (He et al., 2015). Agarose gel analysis confirmed that DTE gene cloned in *E. coli* JM109 using pUC18 cloning vector (Figure 2).

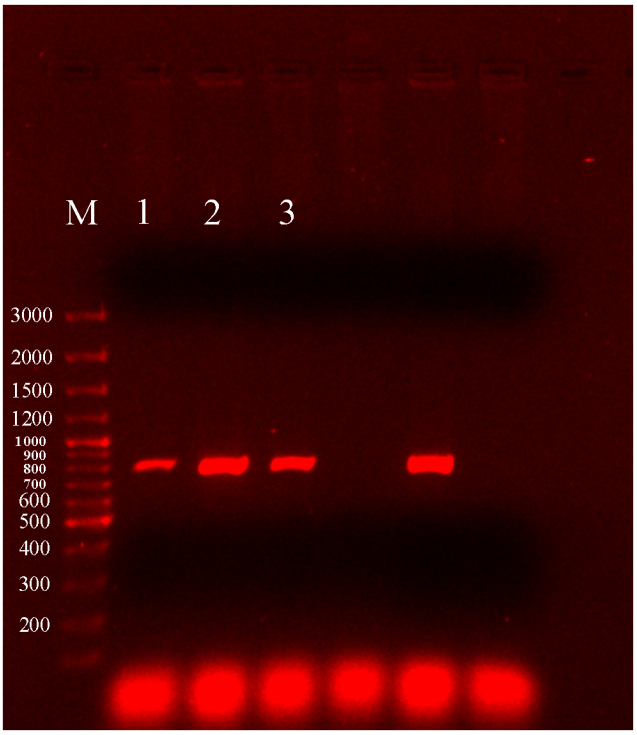


FIGURE 2 Agarose gel image of the DTE gene PCR product

3.2 | Purified of DTE protein

The DTE protein was eluted by 150 mM and then 500 mM imidazole. The eluted fractions were identified by 10% SDS–PAGE. The results showed that the purified DTE protein still contained some higher molecular weight contaminants by the His-select gel column. The eluted fractions (150 mM and 500 mM imidazole) were collected and concentrated. Then the second purification step of fast-protein liquid chromatography (Sephacryl S-200, GE Healthcare, USA) was performed. The results showed that only one peak appeared after the fast-protein liquid chromatography.

3.3 | Conversion rate of D-fructose into D-allulose

As a result of the analysis, the conversion rate of fructose into allulose was found to be 20.76%. The chromatograms and retention times of standard substances of fructose and allulose obtained as a result of HPLC analysis are given in Figure 3, the regression curves of these standard substances are given in Figure 4 and the conversion of fructose to allulose is given in Figure 5.

3.4 | Effects of temperature on DTE enzyme activity

It was determined that DTE enzyme activity was maximum at 60°C and the enzyme activity continued even if it was relatively

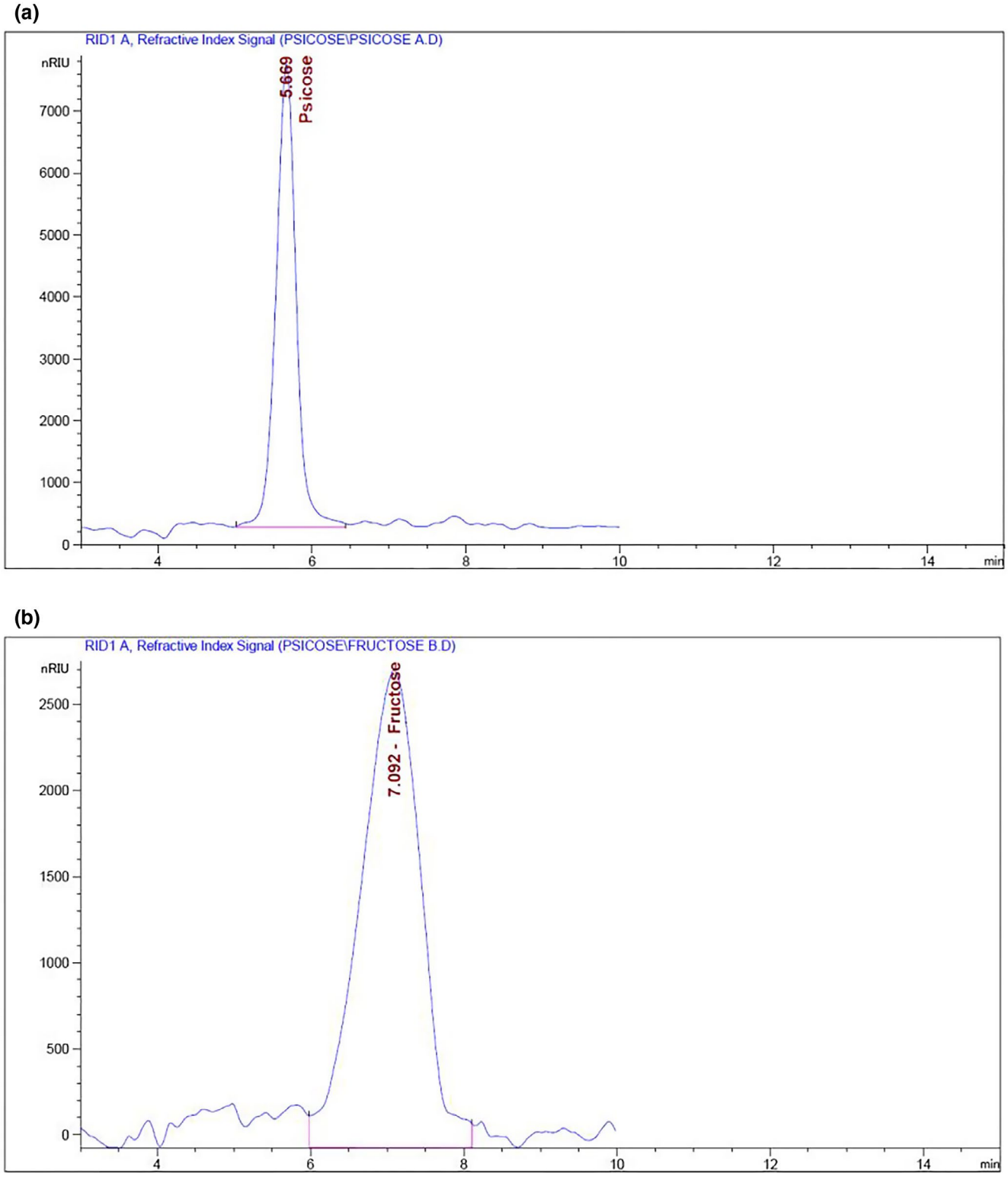


FIGURE 3 The standard substances of fructose and psicose (allulose)

slow at 20–40°C (Figure 6). However, according to the HPLC analysis performed with fructose solutions added the DTE enzyme and kept at 70–80°C, it was observed that the DTE enzyme lost its activity and the color of the reaction mixture turned from white to yellow.

4 | DISCUSSION

In this study, the results showed that D-tagatose-3-epimerase from *E. coli* JM109 was successfully inserted into the pUC18 plasmid and re-cloned into competent *E. coli* JM109 cells. The DTE gene of *E. coli*

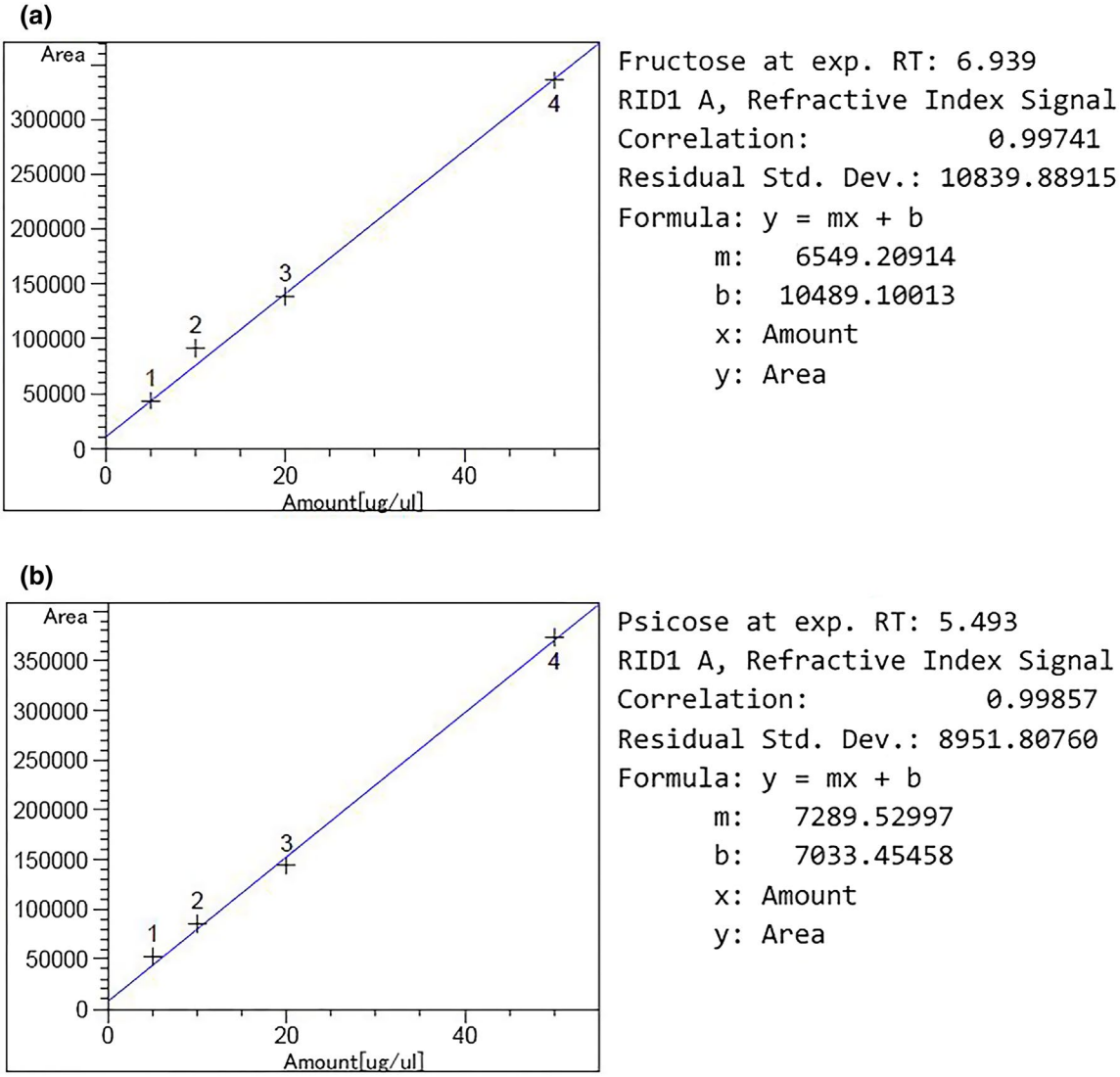


FIGURE 4 The regression curves of the standard substances of fructose and allulose

JM109 was previously cloned and expressed in *E. coli* strains BL21 and Top10, and it has been reported that for the first time, the highly purified DTE protein has been obtained from an *E. coli* strain (He et al., 2015). The amount of enzyme secreted from the native strains quite inadequate for commercial application. Thus, expressing the DTEase enzymes in heterologous organisms is of great significance in enzymatic applications (Jiang et al., 2020). So, in this study, we aimed to produce native and recombinant DTEase enzyme together in *E. coli* JM109 bacteria. *E. coli* JM109 bacterium was decided as a host because it is not pathogenic and suitable for industrial production. *E. coli* expression system also has the advantages of low cost and high expression efficiency. Furthermore, *E. coli* JM109 will be a secondary host as a source of recombinant plasmid in the cloning of the DTE gene to other microorganisms.

Most of DTEase enzymes have been isolated and characterized from certain bacteria including *Agrobacterium tumefaciens* (Kim et al., 2006), *Pseudomonas cichorii* (Itoh et al., 1994), *Rhodobacter sphaeroides* (Zhang et al., 2009), *Clostridium cellulolyticum* (Mu et al., 2011), *Clostridium scindens* (Zhang et al., 2013), *Bacillus subtilis* (Patel et al., 2020), *Arthrobacter globiformis* (Yoshihara et al,. 2017), *Staphylococcus aureus* (Zhu et al., 2019), and *Paenibacillus senegalensis* (Yang et al., 2019).

Recombinant DTEase enzyme also expressed recombinantly in Bacillus subtilis and yeast. It was reported that over-production of DAEase from *Ruminococcus* sp. In recombinant *B. subtilis* could be high efficiency and low cost (Jiang et al., 2020). *A. tumefaciens* DAEase enzyme recombinantly expressed in yeast *Saccharomyces cerevisiae* AN120 via expression vector pRS424-TEFpr-ss-xy/A (Li et al., 2015).

Due to high intake of sugar, especially fructose, is directly related to some diseases such as obesity, insulin resistance, hypertension, and these diseases are known to increase the risk of type 2 diabetes, the interest in the consumption of rare sugars is increasing day by day (Hossain et al., 2011, 2015; Itoh et al., 2015; Kim, Kim, et al., 2017; Kim, Lee, et al., 2017; Oppelt et al., 2017; Thomes et al., 2017). In previous years, D-allulose has been tried to be produced by

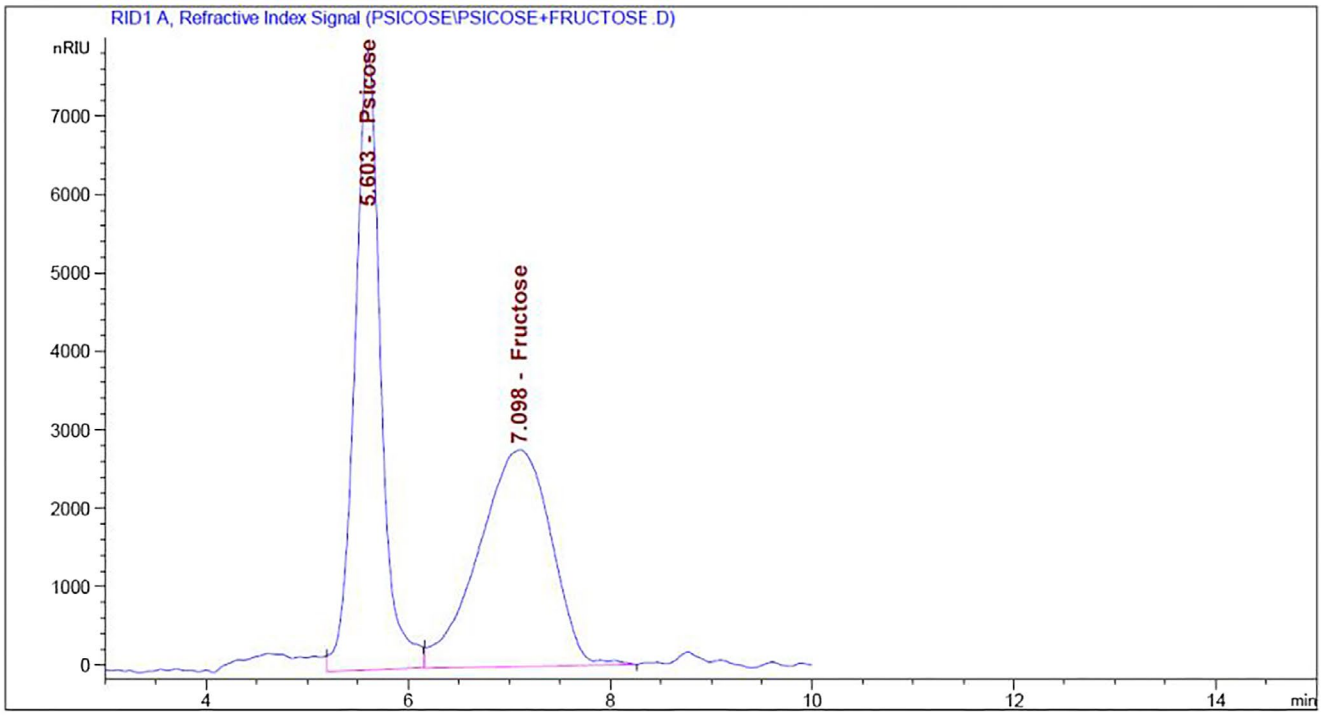


FIGURE 5 HPLC diagram of the conversion of fructose to allulose

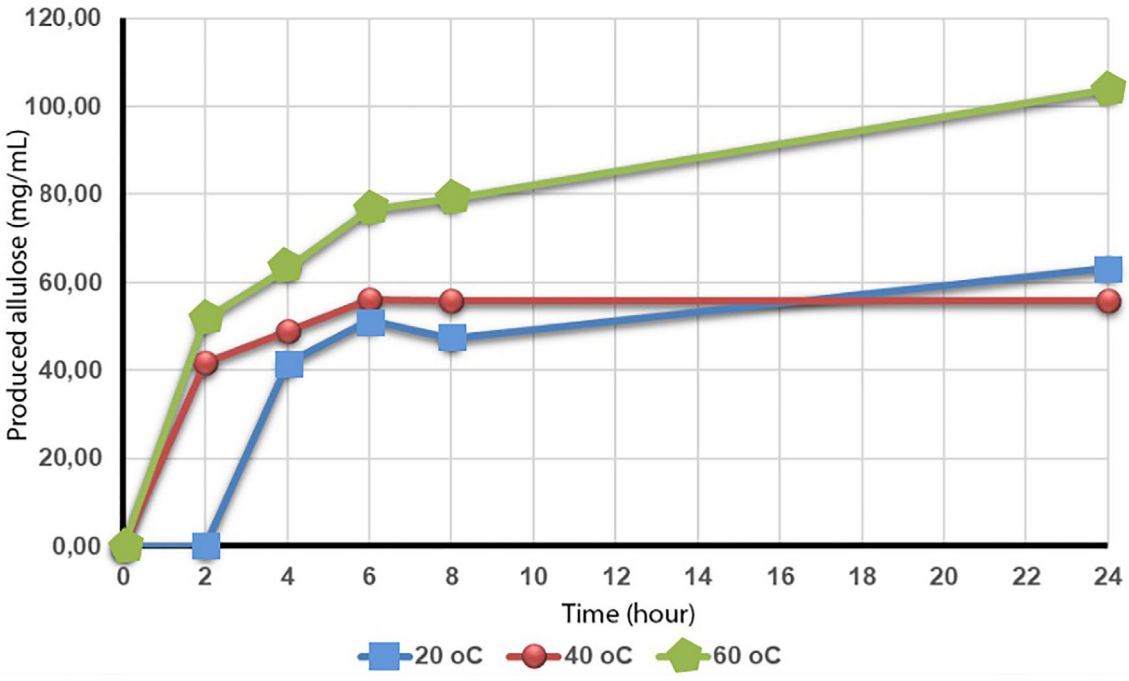


FIGURE 6 DTE enzyme activity at different temperatures

different methods and different raw materials. The efficiency of each method has been analyzed. In one of these methods, chemical synthesis method, D-allulose was produced by the bioconversion of D-fructose through the catalytic effect of molybdate ions in an aqueous and acidic solution (Bilik & Tihlárik, 1973). In another study, D-fructose was converted into D-allulose by boiling in ethanol and triethylamine (Doner, 1979). These chemical methods are inconvenient and expensive. In addition, some waste products come to exist as a result of the process. Due to the use of preservatives in these methods, removal of these preservatives after production, requiring complex and long purification steps chemical methods are not preferred (Takeshita et al., 2000; Wen et al., 2016; Zhang et al., 2016). It

has been reported that D-allulose synthesis is performed more efficiently by the epimerization of D-fructose at C-3 position catalyzed by the D-tagatose 3-epimerase enzyme group (DTEase, EC 5.1.3.-) (Li et al., 2015). In light of all these findings, we prefer enzymatic conversion and fructose as a starting material for D-allulose production. The use of a natural and cheap sugar, D-fructose as a starting material and minimum amount of chemical reagents have provided an economically and environmentally suitable and efficient production.

5 | CONCLUSION

In conclusion, the DTE from *E. coli* JM109 was cloned and expressed in *E. coli*. The optimum conditions for this enzyme including temperature and substrate concentrations were determined. The optimum temperature for DTE enzyme activity was found to be 60°C. The conversion rates of 50% fructose solution kept at this temperature for 2, 4, 6, 8, and 24 hr were 10.32%, 12.66%, 15.32%, 15.78%, and 20.76%, respectively. These results play a significant role in the production of D-allulose by an enzymatic process.

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CONFLICTS OF INTEREST

The authors have declared no conflicts of interest for this article.

AUTHOR CONTRIBUTIONS

Formal analysis; Investigation; Methodology; Resources; Supervision; Writing-original draft; Writing-review & editing: Erva Parıldı. *Conceptualization; Data curation; Project administration; Supervision*: Osman Kola. *Formal analysis; Investigation; Methodology; Resources; Supervision*: Bahri Devrim Özcan. *Conceptualization; Project administration; Software; Validation*: Murat Reis Akkaya. *Formal analysis; Investigation; Methodology; Resources*: Elif Dikkaya.

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