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Article

Kinetic Characterization of β-xylosidase (GbtXyl43A) from Wild-type *Geobacillus thermoleovorans* IT-08 and The Variant GbtXyl43A-D121N

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Abstract:
GbtXyl43A, a β-xylosidase that is isolated from *Geobacillus thermoleovorans* IT-08 and grouped in GH43 family. The substitution of 121Asp residue with Asn in GbtXyl43A caused decrease the enzyme activity. The aim of this study, determine the kinetic characteristics of wild-type GbtXyl43A and D121N variant using V_{max}, K_M, k_{cat}, and k_{cat}/K_M. These parameters indicated catalytic mechanism of GbtXyl43A and its derivative. All of them were produced in *Escherichia coli* BL21 star. The purification of wild-type GbtXyl43A using affinity chromatography, but D121N variant also required anion-exchange chromatography. The specific activity of wild-type GbtXyl43A and D121N variant were 0.471 U mg^{-1} in purity level 55,44 and 0.012 U mg^{-1} in purity level 2,407, respectively. Both enzymes had same molecular weight, ~58 kDa. The kinetic parameters of wild-type GbtXyl43A were K_M: 2.845 mM, k_{cat}: 0.033 s^{-1}, V_{max}: 0.0033 mM $min^{-1}$and k_{cat}/K_M: 0.0115 $s^{-1}mM^{-1}$. Furthermore, the K_M, k_{cat}, V_{max}, and k_{cat}/K_M values of D121N variant were 4.565 mM, 1.01×10^{-4} mM min^{-1}, 0.140×10^{-4} s^{-1}, and 0.0307 $s^{-1}mM^{-1}$, respectively. The K_M value of the D121N variant was higher than its wild type and showed the affinity of D121N variant was lower than GbtXyl43A.

Keywords: *Geobaccilus thermoleovorans* IT-08; β-D-xylosidase; GbtXyl43A; kinetic characteristics

1. Introduction

Xylan is the main component of plant cell walls and one of the most abundant biopolymers on earth. It is composed of the major residues of β-D-xylopyranoside. Three major groups of enzymes involved in xylan degradation are xylanase, α- glucuronidase, and β-xylosidase. β-Xylosidase serves to degrade xylan into free

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xylose [4]. β-D-xylosidase has an activity to hydrolyze xylan, thus β-D-xylosidase widely used by adding to commercial enzyme of hemicellulolytic to increase the xylan hydrolysis [6]. Applying the β-xylosidase enzyme with other glycohydrolases, such as endo-1,4-β-xylanases (EC 3.2.1.8), has increased, especially in biomedical research and industrial processes, such as those for pharmaceutical industries, bio-bleaching and biomechanics, pulping, wine production, baking, fruit, brewing, vegetable processing, and animal feed additives, as well as the production of bioethanol and other bioproducts [5].

Many pulping industries were done at high temperatures (55–70°C) and in the alkaline conditions [6]. Chemical bleaching is generally used to facilitate several processes of pulping; thus, it is potentially toxic for the environment. Hence, alkaliphilic and thermophilic xylanase, which is operationally stable under such conditions, is required. Two types of thermophile β-D-xylosidases have been found from *Geobacillus thermoleovorans* IT-08. These enzymes are grouped into xylanolytic enzyme encoding genes: GbtXyl43A (xyl43A, GenBank No. DQ345777) and GbtXyl43B (xyl43B, GenBank No. DQ387047). Both enzymes exhibit differences in biochemical properties. GbtXyl43B has two modules: the catalytic module (CM), in the form of a five-bladed β-propeller; and the carbohydrate binding module (CBM), of which the kinetic parameters have been studied [12]. In addition, the CBM of GbtXyl43A is not functional; therefore, this enzyme is unable to hydrolyze oat-spelt xylan and has only a CM in the form of a five-bladed β-propeller [6,11,12]. However, the kinetic parameters of wild-type GbtXyl43A and its variants have not been studied. GbtXyl43A is classified into the GH43 enzyme group, which has a catalytic residue of Asp14 as a common base, Glu177 as a common acid, and Asp121 as a pKa modulator that directs the enzyme to the substrate [6,15].

The catalytic reaction of the GH43 enzyme group is based on an anomeric center inversion involving catalytic residues, aspartate, and glutamate, with a single substitution reaction. The effect of single amino acid substitution has been observed in several GH enzymes from *Selenomonas ruminantium* [8], *Bacillus halodurans*

C-125 [15], and *Geobacillus stearothermophilus* [9], revealing changes of enzymatic kinetic parameters. Greater catalytic activities were observed for variant enzymes compare to the wild-type enzyme for the β-D-xylosidase of *S. ruminantium*, at 46%, 71%, and 48%, respectively [8]. A study by Wagschal et al. (2012) on β-D-xylosidase XylBH43 from *B. halodurans* C-125 and the XylBH43-W147G variant demonstrated lowered substrate inhibition for both natural and artificial substrates, whereas the half-saturation constant (K_M) of the substrates increased [15]. Huang reported that for β-xylosidase from *G. stearothermophilus*, substitution of tyrosine 509 into glutamic acid leads to a new exoxylanase activity. The K_M and k_{cat} values of the Y509E variant xylanase was determined using beechwood xylan as a substrate were 5.10 mg/mL and 22.53 s^{-1}, respectively [9].

Kinetic parameters that used were derived from the Michaelis–Menten equation, including the maximum enzyme activity (*V*max) and Michaelis constant (K_M), which has a role to measure the reflecting of the enzyme affinity [1].

The importance of Asp residue as a p*K*a modulator was described by Brux (2006). A similar study was conducted by Hartanti (2016). The substitution of the Asp121 residue from GbtXyl43A with glutamic acid (Glu), asparagine (Asn) and valine (Val) reduced the enzyme activity dramatically. The decrease in activity in the three variants showed a change of conformation due to the mutation. Thus, the substitution of Asp with Glu (D121E), Asn (D121N), and Val (D121V) caused some changes in the characteristics of GbtXyl43A, with respect to its pH and temperature optima [6]. Based on that analysis, it could be predicted that the GbtXyl43A variant had a lower affinity value against the substrate compared with its wild type. Enzymes with good catalytic properties (e.g., a high rate of substrate degradation) and acid–base-tolerance are needed in industrial process to reduce the production cost. Therefore, it is necessary to carry out advanced characterization of GbtXyl43A

by specifying the kinetic parameters of the enzyme, which include V_{max}, K_M, k_{cat}, and k_{cat}/K_M. The current study used the D121N variant because it has a higher pH and activity than those of the D121E and D121V variants.

2. Materials and methods

The materials that used in this study were *Escherichia coli* BL21 containing a wild-type GbtXyl43A gene and the D121N variant, were belong to Proteomic Laboratory, University CoE-Research Center for Bio-Molecule Engineering, Institute of Tropical Diseases, Universitas Airlangga, Surabaya, East Java, Indonesia.

2.1 Expression and production of proteins

The protein of GbtXyl43A was expressed in *E. coli* BL21 (DE3) which contained of pET-xyl plasmid and its purification had established [6]. First, pre-culture was prepared and incubated in 37°C for overnight. This pre-culture was used Luria–Bertani (LB) medium that supplemented with 100 µg/mL of ampicillin and grown by the recombinant plasmid *E. coli* BL21 (DE3)-pET-xyl. It was used 1% for inoculating in 1 L of fresh LB medium contained 100 µg/mL of ampicillin. The production was shaked until OD_{600nm} value between 0.7 and 0.8 (±2.5 h) and induced by 1 mM isopropyl-β-D-thiogalactosidase for 3 h (wild-type GbtXyl43A) and 6 h (GbtXyl43A-D121N). The harvesting of cells was used centrifugation (3,500 rpm) for 30 min at 4°C. The pellet was resuspended by 10 mM phosphate buffer (pH 7.5). The phosphate buffer also contained of 10 mM imidazole and 50 mM NaCl. This solution of cells was sonicated. The supernatants of recombinant protein were taken after centrifugation (10,000 rpm) for 15 min at 4°C.

2.2 Purification of GbtXyl43A

The protein of GbtXYl43A was purified using affinity chromatography while the column containing nickel-nitrilotriacetic acid (Ni-NTA) resin. First, the 1 mL column of Ni-NTA was washed by five column volumes of sterile aquabidest and buffer phosphate pH 8 for equilibration. The buffer phosphate pH 8 that used was

containing of 50 mM phosphate buffer, 250 mM NaCl, and 10 mM imidazole. Next, the supernatant as GbtXyl43A protein was loaded into the column. It was needed incubation at 0°C for 1 h for allowing the binding of protein. The flow through of this loading was collected for 1 h. After that, the resin was eluted using elution buffer. The elution buffer was containing of 50 mM phosphate buffer, 250 mM NaCl, and 30 mM imidazole. Wild-type GbtXyl43A was eluted using increasing imidazole concentration (60, 100, and 300 mM). Moreover, the GbtXyl43A-D121N variant was recovered by the imidazole concentration (10, 15, 20, and 30 mM).

Purification of the GbtXyl43A-D121N variant was continued using anion-exchange chromatography with the type of *DEAE Toyopearl 650 M*. The pre-equilibration was used Tris buffer that contain of 25 mM Tris–HCl buffer of pH 8.0. The fraction of Xyl was applied onto a 1 mL *DEAE Toyopearl 650 M* anion-exchange column. This purification was eluted using linear gradient method of NaCl concentration. The concentration about 0–0.5 M NaCl in Tris buffer. The result of the elution was collected and analyzed using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) by Laemmli method (1970). The concentration of purified protein was also determined using Bradford method (1976).

2.3 Enzymatic activity

The purified enzyme was analyzed about the activity using 2.0 M of *p*NP-X (*p*NP-β-D-xylopyranoside) as a substrate. This analysis was incubated at 50°C. The mixture solution for assay was contained of 450 µL substrate in phosphate buffer and 50 µL enzyme. The enzyme had pre-treatment by incubating at 50°C for 30 min. The analysis was continued by adding 50 µL of 0,4 M Na_2CO_3 and measured at 400 nm. The measurement was calculated in duplicate. the activity of recombinant GbtXyl43A was defined in one unit of activity (IU). This analysis enzyme was required 1 µmol *p*NP per minute under reaction conditions.

2.4 Enzyme kinetic assays

The enzyme kinetic parameters that contained of K_M, V_{max}, k_{cat}, and k_{cat}/K_M were measured by observing the absorbance changes with a UV-Vis spectrophotometer at 405 nm. The hydrolysis reaction was determined by incubating the *p*NP-X substrate with a variety of concentrations (0.5, 2, 4, 6, and 10 mM), as adapted from Wagschal (2009). The substrate was dissolved in phosphate buffer of pH 6. The reaction was initiated by adding an enzyme solution to each substrate, which was incubated at 50°C. Each reaction was observed at 0, 6, 14, 22, 30, 38, 46, and 54 min. The initial velocity (V_0) of each substrate concentration was obtained from the linearity of each curve by plotting the incubation time on an axis and forming the product as an ordinate. Next, K_M, V_{max}, k_{cat}, and k_{cat}/K_M were determined using the Lineweaver–Burk plot.

3. Results and discussion

3.1 Expression and purification of the wild-type GbtXyl43A and the D121N variant

The β-D-xylosidase of wild-type GbtXyl43A and the D121N variant were expressed in *E. coli* BL21 and purified. To elucidate the xylanolytic activity on synthetic substrates, the GbtXyl43A properties were explored. Purified wild-type GbtXyl43A was recovered after an elution of buffer with 100 mM imidazole concentration. A purified D121N variant was obtained using anion-exchange chromatography on four and five fractions. The fractions were analyzed using SDS-PAGE and stained with Coomassie blue. The purified enzyme showed a single band on the electropherogram, with a molecular weight of ~58 kDa (Figure 1).

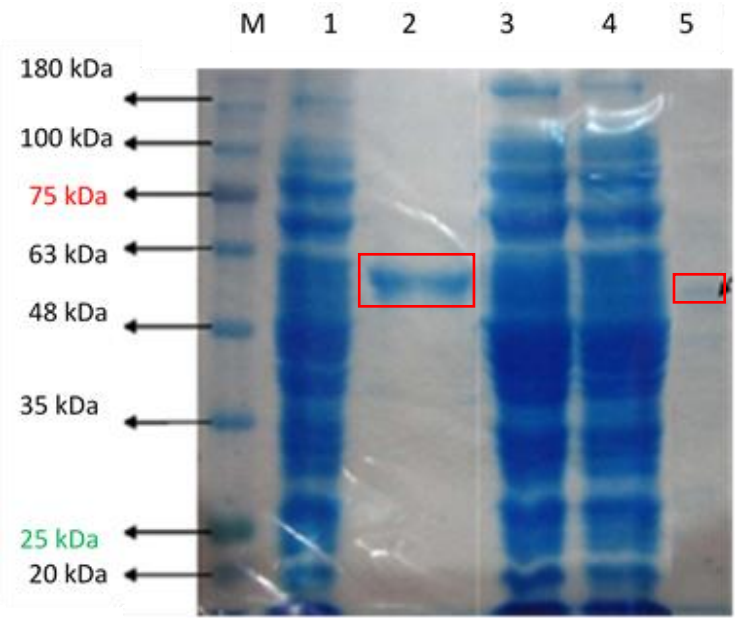

Figure 1. SDS-PAGE analysis of fractions obtained during the purification of GbtXyl43A. Lane M, protein marker; lane 1, crude extract wild type; lane 2, pure wild type; lane 3, crude extract D121N; lane 4, affinity extract D121N; lane 5, pure D121N.

The D121N variant was showed that the expression lower than wild-type GbtXyl43A. Mutations have been known to cause changes in protein folding and stability, protein function, and protein expression. In protein expression, mutations can speed or slow protein synthesis. The lower expression level of the D121N variant was showed that change in its optimum pH of enzyme activity and was significantly higher than the wild-type enzyme [6].

Table 1. Purification table of wild-type (WT) GbtXyl43A and D121N

Sample	Protein (mg mL^{-1})	Activity (mL^{-1})	Specific Activity (mg^{-1})	Fold
WT:				
Crude extract	3.48054	0.02957	0.0085	1
Pure Enzyme	0.39448	0.18584	0.4711	55.44
D121N:				
Crude Extract	5.29751	0.02731	0.00515	1
Pure Enzyme	0.06805	0.00084	0.01241	2.407

3.2 The activity of GbtXyl43A and the D121N variant

The enzyme of β-xylosidase that isolated from *G. thermoleovorans* IT-08 (Xyl) was grouped to the GH43 family. It has a three-dimensional structure that consist of a five-bladed β-propeller fold [13]. β-Xylosidase hydrolyzes the substrate with the use of a mechanism that leads to inversion the structure by the anomeric configuration. It was caused by the displacement of nucleophilic. There are three important residues that have role in catalytic site, they are Glu177, Asp14, and Asp121. The role of Glu177, Asp14, and Asp121 are as general acid, general base, and modulate the p*Ka* of the acid and keeping all of them in the correct orientation toward to the substrate [6,3,13].

In addition, to modulate the p*Ka* of the catalytic acid, the residue had a role to guide the catalytic acid to the substrate. It was shown in the binding of 2-O of the sugar bond and subsite-1. It was influenced in the stabilization by the substrate's transition state. There was a mutation by changing the Asp into Asn (D121N) was decreased the active enzyme than the wild type did [6].

Wild-type GbtXyl43A showed the specific activity of 0.47110 (U mg^{-1}), or 55.44-fold over that of the activity of supernatant, when *p*NP-X was used as a substrate. By contrast, the D121N variant exhibited a specific activity of 0.01241 (U mg^{-1}), or 2.407-fold over that of the activity of its supernatant, when the same substrate was used (Table 1).

The activity of the β-D-xylosidase D121N variant was considerably lower than that of its wild type. The low activity value could have been due to the Asp121 substitution being Asn in the GbtXyl43A-D121N variant. This substitution caused a change in the nature of the enzyme. The GbtXyl43A-D121N variant had an optimal temperature and pH of 90°C and 9, respectively, whereas the optimal temperature and pH of wild-type GbtXyl43A were 50°C and 6, respectively. Asp121 mutation also caused changes in enzyme conformation. Asp121 plays a role as the pKa modulator and also keep it in the correct orientation to the substrate in the

catalytic reaction. Asp substitution with Asn at the catalytic site reduced the enzyme's ability to hydrolyze the substrate.

Asn is a non-charged amino acid. Deep View analysis of the substitution that produce the variant was predicted that it changed into carboxamide function from the carboxylate function and it introduce a new hydrogen bond that showed by the O of Glu177 to HN of Asn121. The new hydrogen bonding had an interaction between these two residues because of the location was closer and also the differences of electronegativity N and O (N<O). It's caused the ability of $N^{\delta 2}$ in Asn121 to donate the proton to $O^{\varepsilon 1}$ in Glu177. Thus, the Glu177 also needs a donation of proton. Besides, the optimal pH of the enzyme was decreased and changed to be alkaline. Otherwise, changing Asp into Asn increased the interaction of hydrophobic that happen on the catalytic area, and thus it also influenced the protein's stability [6].

3.3 Enzyme kinetic assays

The parameters of enzyme activity were obtained from the Michaelis–Menten equation as follows:

$$V_0 = V_{max}\frac{[S]}{K_M+S} \quad (1)$$

It then transforms into a new equation known as the Lineweaver–Burk plot [1].

$$\frac{1}{V_0}=\frac{K_M+[S]}{V_{max}[S]} \quad (2)$$

where V_0 is the velocity in the substrate reaction, *Vmax* is the maximum rate of enzyme activity at a reaction that given temperature, whereas the K_M is the half-saturation constant (Michaelis constant), and $[S]$ is the concentration of the substrate [7].

When the substrate concentration is greater than K_M, the rate of catalysis is equal to k_{cat}. The kinetic constant k_{cat} is the number of substrate molecules that converted into product per unit of time at a single catalytic site when the enzyme is fully saturated with the substrate.

$V_{max} = k_{cat}$ [E]T.....(3)

The ratio of k_{cat}/K_M provides a penetrating probe into enzyme efficiency. To identify the Asp121 as the residue that had an important role to modulate the p*Ka* of the general acid and also guide it in the correct orientation to the substrate, an extensive study was performed by mutating this residue into Asn, Val, and Glu [6]. In this work, we determined the kinetic parameters of the D121N variant by using *p*NP-X as the substrate. We examined the D121N variant because it has the highest activity among the three variants.

The kinetic reaction of the *p*-nitrophenyl-β-D-xylopyranoside substrate by wild-type GbtXyl43A and the D121N variant was performed with a variety of substrate concentrations. An increased rate of product formation occurs with an increase in substrate concentration. In a relatively high substrate concentration, the reaction speed reaches a maximum. Therefore, a continuous monitoring method was applied to ascertain the initial-rate reactions for determining the kinetic parameters of the GbtXyl43A acting on the *p*NP-X substrate.

The kinetic constants for the synthetic substrates were determined using Lineweaver–Burk plots (Figures 2 and 3). The wild-type enzyme exhibited kinetic constants for the hydrolysis of *p*NP-X, of 2.845 mM and 0.0033 mM min^{-1} for K_M and V_{max}, respectively. Furthermore, the D121N variant exhibited kinetic constants for the hydrolysis of *p*NP-X, of 4.565 mM and 0.101×10^{-3} mM min^{-1} for K_M and V_{max}, respectively (Table 2).

The K_M value of the GbtXyl43A-D121N variant was higher compared with its wild type, showing that the affinity of the D121N variant is lower than that of the wild type. The high value of K_M in the D121N variant is due to the amino acid substitution on the catalytic side. The substitution caused an alteration of enzyme conformation, thus decreasing the enzyme affinity for the substrate.

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Table 2. Kinetic parameter analysis for *p*NP-X substrate hydrolysis by wild-type (WT) GbtXyl43A and the D121N variant

Sample	K_M (mM)	V_{max} (mM min^{-1})	kcat (s^{-1})	Kcat/KM (s^{-1} mM^{-1})
WT	2.845	0.0033	0.033	0.6922
D121N	4.565	0.101 x 10^{-4}	0.140 x 10^{-4}	0.037

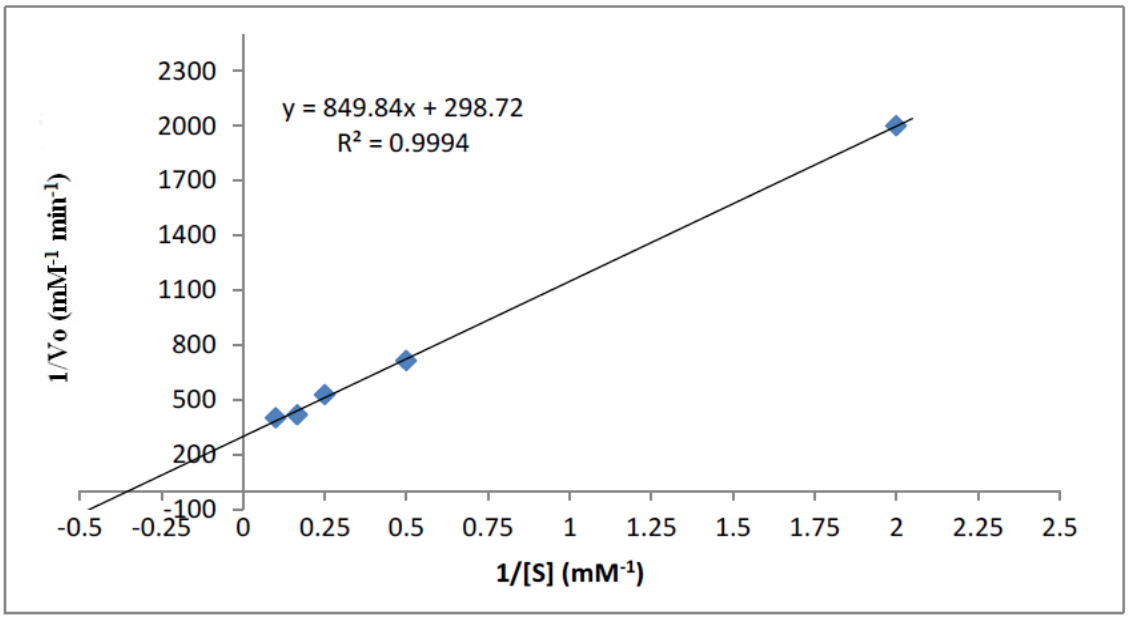

Figure 2. Lineweaver–Burk plot of kinetic parameter determination of the hydrolysis reaction of *p*NP-X catalyzed by wild type GbtXyl43A

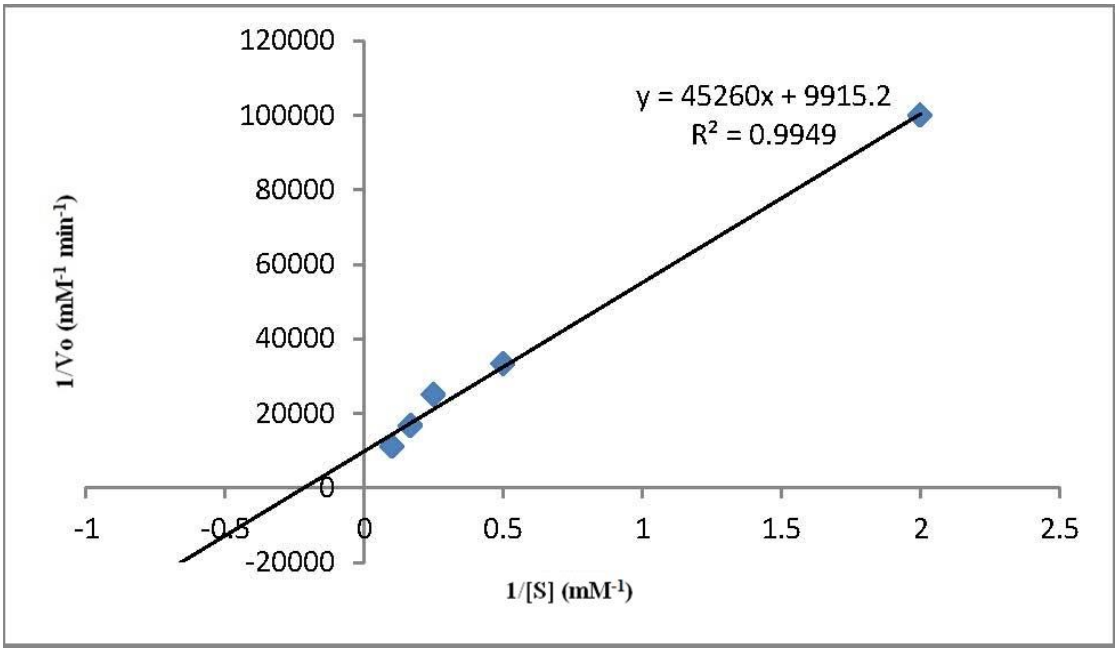

Figure 3. Lineweaver–Burk plot of kinetic parameter determination of the hydrolysis reaction of pNP-X catalyzed by D121N

Asn was thermolabile amino acid; however, the substitution was performed at the important residue at the catalytic site, a decrease in the bonding power of the substrate ensues. The V_{max} value expressed the velocity of the product formation of *p*-nitrophenol. The V_{max} value of the D121N variant was lower than that of its wild type, demonstrating that the speed of enzymatic reactions to produce the *p*-nitrophenol of the variant D121N is slower than that of the wild type.

The k_{cat} values of wild-type GbtXyl43A and the variant D121N were 0.003 s^{-1} (or 1,969 min^{-1}), and 0.140 × 10^{-4} s^{-1} (or 8.416 × 10^{-4} min^{-1}), respectively. Lastly, we determined the catalytic efficiency of the enzyme, expressed as k_{cat}/K_M, which is a measure of substrate specificity. The k_{cat}/K_M value of the wild type and its variants were 0.692 $s^{-1}mM^{-1}$ and 0.0307 $s^{-1}mM$ (Table 2). Lower values of k_{cat} and k_{cat}/K_M were also expected as a result of substitution at the catalytic site. Research by Jordan [7] on the β-SXA enzyme showed that a xylosidase yielded a K_M value of 0.0494 mM, whereas Singh [14] reported a K_M value of XylBH43 at 0.3 mM. The K_M value of the wild type and GbtXyl43A-D121N was higher than that of SXA or XylBH43, indicating that the wild-type and GbtXyl43A-D121N enzyme affinities were lower than those of SXA or XylBH43 against the *p*NP-X substrate.

The wild-type GbtXyl43A k_{cat} values (0.03 s^{-1}) were lower than those of SXA (27.3 s^{-1}) and XylBH43 (17.9 s^{-1}), indicating that the catalytic velocity process of the substrate–enzyme complex to the product on GbtXyl43A is slower than that of SXA or XylBH43. SXA, XylBH43, and wild-type GbtXyl43A had k_{cat}/K_M values of 55.2, 61.7, and 0.692 $s^{-1}mM^{-1}$, respectively. This revealed that the catalytic efficiency of wild-type GbtXyl43A is lower than that of SXA or XylBH43.

The substitution of aspartate (Asp121) with asparagine (Asn121) in β-D-xylosidase GbtXyl43A increased the optimal temperature and pH. These changes were supported by their kinetic properties.

Conclusion

We studied kinetic parameters, such as K_M, V_{max}, k_{cat}, and k_{cat}/K_M, from wild-type GbtXyl43A and the D121N variant on *p*NP-X substrates. The wild-type enzyme exhibits kinetic constants for the hydrolysis of *p*NP-X, of 2.845 mM and 0.0033 mM min^{-1} for K_M and V_{max}, respectively. The k_{cat} value of wild-type GbtXyl43A is 0.033 s^{-1} (or 1,969 min^{-1}), and k_{cat}/K_M is 0.692 $s^{-1}mM^{-1}$. By contrast, the D121N variant exhibits kinetic constants for the hydrolysis of *p*NP-X, of 4.565 mM and 0.101×10^{-3} mM min^{-1} for K_M and V_{max}, respectively. The k_{cat} value of the GbtXyl43A variant is 0.140×10^{-4} s^{-1}, and the k_{cat}/K_M value is 0,0307 $s^{-1}mM^{-1}$. Further study should involve other substrates and analyze possible inhibitors during the catalytic reaction.

Author Contributions: Conceptualization, I.F.J.P., N.N.T.P, S.S. and N.P.; methodology, K.D.A.P., N.N.T.P, I.F.J.P. and S.S.; investigation, I.F.J.P., N.P.; writing—original draft preparation, I.F.J.P.; validation, I.F.J.P., N.N.T.P., S.S., N.P. and K.D.A.P.; writing—review and editing, N.N.T.P.; supervision, N.N.T.P. All authors have read and agreed to the published version of the manuscript.

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