# ASSESSMENT OF ENZYME KINETIC PARAMETERS FOR XYLONIC ACID PRODUCTION

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## ABSTRACT

Equimolar amounts of xylonic acid and sorbitol can be obtained in a reaction catalyzed by the enzymatic complex glucose-fructose oxidoreductase and glucono-δ-lactonase, present in the periplasm of *Zymomonas mobilis* cells. In this study the affinity of the GFOR/GL enzyme complex in relation to the substrates xylose and fructose was evaluated, aiming for the bioproduction of xylonic acid and sorbitol, respectively. In the estimation of the Michaelis Menten parameters 0.085 mol/L for $K_m$ of fructose ($K_F$) and 0.24 mol/L for xylose ($K_X$) were obtained with a maximum velocity ($V_{max}$) of 18 U/g. The values found were compared with different substrates, confirming that the $K_m$ value is unique for each pair of substrates. Glucose is a preferential substrate of GFOR, therefore the data obtained on these operational settings indicate the broad specificity of GFOR in relation to alternative substrates. These results are essential and provide an enzymatic route for the obtention of xylonic acid and sorbitol.

**Keywords:** *Zymomonas mobilis*. Xylonic acid. Kinetic parameters

## 1 INTRODUCTION

*Zymomonas mobilis* is a non-pathogenic bacterium that has aroused technological interest due to its ability to obtain ethanol from the catabolism of sugars such as glucose, sucrose and fructose. In addition to the primary metabolites, the periplasmic enzymes glucose-fructose oxidoreductase (GFOR) and glucono-δ-lactonase (GL) are also obtained[1]. This GFOR/GL enzymatic complex acts on the oxidation of different aldoses to their respective organic acids and the reduction of fructose to sorbitol[2-6].

Xylonic acid is characterized by being a polyhydroxy alcohol, an organic acid that has several hydroxyls in its structure. Xylonic acid has stood out for its different applications, involving the areas of food, pharmaceuticals, construction, and chemistry in general. The production of this compound is most common through chemical synthesis; however, studies have been directed to biotechnological processes [7-10].

Enzyme kinetics is a quantitative description of the influence of experimental variables on reaction rates. The main factors that influence the initial velocity of an enzymatic reaction are substrate concentration, pH and temperature. The simplest and best-known approach for determining the kinetics of enzymatic reactions is the Michaelis-Menten model. The model explains how an enzyme can cause the kinetic rate enhancement of a reaction and describes how the initial velocity depends on the concentration of enzyme and substrate. The linearization by double-reciprocal Lineweaver-Burk method can be used to evaluate experimental results and to determine the values of Michaelis kinetic constant ($K_m$) and maximum velocity ($V_{max}$) kinetic parameters[11].

In this work, the affinity of GFOR/GL enzymatic complex present in free cells of *Z. mobilis* by xylose/fructose as substrates was evaluated considering the assessment of kinetic parameters $K_m$ and $V_{max}$ from Michaelis-Menten equation.

## 2 MATERIAL & METHODS

GFOR was obtained by *Zymomonas mobilis* ATCC 29191. The cultures were maintained in suspension in a liquid medium at 4°C and subcultured monthly to maintain cell viability. The liquid medium used for maintenance, inoculum preparation, cell growth and enzyme production had the following composition (in g/L): glucose, 20 (maintenance), 100 (inoculum), 150 (biomass and enzyme production); $(NH_4)_2SO_4$, 1.0; $MgSO_4 \cdot 7H_2O$, 0.5; $KH_2PO_4$, 1.0; $FeSO_4.7H2O$, 0.01; yeast extract (Prodex Lac®, Prodesa S.A, Brazil), 7.5[3].

In order to prepare the inoculum, the pH of the culture medium was adjusted to 5.5 and maintained with the addition of $CaCO_3$ 5 g/L. The inoculum was carried out in 500 mL anaerobic flasks, with $CO_2$ release filters, with a total volume of 450 mL of culture medium. They were kept under orbital agitation at 200 rpm at 30°C for approximately 10 hours. The cultivation of *Z. mobilis* for the production of enzymes was carried out in a batch mode, in a 5.5-liter bioreactor for approximately 12 hours. The temperature was kept at 30°C, under stirring of 450 rpm, and pH controlled at 5.5 with the addition of 5 mol/L NaOH. The cell mass was centrifuged at 5836 g for 10 min and resuspended in distilled water to a concentration of 50 g/L on dry basis[3,4].

The kinetic parameters for xylose oxidation by GFOR were determined in enzymatic assays. The tests were carried out using a constant fructose concentration of 0.7 mol/L and varying the xylose concentration (between 0.05 and 0.7 mol/L) and keeping the xylose concentration constant at 0.7 mol/L and different concentrations of fructose (between 0.03 and 0.7 mol/L). The tests were carried out in a reactor containing 100 mL of substrate solution and 0.05 g/L of cells/enzymes[12].

The reactor was kept in a thermostatic bath under magnetic stirring. The temperature of 39°C and pH 6.4, conditions defined as standard, were constant throughout the tests. The pH was maintained by adding the 0.1 mol/L NaOH, used to neutralize the organic acid formed[4]. One enzymatic unit of GFOR/GL is defined as the amount of enzyme capable of forming 1mmol of xylonic acid per hour, with activity expressed as a unit per gram of cells on a dry basis (U/g).

The Michaelis-Menten equation was used to predict the reaction velocity as a function of substrate concentration, being demonstrated by a rectangular hyperbolic segment. $K_m$ and $V_{max}$ apparent parameters were estimated for excess substrates, by applying the double-reciprocal plots proposed by Lineaweaver-Burk model (Equation 1) in which $V$ is the initial velocity, $K_m$ is the Michaelis-Menten constant, $V_{max}$ is the maximum velocity, and $[S]$, the substrate concentration. Apparent kinetic parameters were calculated using the Graph Pad Prism 10 program. All the values presented in graphs and tables are the mean values from two replications.

$$\frac{1}{V} = \frac{Km}{Vmax} \cdot \frac{1}{[S]} + \frac{1}{Vmax} \quad (1)$$

## 3 RESULTS & DISCUSSION

Glucose is considered the preferential substrate by the GFOR enzyme, with a $K_G$ value of 0.019 mol/L[12]. However, due to the important applications of xylonic acid, the enzyme kinetics using xylose in the place of glucose was evaluated. In Figure 1, the experimental data obtained in these tests and the adjustments predicted by the Michaelis-Menten equation are shown.

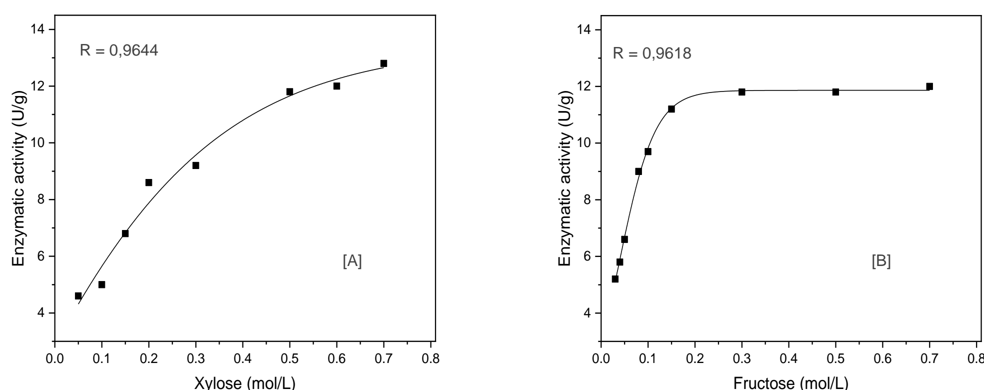


**Figure 1** Variation in enzymatic activity of the GFOR/GL complex, present in permeabilized cells of *Zymomonas mobilis* ATCC 29191, with different concentrations of substrates, at 39°C and pH 6.4. [A] 0.70 mol/L fructose + xylose (0.05 to 0.7 mol/L); [B] xylose 0.70 mol/L + fructose (0.03 to 0.7 mol/L). (•) experimental values; (_) values predicted by the Michaelis-Menten equation.

In tests carried out with 0.7 mol/L fructose, an increment in enzymatic activity was observed according to the increase of xylose concentration (Figure 1A), reaching a value of 12.8 U/g at 0.7 mol/L xylose. When using a constant concentration of 0.7 mol/L xylose, a rise in GFOR/GL activities were observed up to a concentration of 0.3 mol/L of fructose. Between 0.3 and 0.7 mol/L, similar results were achieved, of around 12 U/g (Figure 1B). The kinetics of xylose oxidation by GFOR can be described by a hyperbolic one-substrate Michaelis-Menten equation when the second substrate, fructose, is kept constant.

In Table 1 the results of apparent kinetic parameters $V_{max}$ and $K_m$ obtained in this work from enzymatic tests using xylose and fructose and also by other pair of substrates were compared. These aldose sugars were previously tested as alternative substrates for the oxidizing half-reaction catalyzed by GFOR. In order to evaluate the effect of replacing glucose by xylose and other aldoses on the enzymatic activity of GFOR, the $V_{max}/K_m$ ratio was equally determined and depicted in Table 1.

Considering the results of apparent $K_X$ and $K_F$ of 0.24 mol/L and 0.085 mol/L, respectively, it can be observed that xylose is the occurrence-limiting substrate. The $V_{max}$ value of 18 U/g was estimated, approximately 3.3 times lower than those obtained in previous studies with the glucose/fructose substrates pair[4]. In spite of the results, the difference among the data obtained on these operational settings indicate the versatility of the GFOR/GL enzymatic complex to different substrates.

**Table 1** Apparent kinetic parameters and the $V_{max}/K_m$ ratio for GFOR/GL of *Zymomonas mobilis* and different aldose sugars.

| Kinetic Parameters | Substrates | | | | |
|---|---|---|---|---|---|
| | Xylose/ Fructose* | Lactose/ Fructose[12] | Glucose/ Fructose[12] | Maltose/ Fructose[13] | Galactose/ Fructose[14] |
| $V_{max}$ (U/g) | 18 | 7.7 | 60 | 25 | 7.6 |
| $K_F$ (mol/L) | 0.085 | 0.050 | 0.39 | 0.22 | 0.60 |
| $K_X$ (mol/L) | 0.24 | - | - | - | - |
| $K_L$ (mol/L) | - | 0.39 | - | - | - |
| $K_G$ (mol/L) | - | - | 0.019 | - | - |
| $K_{MA}$ (mol/L) | - | - | - | 0.19 | - |
| $K_{GA}$ (mol/L) | - | - | - | - | 0.60 |
| $V_{max}/K_F$ (U/g/mol/L) | 212 | 154 | 154 | 114 | - |
| $V_{max}/K_x$ (U/g/mol/L) | 75 | - | - | - | - |
| $V_{max}/K_L$ (U/g/mol/L) | - | 20 | - | - | - |
| $V_{max}/K_G$ (U/g/mol/L) | - | - | 3157 | - | - |
| $V_{max}/K_{MA}$ (U/g/mol/L) | - | - | - | 132 | - |
| $V_{max}/K_{GA}$ (U/g/mol/L) | - | - | - | - | 13 |

$V_{máx}$, maximum speed; $K_F$, Michaelis-Menten constant for fructose; $K_X$, Michaelis-Menten constants for xylose; $K_L$, Michaelis-Menten constant for lactose; $K_G$, Michaelis-Menten constant for glucose; $K_{MA}$, Michaelis-Menten constant for maltose; $K_{GA}$, Michaelis-Menten constant for galactose. *data from present work.

In previous studies, carried out under the same conditions presented in this study, the apparent data of Michaelis constant for different aldose sugars were determined and presented in Table 1. The $K_L$ data obtained for lactose was of 0.39 mol/L; $K_G$ of 0.019 mol/L for glucose substrate; $K_{MA}$ of 0.19 mol/L for maltose, and $K_{GA}$ of 0.60 mol/L for galactose [3-4,12-14]. The values of these operational parameters are related to the affinity of the enzyme in relation to substrates, and as expected, the preferential substrates of the GFOR/GL enzyme complex are glucose/fructose. The GFOR enzymatic complex operates in a ping-pong type mechanism and has a single binding site for their preferential substrates[1]. This mechanism is suitable to describe the oxidation and reduction steps of the glucose and fructose substrate pair by the GFOR enzyme. Therefore, according to the substrates evaluated, possible conformational changes in the active site of the enzyme would imply an alteration in the reaction rate.

In view of the values of $V_{max}/K_m$ ratio for different pairs of substrates, it was observed that the catalytic efficiency of the GFOR enzyme is favored using glucose as a substrate ($V_{max}/K_G$ = 3157 U/g/mol/L), being 42 times superior when compared to xylose (Table 1). This preferential behavior was also observed in relation to the other aldose sugars[12-14]. According to the substrates evaluated, the increasing order of GFOR affinity was observed for galactose, lactose, xylose, maltose and glucose, confirming the broad specificity of GFOR in relation to alternative substrates. Taking into account the important results attained for xylose/fructose pair in comparison to the other aldose sugars, the affinity of GFOR by xylose must be considered in studies for the bioproduction of xylonic acid and sorbitol by biotechnological process.

## 4 CONCLUSION

In spite of the relevant affinity of GFOR for xylose and also the requirement for fructose as acceptor substrate, the data obtained in this work provides an enzymatic route for the obtention of xylonic acid and sorbitol derived from xylose and fructose, respectively.

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