EFFECT OF pH AND TEMPERATURE ON XYLONIC ACID FORMATION BY *Zymomonas mobilis*

Caroline R. Corrêa[1*], Vanderson de Lima[1], Ricardo C. Baccin[1], Camila Klein[1], Sabrina Carra[1], Eunice Valduga[2], Jamile Zeni[2], Júlia G. Dick[1], Eloane Malvessi[1]

[1] *Laboratory of Bioprocesses, Institute of Biotechnology, University of Caxias do Sul. Caxias do Sul, RS, Brazil*
[2]*Department of Food Engineering, URI - Erechim, RS, Brazil*
**crcorrea1@ucs.br*

ABSTRACT

Xylonic acid can be obtained by the chemical or enzymatic oxidation of xylose. The enzymatic method has advantages because it is a clean technology, being carried out at milder process conditions. Equimolar amounts of xylonic acid and sorbitol can be obtained in a reaction catalyzed by glucose-fructose oxidoreductase (GFOR) and glucono-δ-lactonase (GL), an enzymatic complex present in the periplasm of *Zymomonas mobilis* cells. In order to provide the best conditions to the enzyme activity, temperature and pH are the main parameters to be controlled. In this context, the catalytic action of GFOR/GL at 34, 37, 39, 43, 47, 50 and 53ºC in pH 6.4, and pH values of 5.8, 6.0, 6.4, 6.8, 7.2 and 7.6 at 39ºC were evaluated. By using 0.7 mol/L of xylose/fructose as substrates and 4 g/L of cell/enzymes suspension, the highest GFOR/GL activities were attained at pH 6.8 to 7.2 and between 47 and 50°C. However, long-term testing is necessary to define the stability of the enzymatic complex of *Z. mobilis* in relation to pH and temperature for the bioconversion process aiming at the production of xylonic acid and sorbitol, compounds that have important applications in chemical and pharmaceutical areas.

Keywords: Xylonic acid. *Zymomonas mobilis*. Periplasmic enzymes.

1 INTRODUCTION

Xylonic acid is characterized by being a polyhydroxy alcohol, an organic acid that has several hydroxyls in its structure[1]. Xylonic acid has a wide range of applications, involving the areas of food, pharmaceuticals, and chemistry in general. The production of this compound is most common through chemical synthesis; however, studies have been focused in biotechnological processes[2,3]. In the fermentation process, the xylonic acid production is performed by using the hemi-cellulosic fraction of vegetal biomass as a substrate[4]. Nevertheless, studies about its attainment by enzymatic route are still scarce.

Zymomonas mobilis is a non-pathogenic bacterium that has aroused technological interest due to its ability to obtain ethanol and carbon dioxide from the catabolism of sugars such as glucose, sucrose and fructose[5]. In addition to the primary metabolites, the periplasmic enzymes glucose-fructose oxidoreductase (GFOR) and glucono-δ-lactonase (GL) are also obtained. This GFOR/GL enzymatic complex acts on the oxidation of different aldoses to their respective organic acids and the reduction of fructose to sorbitol[6]. In previous studies, among these aldoses, GFOR also shows affinity for xylose[7]. Also, a high production level of native organic acids such as lactobionic and maltobionic acids has been achieved by using the enzymatic complex of *Z. mobilis* [8-12].

Considering the promising capability of xylose oxidation, the catalytic action of GFOR/GL in relation to pH and temperature of process were evaluated as an attempt to improve the conversion of xylose and fructose to xylonic acid and sorbitol, respectively.

2 MATERIAL & METHODS

The microorganism used was *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[7].

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 concentrated glucose solution and calcium carbonate were sterilized separately and added to the medium before inoculation. Sterilization of the culture medium and glucose solution occurred in an autoclave at 1 atm for 20 minutes. 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[8,9].

The cultivation of *Z. mobilis* for the production of biomass and 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[12].

The enzymatic assays were carried out aiming at the evaluation of GFOR/GL enzymatic complex activity. The tests were performed using 0.7 mol/L fructose/xylose solution and 4.0 g/L of free *Z. mobilis* cells in 100 mL of reaction volume. The temperature of the jacketed reactor was maintained at the desired value, and the pH was controlled by adding NaOH 1.0 mol/L solution through a pH controller (Provitec 2900, Brazil)[12]. The effects of pH and temperature on GFOR/GL activity were evaluated at 34, 37, 39, 43, 47, 50 and 53ºC at pH 6.4, and then at pH values of 5.8, 6.0, 6.4, 6.8, 7.2, and 7.6 at 39ºC.

Cell concentration was determined by measuring the optical density of cell suspensions from previous *Z. mobilis* cultivation at 560 nm. These values were converted into concentration, mass of dry matter per unit volume, by a calibration curve. Standard GFOR/GL activity was defined by incubation of 4.0 g/L suspension cells in 100 mL of a solution containing 0.7 mol/L each of xylose and fructose at 39ºC for 40 min, with pH controlled at 6.4 (Provitec 2900, Brazil) by using 1mol/L NaOH[9]. One enzyme unit of GFOR/GL (U) was defined as the amount of enzyme that produced 1 mmol of xylonic acid per hour under the assay conditions. Activity was expressed as units per gram of cells, dry basis (U/g).

3 RESULTS & DISCUSSION

The formation of xylonic acid and sorbitol was evaluated in relation to the influence of the pH and temperature of the enzymatic reaction. In the first step, the effect of pH on GFOR/GL activity was evaluated, covering the range from 5.8 to 7.6 values, as observed on Figure 1. Previous works reported the most favorable conditions for catalytic action of GFOR/GL, towards other sugars such as glucose, maltose, galactose and lactose, at pH 6.4[6-8]. As mentioned before, these conditions were adopted as standard for the evaluation of pH and temperature effects on catalysis by using the xylose/fructose pair of substrates.

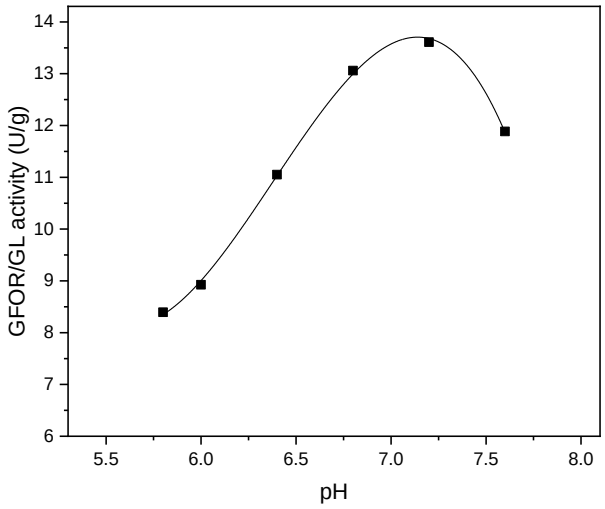

Figure 1 Influence of pH on the enzymatic activity of the GFOR/GL complex present in free cells of *Zymomonas mobilis.*. Reactions were carried out in 0.7 mol L^{-1} equimolar xylose/fructose solution, at 39ºC.

With the use of xylose/fructose substrates, a better catalytic action was observed with an increase in pH between 5.8 and 7.2, as observed in Figure 1. The highest enzymatic activity of 13.6 U/g was achieved under pH 7.2. which is significantly higher than the values normally reported. At a pH value superior to 7.2, it was observed a decline in enzymatic activity. This profile of increase and posterior drop of GFOR/GL activity under a certain range of pH was also reported in a previous pH assessment using other aldoses, such as glucose, maltose, galactose and lactose[9].

In order to evaluate the effect of temperature on GFOR/GL activity, the reactions were carried out between 34 and 53°C, at pH standardized at 6.4. Superior enzymatic activities were observed under an increase of the reaction temperature (Figure 2). Average activities of 15.5 U/g were achieved between 47 and 50°C. However, high temperatures of reaction cannot be immediately assumed as ideal for bioconversions aiming the production of xylonic acid and sorbitol because enzymes can be inactivated in long-term operations. In the case of activity evaluation, the reaction time from 0.5 to 2 hours is necessary, which is considered a short period when compared to bioconversion processes that are carried out during at least 24 hours.

Considering these preliminary but also important results, the best conditions for catalytic action of GFOR/GL using xylose were attained at pH from 6.8 to 7.2 and temperatures between 47 and 50°C. Additionally, tests regarding the evaluation of catalytic thermostability are mandatory for the bioproduction of organic acids. To assess the effects of pH and temperature on the bioconversion process, the standard conditions described in the literature (pH 6.4 and 39ºC) for other organic acids as gluconic, lactobionic and maltobionic acid[10-12] must be compared with those determined as optimal in the present work.

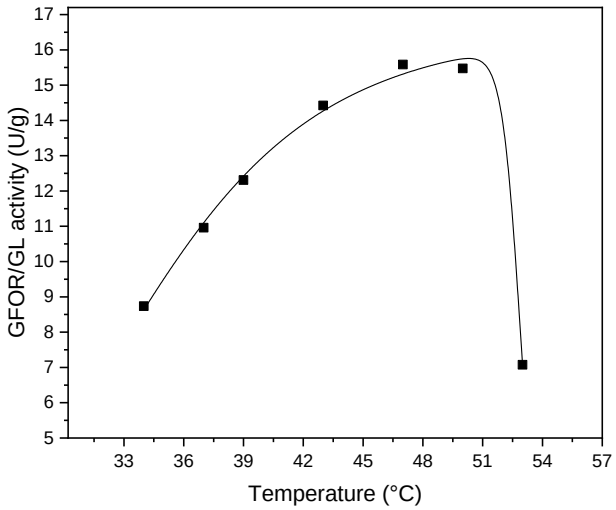

Figure 2 Influence of temperature on the enzymatic activity of the GFOR/GL complex present in free cells of *Zymomonas mobilis*. Reactions were carried out in 0.7 mol L^{-1} equimolar xylose/fructose solution, at pH 6.4.

In summary, the enzymatic activity studies presented here are essential when it comes to defining the operational conditions for the bioproduction of xylonic acid and sorbitol, considering the important applications of these compounds obtained by biotechnological routes, in the chemical and pharmaceutical areas.

4 CONCLUSION

The biotechnological process for xylonic acid and sorbitol production from xylose and fructose, respectively, can become feasible if an efficient and stable enzymatic system is available. By using 0.7 mol/L of xylose/fructose substrates, the highest GFOR/GL activities were attained at pH 6.8 to 7.2 and temperatures between 47 and 50°C. However, long-term testing is necessary to define the stability of enzymatic complex of *Z. mobilis* in relation to pH and temperature for products formation. Thus, designing a process using the GFOR/GL system capable of producing balanced amounts of xylonic acid and sorbitol becomes an important technological alternative.

REFERENCES

1 JOKIC, A., RISTIC, N., JAKSIC, M.M. 1991. J. Appl. Electrochem. 21. 321-326

2 TOIVARI, M. H., NYGÅRD, Y., PENTTILA, M., RUOHONEN, L., WIEBE, M. G. 2012. Appl Microbiol Biotechnol 96. 1-8.

3 MEHTIÖ, T., TOIVARI, M., WIEBE, M. G., HARLIN, A., PENTTILA, M., KOIVULA, A. 2016. Crit. Rev. Biotechnol. 36 (5). 904–916.

4 ZHANG, H. 2017. Bioresour Technol. 224. 573-580.

5 VIIKARI, L. 1986. Crit. Rev. Biotechnol. 7. 237-261.

6 ZACHARIOU, M.; SCOPES, R.K. 1986. J. Bacteriol. 3. 863-869.

7 MALVESSI, E. PhD thesis. 2008. Federal University of Rio Grande do Sul. 235 p.

8 MALVESSI, E., CARRA, S., SILVEIRA, M. M., AYUB, M. A. Z. 2010. Biochem. Eng. J. 54. 1-6.

9 MALVESSI, E., CARRA, S., PASQUALI, F. C., KERN, D. B., SILVEIRA, M. M., AYUB, M. A. Z. 2013. J. Ind. Microbiol. Biotechnol. 40.1-10.

10 DELAGUSTIN, MG. GONÇALVES, E. CARRA, S. 2019. J Pharm Biomed. Anal 174.104-114.

11 FOLLE, A. B.; BASCHERA, V. M.; VIVAN, L. T. 2018. Bioprocess Biosyst. Eng. 41(2). 185-194.

12 CARRA, S., RODRIGUES, D.C., BERALDO, N.M.C. 2020. Bioprocess Biosyst. Eng. 43.1265-1276.

ACKNOWLEDGEMENTS

The authors are grateful to the University of Caxias do Sul, Laboratory of Bioprocess, Post-Grad Program in Biotechnology (PPGBIO), Coordination for the Improvement of Higher Education Personnel (CAPES), National Council for Scientific and Technological Development (CNPq), Foundation for Research Support of the State of Rio Grande do Sul (FAPERGS) for supporting this research.

.