Appl Biochem Biotechnol (2010) 162:1558–1563
DOI 10.1007/s12010-010-8937-9

Contribution of Tris Buffer on Xylitol Enzymatic Production

Ricardo de Freitas Branco · Silvio S. Silva

Received: 9 September 2009 / Accepted: 18 February 2010 /
Published online: 17 April 2010

Abstract Xylitol enzymatic production can be an alternative to chemical and microbial processes, because of advantages like higher conversion efficiency. However, for an adequate conversion, it is necessary to investigate the effect of many parameters, such as buffer initial concentration, pH, temperature, agitation, etc. In this context, the objective of this work was to evaluate xylitol enzymatic production under different Tris buffer initial concentrations in order to determine the best condition for this parameter to begin the reaction. The best results were obtained when Tris buffer initial concentration was 0.22 M, reaching 0.31 g L^{-1} h^{-1} xylitol volumetric productivity with 99% xylose–xylitol conversion efficiency. Although the increase in buffer concentration allowed better pH maintenance, it hindered the catalysis. The results demonstrate that this bioreaction is greatly influenced by involved ions concentrations.

Keywords Enzymatic process · Glucose dehydrogenase · Polyalcohols · Tris buffer · Xylitol · Xylose reductase

Introduction

Xylitol is a polyalcohol which has real applications in the odontological and food industries and interesting properties for pharmaceutical and cosmetic products. It is an alternative sweetener with anti- and noncariogenic characteristics [1, 2]; indeed, it is the sweetest among the polyalcohols [3] with similar sweetener strength as sucrose [4]. Additionally, xylitol does not require insulin for its metabolism [5–7]; hence, it can be consumed by diabetic. The largest application of xylitol is currently in oral products such as toothpaste, gum, mouthwash, and nasal spray among others. This compound is elaborated through a chemical reaction, which requires operational conditions of high temperature and pressure as well as extensive stages of upstream and downstream processing [8–10]. These facts motivated researchers to investigate new alternatives for xylitol production, aiming a

R. F. Branco · S. S. Silva
Grupo de Microbiologia Aplicada e Bioprocessos, Departamento de Biotecnologia,
Universidade de São Paulo—Escola de Engenharia de Lorena, Lorena, São Paulo, Brazil

R. F. Branco (✉)
Estrada Municipal do Campinho, P.O. Box 116, Lorena, São Paulo, Brazil
e-mail: ricardo@debiq.eel.usp.br

reduction of the production costs. A promising option is the microbiological conversion of xylose to xylitol, a process in which mild operational conditions are used and the stages of upstream and downstream are simplified [11, 12]. Despite the intensive research in the last decades, there are some limitations that have not yet been surpassed in the fermentative process such as low xylose uptake, consequently low volumetric productivity, and xylose misuse. The advance of biotechnology made possible a second alternative to produce xylitol, the enzymatic or in vitro path. So the main disadvantages of the traditional processes are surpassed. In this process, xylose is converted into xylitol by the enzyme xylose reductase (E.C. 1.1.1.21) from *Candida guilliermondii* FTI 20037 using the coenzyme nicotinamide adenine dinucleotide phosphate (NADP) in its reduced form (NADPH). In addition, this reaction is assisted by a coupled enzymatic conversion in order to regenerate NADPH using the glucose dehydrogenase (E.C. 1.1.1.119) system. A simple scheme of the process is presented in Fig. 1. It must be mentioned that no literature was found in the last 10 years about this xylitol enzymatic process. This fact emphasized the necessity to investigate this alternative. It is known that ions, and consequently buffers, have great influence on enzymes behavior, in which the effect can be positive or negative depending on the ion, its concentration, and of course, the enzyme. In this context, the present work had as objective to evaluate the xylitol enzymatic production under different Tris buffer initial concentrations aiming to determinate an appropriate concentration to start the reaction. Tris buffer was the only buffer studied because previous experiments with different buffers showed that xylose reductase is negatively influenced by inorganic cations, like Ca^{+2} and Na^{+}. This was also observed by Nidetzky et al. 1996 [13] for xylose reductase from *Candida tenuis*. For this purpose, batch assays for xylitol obtainment were carried out in an enzymatic reactor containing xylose reductase, xylose, NADPH, and an in situ NADPH enzymatic regeneration system composed of glucose dehydrogenase and glucose.

Materials and Methods

Pre-purified Xylose Reductase Extract Preparation

A pre-purification is required in order to separate xylose reductase from xylitol dehydrogenase to avoid xylitol loss by its transformation in xylulose by this last enzyme. The pre-purified xylose reductase extract used in the reactions was prepared in three steps: *C. guilliermondii* FTI 20037 cultivation, cell disruption, and reverse micelle technique. The first step, cell cultivation, was performed in a batch system in a bioreactor BIOFLO III of 1.25 L (New Brunswick Scientific Co. Inc., Edison, New Jersey, USA). The fermentation media total volume was 1.0 L, containing 50 g L^{-1} xylose, 3 g L^{-1}

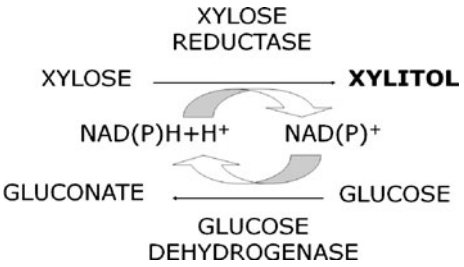

Fig. 1 Xylitol enzymatic production with a coupling coenzyme enzymatic regeneration system

ammonium sulfate, 10% $v\ v^{-1}$ rice bran extract, 0.1 g L^{-1} calcium chloride, and 0.1% $v\ v^{-1}$ antifoaming (silicone base). The pH, dissolved oxygen, and temperature were kept at 5.50, 50%, and 30°C, respectively. Initial concentration of *C. guilliermondii* FTI 20037 was 1.0 $g_{dryweight}\ L^{-1}$. The cells were previously revived in Erlenmeyer flasks using a rotary movement shaker at 200 rpm and 30°C. Revive media consisted of the same compounds as described above but using a xylose concentration of 30 g L^{-1}. The crude xylose reductase extract was obtained by the microbial cells disruption under vortex agitation using glass pearls (0.5-mm diameter). The used conditions for the disruption were previously determined by Gurpilhares et al. [14]. Finally, the pre-purified xylose reductase extract was produced by reverse micelle technique using Cetyl trimethylammonium bromide (CTAB)-reversed-micelles in isooctane, hexanol, and butanol by a two-step procedure [15]. Firstly, 3.0 ml of the crude extract was mixed with an equal volume of micellar microemulsion (CTAB in isooctane/hexanol/butanol/water). This mixture was agitated on a vortex for 1 min to obtain the equilibrium phase and separated into two phases by centrifugation at 6,570×*g* for 10 min (Jouan Centrifuge model 1812, Saint-Herblain, France). Afterwards, 2.0 mL of CTAB-micellar phase was mixed with 2.0 mL of fresh aqueous phase (acetate buffer 1.0 M at pH 5.5 with 1.0 M NaCl), in order to transfer xylose reductase from the micelles to this fresh aqueous which was finally collected by centrifugation (6,570×*g*; 10 min).

Enzymes Assays

Xylose reductase activity was determined by spectrophotometric analysis using NADPH as the detecting parameter at 25°C and 340 nm in a medium composed of 350 μL Tris buffer (70 mM, pH 7.2), 50 μL NADPH (1.2 mM), 50 μL xylose (2.0 M), and 150 μL of the enzymatic extract. The glucose dehydrogenase activity was also determined by spectrophotometric analysis using the same conditions. The medium was composed of 350 μL Tris buffer (70 mM, pH 7.2), 50 μL NADP (1.2 mM), 50 μL glucose (1.5 M), and 150 μL of the enzymatic extract. The absorbance variation at 340 nm of the assay against a blank without enzyme was monitored for 1 min. The activity was calculated from the slope of the absorbance versus the time curve by using the apparent molar extinction coefficient of 6.22 $mmol^{-1}\ cm^{-1}$ for NAD(P)H. One xylose reductase or glucose dehydrogenase unit (U) was defined as the amount of enzyme catalyzing the formation of 1 μmol of NADP or NADPH per minute at the assay conditions. The volumetric activity was expressed as unit per milliliter (extract volume).

Enzymatic Reactions

The enzymatic reactions were carried out in an adapted module BIOFLO III (New Brunswick Scientific Co. Inc., Edison, New Jersey, USA). In all assays, the temperature and pH were kept at 25°C and 7.0, respectively. The xylose, glucose, and NADPH were 11 g L^{-1}, 15 g L^{-1}, and 0.2 mM, respectively. The xylose reductase and glucose dehydrogenase volumetric activities were 0.2 U mL^{-1}. The xylitol enzymatic production was evaluated under 0.07, 0.22, 0.50, and 1.00 M Tris buffer initial concentrations. Assays were done in triplicate.

Quantification of Xylose and Xylitol

Xylose and xylitol concentrations were determined by high performance liquid chromatography using Waters equipment (Waters, Milford, MA, USA) with a refractive index

detector (model 2414) and a HPX-87H column (Bio-Rad, Hercules, CA, USA). The operational conditions were temperature of 45°C, 0.005 M sulfuric acid as eluent, flow of 0.6 mL min^{-1}, and injection volume of 20 μL.

Results and Discussion

Since the beginning of enzymology, studies have observed that free ions influenced enzymes activity. Most recently, the application of enzymes in bioproduction showed how important it is to know the most appropriate buffer and its concentration for a successful catalysis [13]. Therefore, batch bioreactions under different Tris buffer initial concentration were carried out to study its influence on xylitol enzymatic process. Four different concentrations of Tris buffer were tested (0.07, 0.22, 0.50, and 1.0 M). Experiments were performed in triplicate and only the arithmetic means of the results are presented. The concentration of xylose and xylitol during the assays are shown in Figs. 2 and 3 and presents the pH value as a function of the reaction time. Figure 2 shows that xylitol is formed in all experiments, however, at different rates. In fact, a decay can be observed at xylitol formation rate after 12 h for all the experiments, probably due to the decrease of available NADPH. The volumetric productivity varied between 0.08 and 0.31 g L^{-1} h^{-1} after 12 h. In all experiments, the efficiency values during the entire reaction course were near the stoichiometric, demonstrating that there were no side reactions between the main catalysis and the coenzyme regeneration system. The pH value decreased in all experiments as shown in Fig. 3 because of gluconic acid production from the coenzyme regeneration system. Further, Fig. 3 shows that an increase in the buffer concentration resulted in a better maintenance of the pH value (around 7.0), which would be the adequate for the process, since (1) it is the optimum pH for xylose reductase and (2) due to pH maintenance in that optimum. However, the results indicated that for this condition, almost no xylitol was formed as seen in Fig. 2. This fact can be explained by the excess of ions which can interact with the enzyme(s) polar parts, changing its form, hindering the conversion, or even inactivating the enzyme(s). A high ionic force can also reduce the available water and

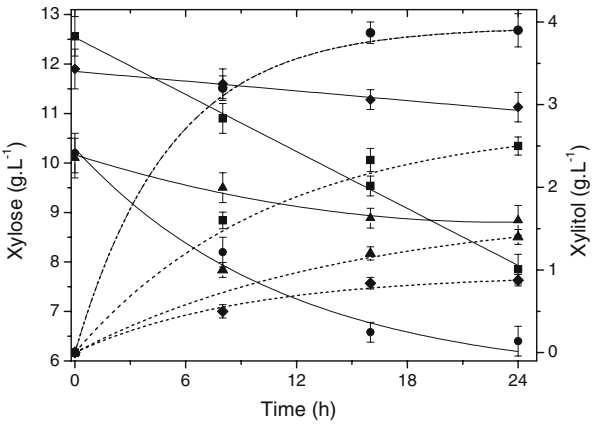

Fig. 2 Xylose (*black*) and xylitol (*dash*) concentrations as a function of the reaction time for the different used Tris buffer initial concentration, (*filled square*) 0.07, (*filled circle*) 0.22, (*filled triangle*) 0.50, and (*filled diamond*) 1.0 M. Error bars for each experimental data are also shown

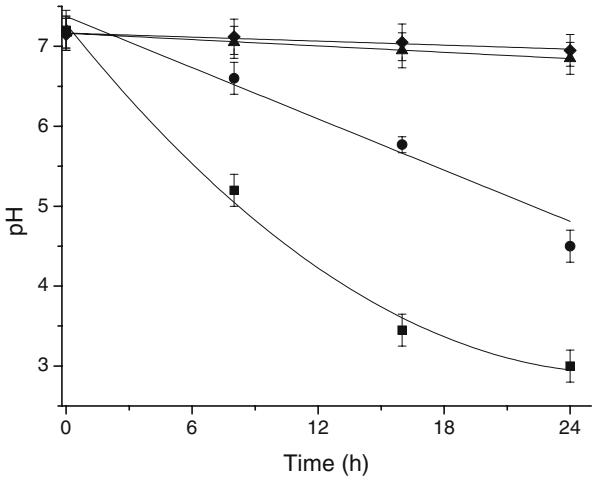

Fig. 3 pH as a function of the reaction time for the different used Tris buffer initial concentration, (*filled square*) 0.07, (*filled circle*) 0.22, (*filled triangle*) 0.50, and (*filled diamond*) 1.0 M. Error bars for each experimental data are also shown

consequently affect enzyme(s) solubility, therefore, compromising the biocatalysis. The best buffer concentration found was 0.22 M. At this condition, 3.7 g L^{-1} xylitol were produced after 12 h of reaction, which corresponded to a 0.31 g L^{-1} h^{-1} xylitol volumetric productivity. This result was six times higher than the experiment using 1.0 M Tris buffer. This was the best experimental condition, probably due to an appropriate ion concentration, which made possible the control of pH in an adequate region (5.0–7.2) for longer and did not interfere in the enzyme(s) activity. According to the literature, the chemical and microbial processes have an average efficiency of 55% [8, 13] and 70–80% [16], respectively. In the enzymatic way, it was obtained in 99–102%. By comparing the volumetric productivity, the best result showed in this work is still lower than that of microbial process, which can reach values of 1.50 g L^{-1} h^{-1} [17, 18] using batch fermentations in synthetic media. However, it must be considered that the results presented in this work are merely preliminary and can still be greatly improved.

Conclusions

The results presented in this work showed the importance of the buffer concentration in an enzymatic reaction. It was obvious that xylitol enzymatic conversion is highly dependent on the Tris buffer concentration. The best volumetric productivity result was observed when the initial Tris buffer concentration was 0.22 M. An increase in the concentration from this point hindered the biocatalysis. Finally, a comparison between the results observed in this work and in the literature demonstrated the potential of the xylitol enzymatic conversion as a new alternative for the conventional production.

Acknowledgements The authors gratefully acknowledge the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) (grant nº 2005/02840-0 and 2005/02866-9), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) for the financial support. Also, the authors gratefully acknowledge Lilian C. M. Robin for the English text review.

References

1. Alanen, P., Isokangas, P., & Gutmann, K. (2000). *Community Dentistry and Oral Epidemiology, 28*, 218–224.
2. Massoth, M. (2006). *Dental Abstracts, 51*, 366–367.
3. Gliemmo, M. F., Calvin, A. M., Tamasib, O., Gerschensona, L. N., & Camposa, C. A. (2008). *LWT—Food Science Technology, 41*, 611–619.
4. Parajó, J. C., Domínguez, H., & Domingínguez, J. M. (1998). *Bioresource Technology, 65*, 191–201.
5. Forster, H., Boecker, S., & Walther, A. (1977). *Fortschritte der Medicin, 95*, 99–102.
6. Nigam, P., & Singh, D. (1995). *Process Biochemistry, 30*, 117–124.
7. Grillaud, M., Bandon, D., Nancy, J., Delbos, Y., & Vaysse, F. (2005). *Archives of Pediatrics, 12*, 1180–1186.
8. Jaffe, G. M., Szkrybalo, W., & Weinert, P. H. (1974). US Patent 3.784.408.
9. Silva, S. S., Ribeiro, J. D., Felipe, M. G. A., & Vitolo, M. (1997). *Applied Biochemistry and Biotechnology, 63*, 557–563.
10. Winkelhausen, E., & Kusmanova, S. (1998). *Journal of Fermentation and Bioengineering, 86*, 1–14.
11. Santos, J. C., Mussatto, S. I., Dragone, G., Converti, A., & Silva, S. S. (2005). *Biochemical Engineering Journal, 23*, 1–9.
12. Branco, R. F., Santos, J. C., Murakami, L. Y., Mussatto, S. I., Dragone, G., & Silva, S. S. (2007). *Process Biochemistry, 42*, 258–262.
13. Nidetzky, B., Neuhauser, W., Haltrich, D., & Kulbe, K. D. (1996). *Biotechnology and Bioengineering, 52*, 387–396.
14. Gurpilhares, D. B., Pessoa, A., Jr., & Roberto, I. C. (2006). *Process Biochemistry, 41*, 631–637.
15. Cortez, E. V., Pessoa, A., Jr., Felipe, M. G. A., Roberto, I. C., & Vitolo, M. J. (2004). *Chromatography B, 807*, 47–54.
16. Silva, S. S., & Afschar, A. S. (1994). *Bioprocess Engineering, 11*, 129–134.
17. Branco, R. F., Santos, J. C., Pessoa, A., Jr., & Silva, S. S. (2009). *Journal of Chemical Technology and Biotechnology, 84*, 326–330.
18. Sampaio, F. C., Faveri, D., Mantovani, H. C., Lopes, F. M. P., Perego, P., & Converti, A. (2006). *Journal of Food Engineering, 76*, 376–386.