ISSN 2348-6201
Volume 5 Number 3
Journal of Advances in Biotechnology

EVALUATION OF CANDIDA GUILLIERMONDII CELL GROWTH AND ONE-POT FERMENTATION FOR XYLITOL BIOPRODUCTION

Luiz Felipe A. Modesto, Manuela Temtemples, Peter Rudolf Seidl & Nei Pereira Jr.
Laboratories of Bioprocess Development, School of Chemistry, Federal University of Rio de Janeiro, Brazil, 21949-900
luiz.felipe@eq.ufrj.br
nei@eq.ufrj.br

ABSTRACT

Xylitol is a polyalcohol with a high sweetener power that presents healthy benefits when compared to sucrose, such as prevention of caries and metabolism only partially dependent on insulin. Its bioproduction, besides being within the context of Biorefiney and Green Chemistry, presents advantages when compared to the traditional chemical process. This study analyzed *Candida guilliermondii* IM/UFRJ 50088 cells growth for three different operational strategies, two of them conducted on shake flasks and the other one held on instrumented bioreactor. The latter provided the best results, achieving a maximum cell productivity ($Q_{x\ max}$) of 0.61 g/L.h while reaching cell concentrations higher than 20 g/L. For the one-pot fermentation assay, despite low xylitol yield ($Y_{P/S}$≈0,1g/g) obtained, which shows that further research is necessary, the propagation steps were successful.

Indexing terms/Keywords

Xylitol; cell growth; bioprocess; *Candida guilliermondii*.

Academic Discipline And Sub-Disciplines

Science, Biotechnology

SUBJECT CLASSIFICATION

Biotechnology, Bioprocess Engineering

TYPE (METHOD/APPROACH)

Cell Growth, Fermentation, Bioproduction, Bioprocess Engineering

INTRODUCTION

Xylitol is a five-carbon polyalcohol naturally found in some fruits and vegetables. It is also an intermediate of the human metabolism, though in small quantities, which makes its extraction not cost-efficient [1,2]. Xylitol is usually regarded because of its high sweetener power – similar to the one of sucrose, but with less calories - , reason why it is commonly used in food industry. Besides, it presents some health benefits, such as anticariogenic properties and low glycemic rates, being referred as “glucose with delay” [3,4]. More recently, due to increasing interest on the concepts of Green Chemistry and Biorefinery, xylitol has received more attention. In 2004, the Department of Energy of the United States identified building blocks, fundamental substances for establishing an economy based on renewable sources and this polyalcohol was amongst them [5]. In 2010, the list of these important compounds was reviewed and xylitol had, once again, its relevance confirmed [6].

Industrially, xylitol is produced under a catalytic process at high temperature and pressure. Since non-specific catalysts are employed, some byproducts are formed and downstream steps become costly.

Several species of microorganisms have been evaluated for the bioproduction of xylitol, especially yeasts from the genus Candida [4]. On this case, the biochemical pathway comprehends two main steps and xylitol is formed as an intermediate of the D-xylose metabolism. On the first stage, D-xylose is reduced to xylitol by a NADPH-dependent xylose reductase, while on the second one occurs the oxidation of xylitol to D-xylulose by NAD^+-dependent xylitol dehydrogenase. Once D-xylulose is converted to D-xylulose 5-phosphate, it can be catabolized whether by pentose-phosphate pathway, Embden-Meyerhof-Parnas or phosphoketolase routes. In order to accumulate xylitol, aeration plays an important role on the imbalance of the NAD^+/NADH redox system. An oxygen limited condition is, therefore, desired to favour the formation of xylitol. On the other hand, for cell growth, an excess of oxygen is required [7].

In this work, three different strategies for the propagation of *C. guilliermondii* IM/UFRJ 50088 cells - two of them in shake flasks and the other one in instrumented bioreactor - were evaluated. Obtaining high cell densities is important to avoid inhibition problems related to fermentation using hemicellulosic hydrolyzates [8]. Besides, a one-pot fermentation, comprising both propagation and fermentation steps consecutively on the same bioreactor, was performed.

MATERIALS AND METHODS

Microorganisms, Inoculum and Media

Candida guilliermondii IM/UFRJ 50088 cells were maintained on agar plates containing 5 g/L of D-xylose, 5 g/L of peptone and 2 g/L of yeast extract and stored at 4°C. Three loopfulls of these stock cultures were then transferred to 500 mL

Erlenmeyer flasks with 200 mL of activation media containing 10 g/L of D-xylose, 1,25 g/L of urea, 1,10 g/L of KH_2PO_4, 2 g/L of yeast extract and 40 mL/L of mineral salt solution [8]. The inoculated flasks were kept on rotary shakers (New Brunswick Scientific Innova 44) at 200 rpm and 30 °C. After 30 hours of activation, propagation media - whether in Erlenmeyers flasks or in bioreactors and with the same composition as the activation ones - were inoculated with 10% of its total volume with the culture grown on the activation stage. For one-pot fermentation experiment, which comprised propagation and fermentation steps, during growth phase, the medium composition was the same of the propagation media above commented, while, for the fermentation stage, the initial concentrations of urea and D-xylose were, respectively, 1,0 g/L, as optimized by a previous study [8], and approximately 90 g/L [9], since xylitol is a compatible solute. All the media had its pH adjusted to 6.0 and were sterilized for 15 min at 110°C.

Propagation Conditions

Three different strategies for propagation of *C. guilliermondii* 50088 cells were adopted. Propagation I and Propagation II were carried out in 1L Erlenmeyer flasks at 200 rpm and 30°C. However, for Propagation I, the volume of culture media on the flasks was 400 mL, while, for Propagation II, it was 200 mL. Propagation III was held on a bioreactor (New Brunswick Scientific New Bioflo 310) with controlled temperature (30°C), pH (6,0 - 7,0) and dissolved oxygen concentration (DO at 30% of saturation level). Once the xylose present in the initial batch was depleted, extra feedings were added for achieving higher cell densities, always keeping xylose levels in a range from 10 g/L to 15 g/L. Not only D-xylose was fed, but also the other components of the medium, for which were prepared concentrated solutions to avoid dilution of the substances in the culture medium.

One-pot Fermentation Conditions

One-pot fermentation strategy comprised a propagation phase, including the initial batch and an extra feeding, and the fermentation stage, all of them held at pH = 6,0 to 7,0 and 30°C. During the growth steps, the dissolved oxygen levels were kept at 10 % of saturation, and the initial substrate concentrations – for first batch and extra feeding - were lower, about 10 g/L of D-xylose. On the other hand, along fermentation, DO concentration was maintained lower and initial substrate concentration was higher, in order to meet biochemical requirements.

Analytical Methods

Along the process, samples were withdrawn from the flasks and bioreactor for analytical determinations. Whenever xylose was depleted and an extra feed was added, a new sample was taken, making it possible to measure the initial substrate concentration in the new batch. Xylose and xylitol were analyzed by high-performance liquid chromatography using a Shimadzu chromatograph linked to a High-Plex-H column at 60°C and H_2SO_4 (0,005 mol/L) as mobile phase at a flow rate of 0,6 mL/min. To quantify cell growth, once centrifuged, cells were washed, resuspended in distilled water and then, by turbidimetry at 570 nm and using a standard curve relating absorbance and dry-cell weight, provided an estimative for cell density.

RESULTS AND DISCUSSION

Propagation Strategies

Figure 1 shows results for Propagation I. Depletion of substrate in the initial batch lasted about 40 hours, with a growth yield factor on xylose consumed ($Y_{X/S}$) equivalent to 0.33 g/g and a xylose uptake rate (Q_s) of 0.28 g/L.h. At the end of this stage, cell concentration in the system was approximately 4 g/L. In order to reach even higher cell densities, an extra feeding was added, but after 10 hours, almost no cell growth was observed. Hence, the assay was interrupted, since its main objective – reaching high cell concentrations in short time – was not achieved. Along the process, xylitol, even though in small quantities (≈ 1 g/L), was formed, probably due to metabolic deviations.

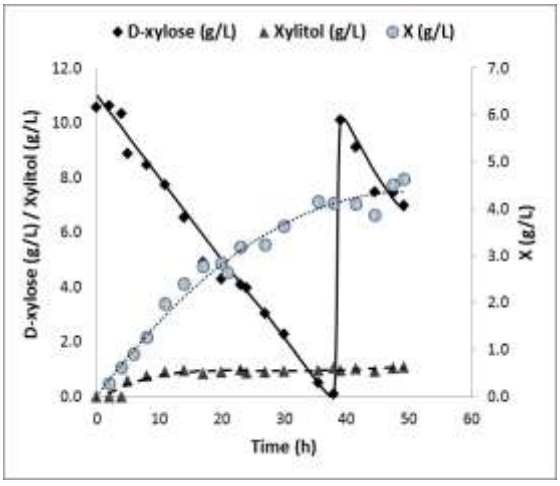

Fig. 1: Propagation I (400 mL of propagation medium in a 1L shake flask at 200 rpm and 30°C)

For Propagation II (Figure 2), a great reduction in the initial batch time (≈ 50%), along with a higher cell concentration – more than 5 g/L –, was noticed when compared to Propagation I, resulting in a $Y_{X/S}$ factor of 0.36 g/g. Also, a xylose uptake rate of 0.73 g/L.h was achieved. This might be associated to the degree of superficial aeration. For Propagation II, the lower medium volume allowed a greater superficial area and, therefore, a higher oxygen transfer, which promotes higher *Candida guilliermondi* growth and xylose uptake rate. Both extra feedings, however, did not provide proportional growth. For the first additional feeding, besides a lowering in Q_s (0.56 g/L.h), $Y_{X/S}$ factor was also reduced to 0.20 g/g, while for the second one, almost none cell growth was observed.

Propagation III (Figure 3), performed in bioreactor, promoted the most interesting results. The depletion time of xylose in the first batch (14h) was even lower than for Propagation II and, by the end of this stage, there were 5.2 g/L of cells, corresponding to a $Y_{X/S}$ factor of 0.42 g/g. Also, for the extra feedings, increasing xylose uptake rates - which should be expected, since a greater population of microorganisms demands more substrate - and $Y_{X/S}$ factors – superior to 0.6 g/g - were observed. Although after 33h a very high cell density (ca. 20 g/L) was achieved, the requirement to maintain high dissolved oxygen levels by managing air supply and agitation led to foam problems and cell adherence to the bioreactor walls, which explains the reduction of the values of cell concentration.

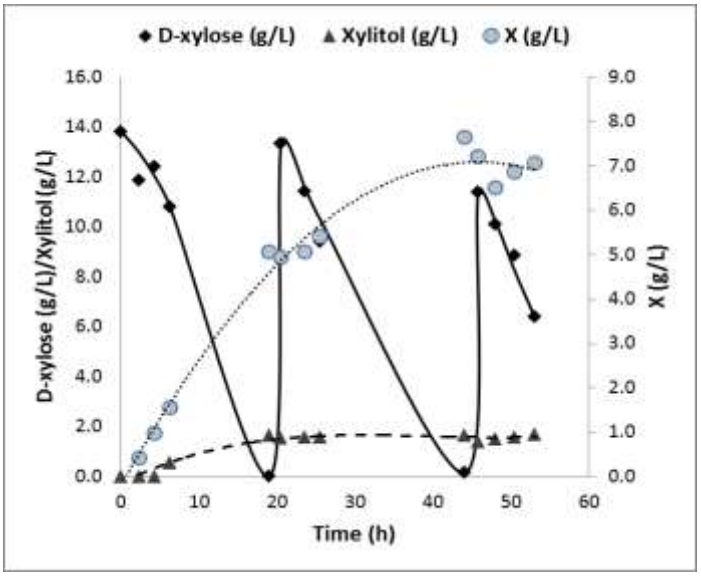

Fig. 2: Propagation II (200 mL of propagation medium in a 1L shake flask at 200 rpm and 30°C)

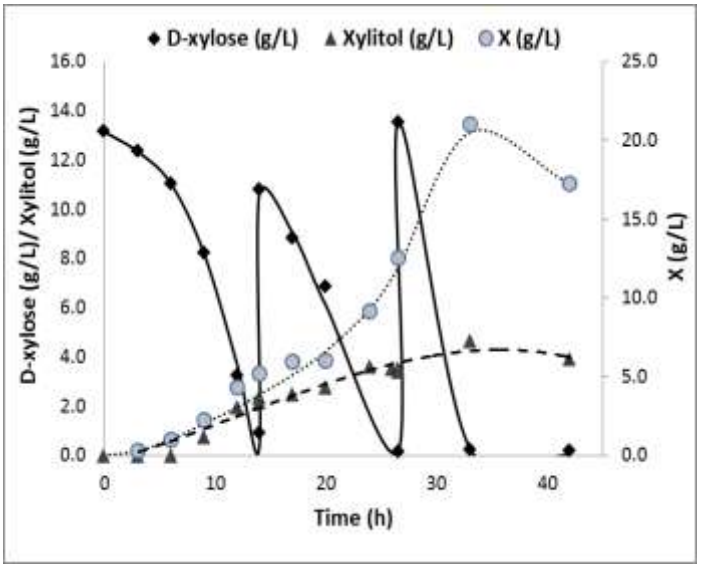

Fig. 3: Propagation III (Bioreactor; DO at 30% of saturation)

Table 1 summarizes the results for Propagations I, II and III.

Table 1. Calculated response variables from each propagation strategy

	$(Y_{X/S})_i$ (g/g)	$Y_{X/S\ F1}$ (g/g)	$Y_{X/S\ F2}$ (g/g)	$Q_{s\ I}$ (g/L.h)	$Q_{s\ F1}$ (g/L.h)	$Q_{s\ F2}$ (g/L.h)
Propagation I	0.33	≈ 0.0	-	0.28	0.09	-
Propagation II	0.36	0.20	≈ 0.0	0.73	0.56	0.68
Propagation III	0.42	0.69	0.64	0.88	0.84	2.10

Where: $Y_{(X/S)}$= Cell growth yield factor (g/g); Qs= Xylose uptake rate (g/L.h)

Index: I= Initial batch; F1=First extra feeding; F2=Second extra feeding

Comparison Between Propagations

In order to compare and select the best cell propagation strategy, results of the three approaches were compared in terms of maximum cell productivity (Q_{Xmax}) reached. Analyzing Figure 4, it is clear that Propagation III, performed in instrumented bioreactor, provided the best results. Its Q_{Xmax} was equivalent to 0.61 g/L.h, a value more than six times higher than that from Propagation I (0.09 g/L.h). Besides, at 26 hours of experiment, cell concentration in the bioreactor had already reached 12 g/L. The reason for such result is inherent to the bioreactor system, which provides a more intense air supply, along with agitation and pH control.

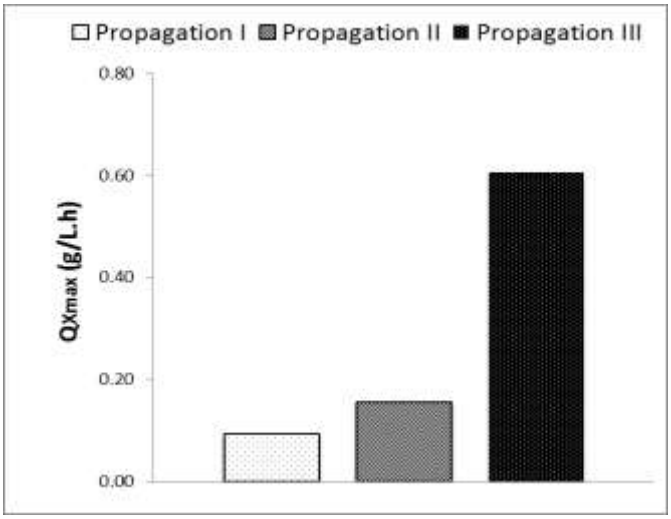

Fig. 4: Comparison between propagation strategies in terms of maximum cell productivity (Q_{Xmax})

One-pot Fermentation

Due to the fact that growing *C. guilliermondii* cells in the bioreactor provided the best results, a one-pot fermentation approach to xylitol production was intended. The DO concentration level during the propagation steps, however, was kept lower than during Propagation III to avoid foam issues. Figure 5 shows the results for this assay. The first two steps comprised the growth stage. The initial batch lasted for 17.5 hours and provided a $Y_{X/S}$ factor equivalent to 0.40 g/g, while the first extra feeding was about 23 hours long, with a yield on cells of 0.36 g/g. At the end of the propagation steps, cell density in the system was roughly 6.0 g/L. Once the fermentation stage started, because of the addition of such a high D-xylose content to support fermentation, cell concentration values were diluted. Although the propagation was well succeeded, during the fermentation there was an interruption on the consumption of D-xylose and, hence, almost no accumulation of xylitol. This behavior might be related to the sudden change of conditions, from excess of oxygen and low levels of substrate (propagation) to restriction of aeration and high concentrations of D-xylose (fermentation). Perhaps, a more gradual transition should be done to avoid what seems to be an inhibitory effect. Earlier studies [10,11] did not mention this inhibition; on the contrary, as the biochemistry of the process would suggest, high D-xylose concentrations provided better yields on xylitol. Nonetheless, these other works were not carried out under a one-pot fermentation strategy and it can be supposed that an accumulation of oxidized cofactors during initial steps – excess of aeration - may have occurred, therefore leading to low xylitol bioconversion.

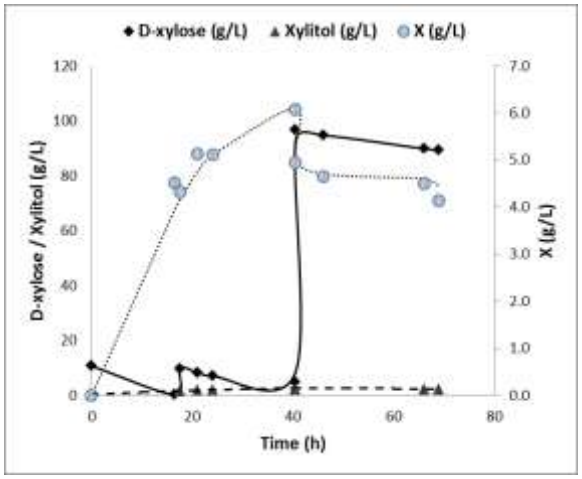

Fig. 5: One-pot fermentation experiment (bioreactor)

CONCLUSIONS

From this work, it is clear to see different behaviors of *C. guilliermondii* IM/UFRJ 50088 cells for each propagation strategy adopted. Propagation III, carried out in bioreactor, promoted higher cell growth when compared to propagations (I and II) performed in shake flasks. This approach led to a higher maximum cell productivity ($Q_{x\ max}$ = 0.61 g/L.h), more than six times greater than the one obtained from Propagation I, and reached cell concentrations near 20 g/L, despite its foam formation. This result might be due to the fact that the equipment used ensures control of agitation and aeration, providing a greater supply of O_2, which is essential for cell growth in *Candida guilliermondii* cells.

Since propagation achieved better results when performed in a bioreactor, it was proposed a one-pot fermentation method, which showed a maximum yield on xylitol ($Y_{P/S\ max}$) of 0.1 g / g, , meaning there is still room for improvement. Although the propagation stage was successful, there was a disruption in the consumption of D-xylose during fermentation resulting in a non-significant accumulation of xylitol. Probably, this is related to the abrupt change of conditions between the propagation and fermentations steps.

ACKNOWLEDGMENTS

The authours would like to thank CAPES for financial support.

REFERENCES

[1] Parajó, J. C., Dominguez, H., Dominguez, J. M. 1998a. Biotechnological production of xylitol. Part 1: Interest of xylitol and fundamentals of its biosynthesis. Bioresource Technology, 66, 25-40

[2] Prakash, G., Varma, A. J., Prabhune, A., Shouche, Y., Rao, M. 2011. Microbial production of xylitol from d-xylose and sugarcane bagasse hemicellulose using newly isolated thermotolerant yeast *Debaryomyces hansenii*. Bioresource Technology, 102, 3304–8.

[3] Dills JR., W. L. 1989. Sugar alcohols as bulk sweeteners. Annual Review of Nutrition, 9, 161-86

[4] Albuquerque, T. L., da Silva JR., I. J., Macedo, G. R., Rocha, M. V. P. 2014. Biotechnological production of xylitol from lignocellulosic wastes :A review. Process Biochemistry, 49, 1779-1789

[5] Pacific Northwest National Laboratory, National Renewable Energy Laboratory 2004. Top value added chemicals from Biomass: Results of screening for potential candidates from sugars and synthesis gas. Department of Energy, OakRidge: TN.

[6] Bozell, J. J., Petersen, G. R. 2010. Technology development for the production of biobased products from biorefinery carbohydrates—the US Department of Energy's "Top 10" revisited. Green Chemistry, 12, 539-554

[7] Arruda, P. V., Rodrigues, R. C. L. B., da Silva, D .D. V., Felipe, M.G.A. 2011. Evaluation of hexose and pentose in pre-cultivation of *Candida guilliermondii* on the key enzymes for xylitolproduction in sugarcane hemicellulosic hydrolysate. Biodegradation, 22, 815-822

[8] Fogel, R. 2004. Otimização da hidrólise ácida de bagaço de cana-de-açúcar e utilização do hidrolisado na produção de xilitol por *Candida guilliermondii.* Msc thesis, Post Graduate Program in Chemical and Biochemical Technology, School of Chemistry, UFRJ, Rio de Janeiro, Brazil

[9] Faria, L.F.F. 2000. Produção de xilitol a partir de D-xilose em um biorreator a membrana. Dsc thesis, Post Graduate Program in Chemical and Biochemical Technology, School of Chemistry, UFRJ, Rio de Janeiro, Brazil

[10] Nigam, P., Singh, D. 1995. Processes for fermentative production of xylitol – a sugar substitute. Process Biochemistry, 30 (2), 117-124

[11] Gimenes, M. A. P., Carlos, L. C. S., Faria, L. F. F., Pereira JR., N. 2002. Oxygen uptake rate in production of xylitol by *Candida guilliermondii* with different aeration rates and initial xylose concentrations. Applied Biochemistry and Biotechnology, 98-100, 1049-1059