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Real-time monitoring of xylitol production in a bioreactor by *Candida tropicalis* IEC5-ITV using Near-Infrared Spectroscopy (NIRS)

Monitoreo en tiempo-real de la producción de xilitol en biorreactor con *Candida tropicalis* IEC5-ITV utilizando Espectroscopia de Infrarrojo Cercano (NIRS)

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Abstract

The use of Near-Infrared Spectroscopy (NIRS) and Chemometrics in-situ or in-line monitoring of xylitol fermentation process by *Candida tropicalis* IEC5-ITV was investigated in a bioreactor and in a complex analytical matrix. Xylose, xylitol, biomass, and glycerol determinations were performed by a transflection fiber optic probe, immersed in the culture broth and connected to a Near-Infrared (NIR) process analyzer. The NIR spectra recorded between 800 and 2,200 nm, these NIR Spectra were pretreated using Savitzky-Golay smoothing and second derivative to perform a partial least squares regression (PLSR) and generate the calibration models. These calibration models were tested by external validation and then used to predict concentrations of xylitol fermentations in batch culture. The standard errors of calibration (SEC) and determination coefficients (R^2) for xylose, xylitol, biomass, and glycerol were 0.234 (R^2 = 0.991), 0.220 (R^2 = 0.999), 0.234 (R^2 = 0.991) and 0.015 (R^2 = 0.999) gL^{-1} and standard errors of prediction (SEP) were 1.771, 0.192, 0.011, 0.503 g/L, respectively. Calibration and validation criteria were defined and evaluated to generate robust and reliable models of a xylitol fermentation process. For validation models, SEV and SEP were ≤ 10 % of initial concentration of xylose and $R^2 \geq 0.96$ were obtained. These results indicate that in situ NIRS probe is suitable for real-time monitoring of xylitol production.

Keywords: Near Infrared Spectroscopy (NIRS); xylose; *Candida Tropicalis*; real-time monitoring; xylitol.

Resumen

Se investigó el uso de la Espectroscopia de infrarrojo cercano (NIRS por sus siglas en ingles) y la quimiometría en la medición en tiempo real del proceso de producción de xilitol utilizando *Candida tropicalis* IEC5-ITV en biorreactor y en una matriz analítica compleja. Las determinaciones de xilosa, xilitol, biomasa y glicerol se realizaron mediante una sonda de fibra óptica de transflexión, sumergida en el caldo de cultivo en el biorreactor y conectada a un analizador de procesos de infrarrojo cercano (NIR). Los espectros NIR registrados fueron entre 800 y 2200 nm, estos espectros NIR fueron pretratados utilizando el suavizado de Savitzky-Golay y la segunda derivada para realizar una regresión de mínimos cuadrados parciales (PLSR) y generar los modelos de calibración. Estos modelos de calibración se probaron mediante validación externa y luego se utilizaron para predecir las concentraciones del proceso de fermentación de xilitol en un cultivo por lote. Los errores estándar de calibración (SEC) y los coeficientes de determinación (R^2) para xilosa, xilitol, biomasa y glicerol obtenidos fueron 0.234 (R^2 = 0.991), 0.220 (R^2 = 0.999), 0.234 (R^2 = 0.991) y 0.015 (R^2 = 0.999) g/L y los errores estándar de predicción (SEP) fueron 1.771, 0.192, 0.011, 0.503 gL^{-1}, respectivamente. Se definieron y evaluaron criterios de calibración y validación para generar modelos robustos y confiables de un proceso de producción de xilitol. Para los modelos de validación, el SEV y SEP fueron <10 % de la concentración inicial de xilosa y se obtuvieron R^2 >0.96. Estos resultados indican que la sonda NIRS en línea es adecuada para el monitoreo en tiempo real del proceso de producción de xilitol.

Palabras clave: Espectroscopia de infrarrojo cercano, xilosa, *Candida tropicalis*, monitoreo en tiempo real, xilitol.

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1 Introduction

Xylitol is a five-carbon sugar alcohol that can be found in nature in small quantities. It has attracted global attention because of its sweetening power like that of sucrose but provides much fewer calories.

Xylitol is known to metabolize through insulin-independent pathways in human body and therefore it can be used as sugar substitute for diabetics. Moreover, a significant property that has been found in xylitol is to be anticariogenic, which can help promote oral health and prevent caries (Prakasham *et al*., 2009; Mussatto *et al*., 2012; Hernández Pérez *et al*., 2019). The Xylitol market continues to see strong demand and rapid growth worldwide, due to an increasing health-conscious consumer and fast growth in chewing gum sales (Franceschin *et al*., 2011). To produce this chemical in a more environmental-friendly manner, research has been conducted on alternative strategies that utilize microorganisms for conversion of Xylose to Xylitol from hemicellulosic hydrolysates (Seonghun, 2019; Reshamwala & Lali, 2020). The ability to produce Xylitol as a normal metabolic product has been frequently observed for diverse yeasts, and particularly *Candida* species have been reported to produce a high yield of Xylitol under oxygen-limited conditions (Walther *et al*., 2001; Ping *et al*., 2013; Castañón-Rodríguez *et al*., 2019; Carneiro *et al*, 2019; Martínez-Corona *et al*., 2020). Some investigators have published studies in different processes using glucose and xylose as carbon source to produce biotechnology products (Marison *et al*., 2013; Pérez *et al*., 2013; Goldfeld *et al*., 2014; Tamburini *et al*., 2014; Corro-Herrera *et al*., 2016; Corro-Herrera, *et al*., 2018; Pérez-Cadena *et al*., 2018; Haq *et al*., 2020; Hamid *et al*., 2021). Industrial level monitoring of Xylitol production is another challenge to be overcome due to the lack of a methodology that allows the capacity to ascertain real-time fermentation conditions and to use this information for taking decisions. Furthermore, reliable monitoring can help to improve fundamental understanding of cellular metabolism and thus be able to optimize the bioprocess (Vaidyanathan *et al*., 1998; Morita *et al*., 2011; Fazenda *et al*., 2013; Xu *et al*., 2019; Pessoa-e-Silva *et al*., 2020). Development and bioprocess optimization are highly dependent on accurate real-time monitoring of chemical and physical process variables (Blanco & Peinado, 2004; Arnold *et al*., 2012; Alves-Rausch *et al*., 2014; do Nascimento *et al*., 2017). Hence, Near Infrared Spectroscopy (NIRS) can be applied as real-time fermentation monitoring methodology using rapid and non-destructive multi-constituents' analyses, without involving sample pretreatment, which leads to effective bioprocess control, a tool for increased yield, productivity and reproducibility (Wold *et al*., 2001; Scarff *et al*., 2006; Workman, 2008; Liebman *et al*., 2009; Lourenço *et al*., 2012; Li *et al*., 2020). In the present study, the utility of NIR spectrometry for the real-time monitoring of Xylitol production by *Candida tropicalis* IEC-5 using xylose as a carbon source was investigated.

2 Material and methods

2.1 Strain

Candida tropicalis IEC5-ITV a strain isolated from sugarcane bagasse, in Bioengineering Laboratory of National Technology Institute (TecNM)-Technological Institute of Veracruz (ITVer). This strain was stored at 4 ºC and maintained in semi synthetic medium agar plates consisting of (gL^{-1}): bacteriological agar, 25.0 (Bioxon, Mexico); xylose, 20.0 (J.T. Baker, Mexico); yeast extract, 10.0 (Bioxon, Mexico).

2.2 Inoculum preparation and batch cultures

A defined medium was used for both inoculum preparation and batch cultures, which contained xylose, 20 gL^{-1}; KH_2PO_4, 5.0 gL^{-1}; Urea, 3.0 gL^{-1}; $MgSO_4 \cdot 7H_2O$ 0.4 gL^{-1}; yeast extract, 1.0 gL^{-1}. Incubated at 30 °C for 24 h at 250 rpm (incubator-shaker Daihan LabTech CO., LTD, model: LSI ? 3016A). Batch cultures were carried out in a 14 L New Brunswick bioreactor (BioFlo 3000, USA) with a 5 L working volume. Process conditions were 30 ºC, 150 rpm, pH 5.5, inoculum size 6×10^6 cell/mL with 99 % viability (Viability was assessed by the methylene blue staining method proposed by Lange *et al*. (1993)). Samples were taken periodically. Near-Infrared (NIR) monitoring was made online using transflection probe.

2.3 Reference analytical methods

2.3.1 Biomass

Culture (5.0 mL) was filtered onto a pre-weighed Whatman Glass Filter grade (GF/C) 0.2 μm (Whatman, England). The cells were then washed twice with distilled water and the filter cake dried to a constant weight in an oven (Yamato Scientific Co. Ltd, USA) at 60 ºC.

2.3.2 Xylose, Xylitol and Glycerol

Xylose, xylitol and glycerol concentrations were determined by high performance liquid chromatography (HPLC) (Waters 600, TSP Spectra System, Waters, Milford, MA, USA), with a Waters 2414 index detector (TPS Refracto Monitor V Waters, Milford, MA, USA) at 50 °C. A Shodex SH 1011 column (8 x 300 mm) (Waters, Milford, MA, USA) was used to separate sugars by size exclusion and organic acids and alcohols by ion exclusion mode, using 5 mM H_2SO_4 as mobile phase at a 0.6 mL/min flow rate. The analysis was carried out in duplicate.

2.3.3 NIRS measurements

Spectra of whole matrix were acquired with a Near-Infrared Spectrophotometer XDS Process Analytics (Foss-NIRSystems, Silver Spring, USA) using an in-situ fiber optic transflection probe, 3 mm path length. The fiber optic probe is made of 316 L stain-less steel with the corresponding corrosion resistance against sulfuric and acids. The samples were scanned in duplicate over the whole NIR range (800-2200 nm) every 3 hours until carbon source depletion. The spectra were then averaged and derivatized (second derivative) with a segment size of 10 nm and gap size of 2 nm to reduce the relation sample/instrument noise (Williams, 1987).

2.4 Spectra pretreatment

First, for all analytes, spectra were averaged and the second derivative with Savitzky-Golay smoothing was applied to 10 nm segment sizes and 2 nm gap sizes. Segment sizes describe the number of data points involved in the degree of smoothing (reducing sample/instrument noise), with a specific gap size between the segments (Williams, 1987). Second derivative was used to deconvolute any broad overlapping peaks and reduce any baseline shift (Crowley *et al*., 2005).

Table 1. Wavelength regions used for calibration and validation.

Analyte	Wavelength (nm)
Xylose	1182 - 1234; 1439 - 1489; 1674 - 1731; 1839 - 1889; 1903 - 1944; 1999 - 2080; 2094 - 2121; 2168 - 2183
Xylitol	1179 - 1223; 1463 - 1489; 1677 - 1716; 1834 - 1886; 1925 - 1944; 2000 - 2056
Biomass	824 - 930
Glycerol	892 - 958; 1095 - 1118; 1175 - 1324; 1680 - 2061

2.5 Model development and validation

Xylose, xylitol, biomass, and glycerol were modeled using the whole bioreactor sample and its spectral region used to construct the model was showed in Table 1. Selections of these spectral regions were supported by spectral second derivative analysis. Analytical models were constructed using partial least square regression (PLSR) in Vision v3.5 (Foss-NIRSystems, Silver Spring).

External validation was performed using random subsets technique, standard error in calibration and prediction/external validation (SEC and SEP, respectively) and determination coefficient (R^2) were used as chemometry parameters to assess the quality of the models.

3 Results and discussion

3.1 Kinetic of xylitol production with Candida tropicalis IEC5-ITV

The batch process to produce xylitol by xylose using *Candida tropicalis* IEC5-ITV is presented in Figure 1, typical profiles of the key analytes (xylose, xylitol, biomass, and glycerol) measured with reference analytical methods (HPLC and VIS Spectroscopy) are showed. During the first five hours of the process, xylose consumption was low due to the lag phase; from the sixth hour, exponential phase starts, and fermentation time is approximately 45 h and xylitol concentration achieved 6 gL^{-1} with a yield of 0.35 g xylitol par g xylose. Xylitol is a growth associated metabolism, by this, higher biomass concentrations means, higher xylitol in medium.

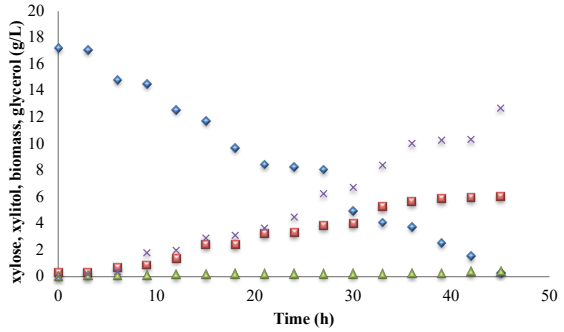

Fig. 1.

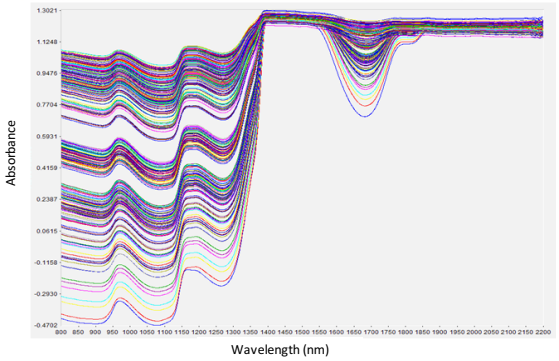

Fig. 2.

Figure 2 shows in-line NIR spectra of xylitol production during xylose fermentation. Absorbance line increasing in the graph is due to the biomass scattering effect. For calibration model construction, is necessary to extract all useful information of the NIR canonical spectra applying chemometrics.

Another useful information to take in consideration is the intense shift shows in wavelength window between 1350 - 1450 nm, due to the strength vibration of -OH functional group in water. This window must be extracted of the wavelength analysis using a spectral subtraction, because there is not useful information for the calibration of any analyte and contribute to avoiding model overfitting.

3.2 *Calibration model construction*

Firstly, to build a NIRS calibration model, analysis and definition of the wavelength regions must be performing (Table 1). As has been stated, NIR spectra have as common features, weak and broad-overlapping absorption bands compared to middle IR spectra. The organic molecular bonds are absorbers of NIR radiation, and these types of bonds can be present in the different analytes of a biological

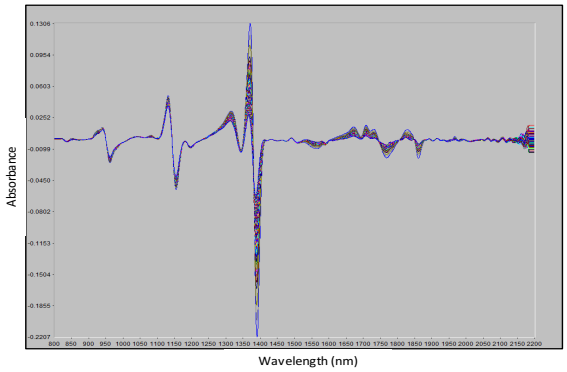

Fig. 3.

sample, transforming in a complex matrix, whose zero order or raw NIR spectrum (Figure 2) with its known overlapping characteristic, in most cases, cannot provide for each analyte the above mentioned specific wavelengths. In the present case, all the zero order spectra underwent second order derivatization to enhance spectral features, overcoming or decreasing drawbacks (broad-overlapping peaks) and biomass baseline shift changes (Figure 3). All peaks appear in zero order spectra, deconvolute and appear as depression in second derivative spectra.

In a typical fermentation process, second derivative of the zero order spectra have four spectral regions of analysis. First region (900 - 950 nm) is where the most of yeast interact with the NIR radiation. Second and third regions (1150 - 1250; 1350 - 1450 nm) represents the absorption of the water. Finally, the fourth region (1700 - 1900 nm), is where all the interest analytes have the most intense interaction with the radiation. According to Cavinato *et al.*, (1990), it is important for the calibration to eliminate the water regions to construct reliable prediction models.

3.3 *Biomass model*

Typically, biomass is measured off-line by gravimetric and optical methods with the corresponding delay in results. NIRS is applicable as invasive *in-situ* or *in-line* monitoring, allowing the possibility of real-time biomass monitoring using correlations with dry cell weight (Williams, 1987; do Nascimento *et al.*, 2017).

Table 2, shows the values of SEC, SEP and R^2 for biomass modeling and Figure 4 shows curve for calibration and validation of the biomass. R^2 value was close to one, indicating a fine correlation between laboratory data and NIRS data.

Table 2. Models quality parameters f: number of factors in the calibration model; SEC: standard error in calibration; SEP: standard error in validation/prediction; R^2: determination coefficient. Units for SEC and SEP are g/L.

		Calibration		Validation	
Analyte	f	**SEC**	R^2	**SEP**	R^2
Biomass	**8**	**1.396**	**0.95**	**1.191**	**0.974**
Xylitol	**8**	**2.365**	**0.95**	**1.166**	**0.977**
Xylose	**8**	**4.803**	**0.96**	**1.778**	**0.984**
Glycerol	**8**	**0.134**	**0.95**	**0.288**	**0.96**

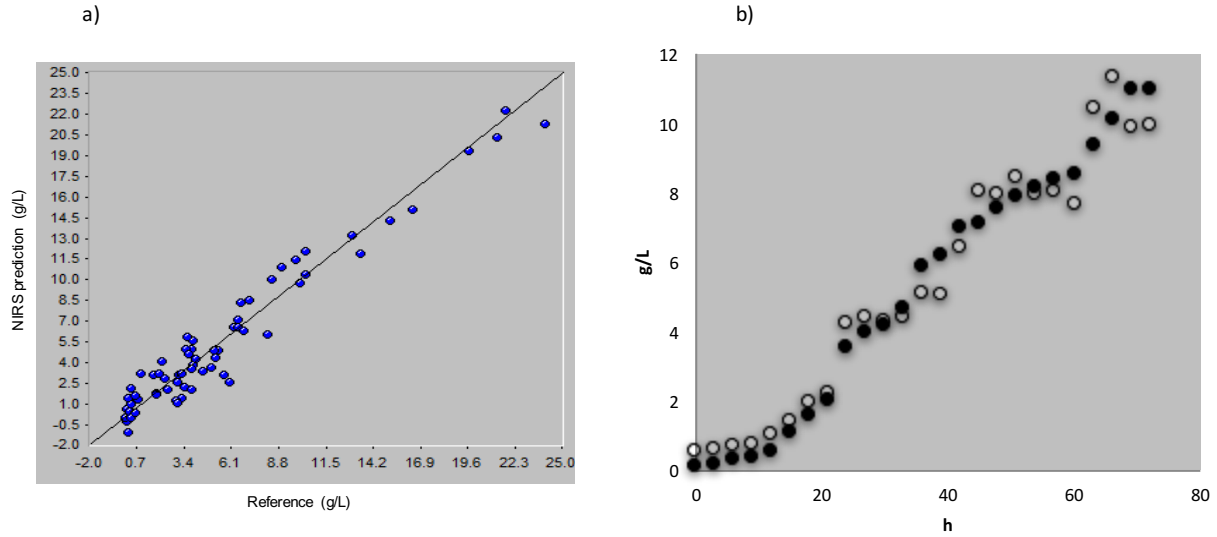

Fig. 4

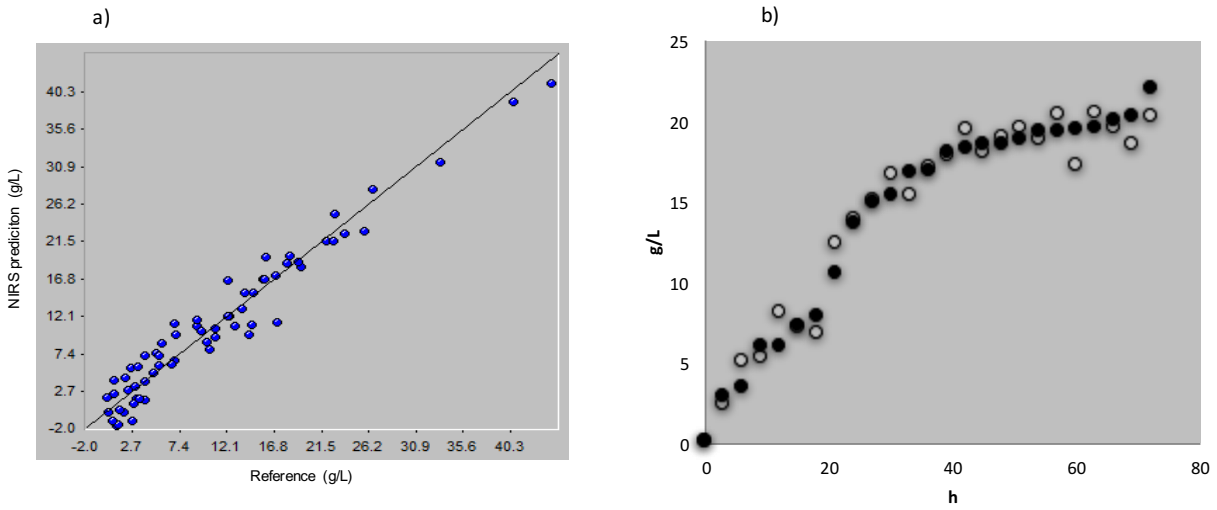

Fig. 5.

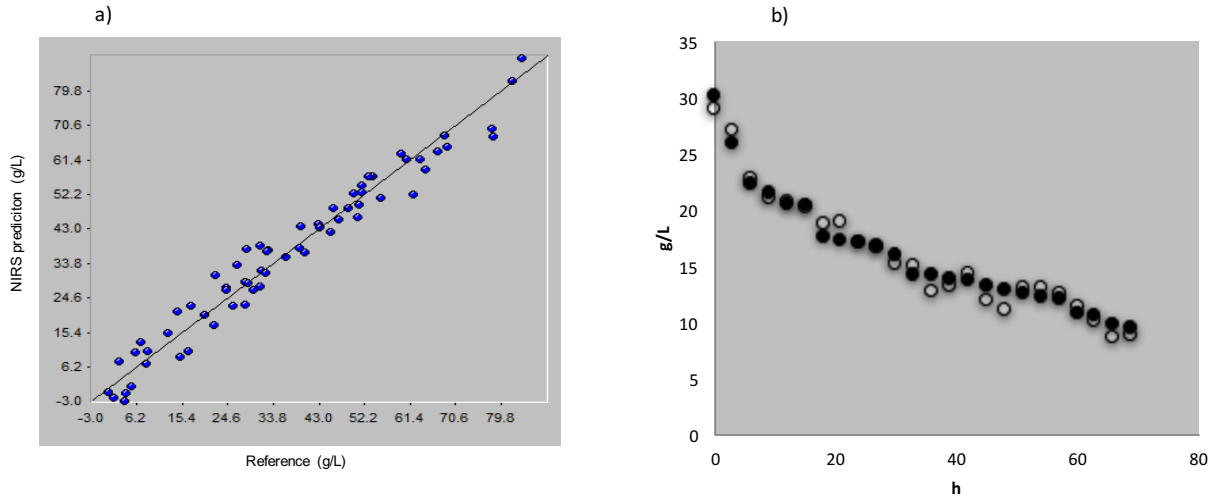

Fig. 6.

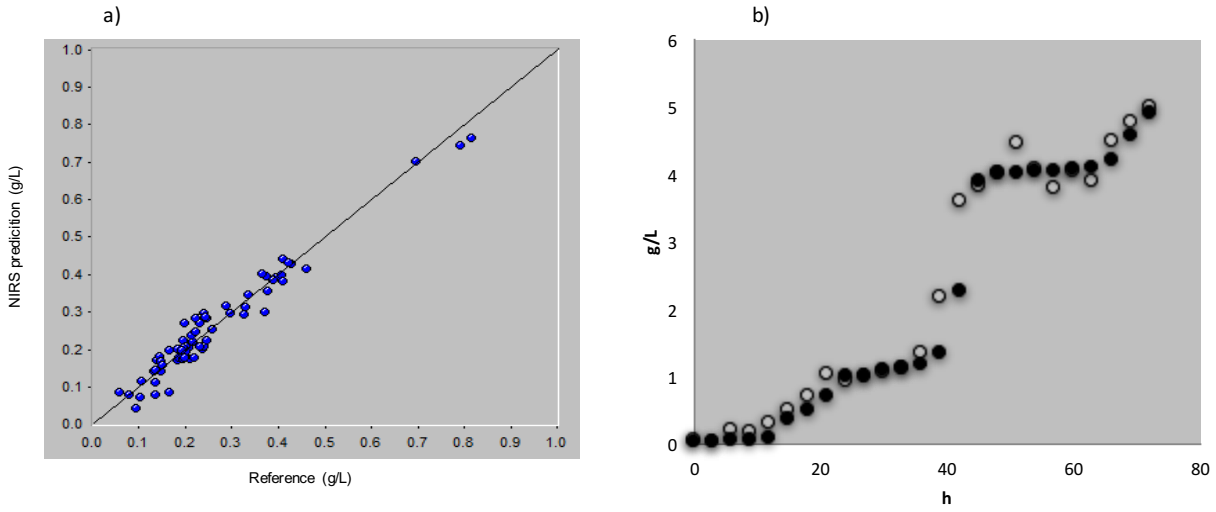

Fig. 7.

The standard error of calibration (SEC) for biomass model being 1.396 g/L. Biomass standard error of external validation or standard error of prediction (SEP) was 1.191 g/L. In case of the validation curve, the prediction adjusts very well (R^2 = 0.974) to the natural kinetic behavior of the process. Furthermore, considering the inherent difficult for the biomass calibration due to all spectral features to take in consideration, the variability of the prediction is consistent to the SEP and SEC values. In case of the calibration curve, accumulation of points in the lower scale represents the phenomena of lag phase and low growth during the yeast adaptation to the media. There are reports for this low growth of this yeast (Castañón-Rodríguez *et al.*, 2019). Despite this, the prediction capacity of the model adjusts to the variability of the growth during fermentation, as shows the plot.

3.4 Xylitol model

In this study, the selection of the wavelength regions for xylitol was based on the second derivative analysis (Figure 3). Table 1 incorporates the spectral windows selected for xylitol. Figure 5 shows the calibration and validation datasets for xylitol model that were generated in the present investigation.

Table 1 shows that xylitol was successfully modeled using three wavelength regions, with an R^2 value of 0.95 and low SEC and SEP (2.365 and

1.166 g/L, respectively). According to the quality parameters and the information in the validation curve, the perform of xylitol prediction model, adjusts to the variability of the process. Although, xylitol and xylose are similar from the molecular perspective, the wavelength selections prove to be effective for the spectral identification of both analytes. Furthermore, the prediction shows robust, adjusting to the smooth variations during process. For example, the production gap around 20 h. Chemometrically speaking, this due the high concentration of samples in the low and medium of the calibration curve, assuring a robust prediction. This is a very desirable condition, considering the dynamic of the process and further real-time in line prediction.

3.5 Xylose model

The NIR wavelength selected for xylose monitoring (Table 1) was based on the analysis of second derivative spectra. Xylose NIRS monitoring becomes a challenge in time, due to the increase of biomass in medium and the correlation between xylose consumed and xylitol-glycerol production. A strategy such as adaptive calibration by spiking experiments breaks this correlation and light scattering caused by biomass (Agbogbo and Coward-Kelly, 2008; Princs *et al*., 2014; Corro-Herrera, *et al*., 2018). During fermentation, samples were filtered and spiked with known concentrations of xylose (Tanino *et al*., 2010). Figure 6 shows the calibration and validation datasets for the glucose model.

The values of SEC and SEP were 4.803 and 1.778 g/L respectively (Table 1). Efficiency of this prediction model was the best of the study. Calibration curve shows a more homogeneous distribution of points, and in consequence, there is a quite good adjust in the prediction. This due, the application of adaptive calibration. Despite the xylose consume was constant with no important gaps in the points, prediction power adjusts to the variation during the process.

3.6 Glycerol model

Glycerol is another product present in xylose fermentation due to cell stress induced by culture conditions or lack of nutrients. The errors for the calibration set and for external validation were 0.134 g/L and 0.288 g/L, respectively (R^2 = 0.95). Figure 7 shows the calibration and validation datasets for this analyte. As shown in the calibration curve, there is a breach between data. A big cluster of data is in the lower position of the curve and there are three points (not outliers) that are in upper position. This situation is not rare but usually, modeling analytes with lower production during the process. For avoiding this problem, the use of Standard Normal Variation (SNV) is recommended. Despite this, the validation curve shows an acceptable adjusts of the prediction data vs reference data.

Conclusions

The technical feasibility of monitoring the xylose fermentation by *Candida tropicalis* IEC5 for xylitol production employing NIRS and Chemometrics has been demonstrated. This affirmation is based on the production employing NIRS and Chemometrics has been demonstrated. This affirmation is based on the generation of functional prediction models for biomass, xylitol, xylose, and glycerol, all with R^2 values close to 1 and low SEC and SEP. The models are based on large datasets compared to previous studies, contributing to likely operational robustness.

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