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Individual and interaction effects of vanillin and syringaldehyde on the xylitol formation by *Candida guilliermondii*

Daniela Vieira Cortez, Inês Conceição Roberto *

Department of Biotechnology, College of Chemical Engineering of Lorena, P.O. Box 116, Lorena, São Paulo, Brazil

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ABSTRACT

The effect of lignin degradation products liberated during chemical hydrolysis of lignocellulosic materials on xylose-to-xylitol bioconversion by *Candida guilliermondii* FTI 20037 was studied. Two aromatic aldehydes (vanillin and syringaldehyde) were selected as model compounds. A two-level factorial design was employed to evaluate the effects of pH (5.5–7.0), cell concentration (1.0–3.0 g l^{-1}), vanillin concentration (0–2.0 g l^{-1}) and syringaldehyde concentration (0–2.0 g l^{-1}) on this bioprocess. The results showed that in the presence of vanillin or syringaldehyde (up to 2.0 g l^{-1}) the cell growth was inhibited to different degrees with a complete inhibition of the yeast growth when the mixture of both (at 2.0 g l^{-1} each) was added to the fermentation medium. The xylitol yield was not significantly influenced by vanillin, but was strongly reduced by syringaldehyde, which showed a more pronounced inhibitor effect at pH 7.0. The yeast was also able to convert vanillin and syringaldehyde to the corresponding aromatic acids or alcohols and their formation was dependent of the experimental conditions employed.

1. Introduction

Xylitol is a naturally occurring sweetener found in the fibers of many fruits and vegetables. This polyol has sweetener properties similar to sucrose, but has 40% fewer calories. It is an important substitute for sucrose and has many applications in the food and drink industries. Xylitol stands out among other sweeteners because it can be used to reduce the incidence of dental caries; to treat illnesses such as diabetes, disorders in lipid metabolism and parenteral and renal lesions; and to prevent lung infection, otitis and osteoporosis (Mussatto and Roberto, 2002).

Commercial production of xylitol is based on the chemical reduction of xylose present in hemicellulosic hydrolysates from a variety of lignocellulosic raw materials. However, the biological method, based on microbial fermentation, is being researched extensively due to its lower environmental impact as compared with the chemical process. Xylitol can be synthesized by various yeast species under appropriate growth conditions (Barbosa et al., 1988; Mayerhoff et al., 1997). Among the various yeast species evaluated, *Candida guilliermondii* has been shown to be a good producer of xylitol from lignocellulosic materials (Mussatto and Roberto, 2008; Rodrigues et al., 2003; Silva et al., 2006). These materials usually have a complex chemical composition and it fractionating by dilute sulfuric acid is often the first step to produce monomeric sugars such as xylose (Mussatto and Roberto, 2004). Nevertheless, the acid hydrolysates obtained contain not only fermentable sugars but also some furan compounds such as furfural and 5-hydroxymethylfurfural, which are formed by the degradation of sugars, and various phenolic compounds such as vanillic acid, syringic acid, vanillin and syringaldehyde, which are formed by degradation of lignin (Mussatto and Roberto, 2004; Olsson and Hahn-Hägerdal, 1996). The degradation products formed by pretreatment of lignocellulose depend on both the biomass and the pretreatment conditions such as temperature, time, pressure, pH, redox conditions, and addition of catalysts (Klinke et al., 2004).

Clark and Mackie (1984) identified a large number of potentially toxic compounds present in dilute acid hydrolysate from *Pinus radiata*, and found a total concentration of low molecular weight phenolics of about 2 g l^{-1}. Buchert et al. (1990) also identified a variety of aromatic monomeric compounds in steamed hemicellulose hydrolysate of birch wood. Vanillin, syringaldehyde, 4-hydroxybenzaldehyde and 5-hydroxymethylfurfural were the major aromatic constituents of this hydrolysate. The inhibitory effects of these four compounds in the range 0.5–2.0 g l^{-1} were evaluated by Buchert and Niemelä (1991) using *Gluconobacter oxydans* as a model organism. These concentration levels have been frequently employed as the reference values to evaluate the influence of different lignin degradation products, including vanillin and syringaldehyde as model compounds, on xylose fermentation (Delgenes et al., 1996; Preziosi-Belloy et al., 1997; Zaldivar et al., 1999).

* Corresponding author. Tel.: +55 12 31595026; fax: +55 12 31533165.
E-mail address: ines@debiq.eel.usp.br (I.C. Roberto).

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In the present study, experimental design methodology was used to determine the individual and interaction effects of two aromatic aldehydes (vanillin and syringaldehyde) on the formation of xylitol by *C. guilliermondii*. The statistical procedure not only helps understand the interrelated biochemical phenomena, but also designs a novel bioprocess for xylitol production from biomass waste. The effect of these compounds on xylose-to-xylitol bioconversion has been little studied. In addition the use of statistical tools to evaluate the influence of lignin degradation products on this bioconversion has not been documented. In this context, the present work aims to evaluate the effect of vanillin and syringaldehyde (as model compounds of lignin degradation products) on xylose-to-xylitol conversion by *C. guilliermondii* FTI 20037 yeast under different cultivation conditions.

2. Methods

2.1. Microorganism and inoculum

C. guilliermondii yeast FTI 20037 (ATCC 201935) was maintained on malt-extract agar slants at 4 °C. All the inocula were prepared by transferring a loopful of cells to test tubes containing sterile distilled water (5 ml). Aliquots of 1 ml of this suspension were transferred to 125 ml Erlenmeyer flasks containing 50 ml of medium composed of (g l^{-1}): xylose, 30.0; glucose, 5.0; $(NH_4)_2SO_4$, 3.0; $CaCl_2{\cdot}2H_2O$, 0.1; KH_2PO_4, 68.9, K_2HPO_4, 22.1 and rice bran extract, 20.0% (v/v). Concentrated solutions of all the nutrients were prepared separately and sterilized at 121 °C for 20 min, except the xylose and glucose solution, which was autoclaved at 112 °C for 15 min. The rice bran extract (source of vitamins and amino acids) was prepared as follows: a 10% suspension of rice bran was autoclaved at 121 °C for 20 min and cooled to room temperature. This suspension was then aseptically centrifuged at 2000*g* for 20 min. The liquid fraction (rice bran extract) was stored at 4 °C for no more than one week. All the nutrients were then added, filling with sterilized water until 50 ml of total volume. All reagents used were of analytical grade. The culture was incubated in a rotary shaker at 30 °C for 30 h with shaking at 200 rev/min.

2.2. Medium and fermentation conditions

The experiments were carried out in 250 ml Erlenmeyer's flasks containing 100 ml of the fermentation medium composed of (g l^{-1}): xylose, 90.0; glucose, 15.0; $(NH_4)_2SO_4$, 3.0; $CaCl_2{\cdot}2H_2O$, 0.1 and rice bran extract, 20.0% (v/v). Potassium phosphate salts were used according to the desired pH values: pH 5.5 (KH_2PO_4 68.9 g l^{-1} + K_2HPO_4 22.1 g l^{-1}), pH 6.3 (KH_2PO_4 43.1 g l^{-1} + K_2HPO_4 55.1 g l^{-1}) and pH 7.0 (KH_2PO_4 17.2 g l^{-1} + K_2HPO_4 88.2 g l^{-1}). All the nutrients were prepared separately as described in the inoculum preparation. The desired mass of each phenolic compound was added to the previously sterilized medium, immediately before the inoculation. Flasks were inoculated to provide the initial cell concentration desired and incubated in a rotary shaker at 30 °C for 96 h with shaking at 200 rev/min.

2.3. Analytical methods

Glucose, xylose and xylitol concentrations were determined by high-performance liquid chromatography (HPLC) using a WATERS instrument, in the conditions: a BIO-RAD Aminex HPX-87H (300 × 7.8 mm) column at 45 °C, 0.005 M sulfuric acid as eluant, flow rate of 0.6 ml min^{-1}, refraction index (RI) detector and 20 μl sample volume. Vanillin, vanilyl alcohol, syringaldehyde, syringic acid concentrations were determined by HPLC using a WATERS instrument with a UV detector (at 276 nm), in the following conditions: a Waters Resolve C18 5 μm (300 × 3.9 mm) column at ambient temperature, acetonitrile/water (1/8 with 1% of acetic acid) with addition of the phosphoric acid for pH correction to 2.5 as eluant, flow rate of 0.8 ml min^{-1} and 20 μl sample volume. Standard solutions of each compound at concentrations which varied of 0.001–0.42 g l^{-1}.

The cell concentration was determined by optical density (OD) at 600 nm using a BECKMAN DU 640 model spectrophotometer and correlated with dry matter. An optical density (OD) of 1 unit is equivalent to 0.58 g of dry cells per litre. For the calibration curve, cells were grown in inoculum medium in a rotary shaker at 200 rev/min, 30 °C, for 30 h. Xylitol yield values were calculated from the slopes of xylitol vs. xylose concentration plots.

2.4. Experimental design and statistical analysis

To determine the influence of vanillin, syringaldehyde, initial cell concentration and pH on the xylose-to-xylitol bioconversion, a 2^4 two-level factorial design experiment with three central points was performed (Table 1). For each of the four factors, high (coded value: +1), center (coded value: 0) and low (coded value: −1) set points were selected. Fermentations representing all 16 set point combinations as well as three assays representing the center point (coded value: 0) were conducted randomly. The statistical software STATGRAPHICS v.6.0 was used to analyze the significance of the experiment results.

3. Results and discussion

3.1. Effect of vanillin, syringaldehyde, initial cell concentration and pH on growth and xylitol production by C. guilliermondii

In order to study the inhibitory effects of vanillin (0–2.0 g l^{-1}) and syringaldehyde (0–2.0 g l^{-1}) on the growth and xylitol production by *C. guilliermondii*, assays were conducted under different conditions of pH (5.5–7.0) and initial cellular concentration (1.0–3.0 g l^{-1}) according to the experimental design. The experimental matrix with the real levels of the variables, as well as the obtained results is shown in the Table 1. Independently of the pH and initial cellular concentration evaluated, the media containing a mixture of vanillin (2.0 g l^{-1}) and syringaldehyde (2.0 g l^{-1}) negatively affected the bioprocess (assays 4, 8, 12 and 16). Under these conditions a complete inhibition of the cell growth was observed and therefore neither xylose consumption nor xylitol production was detected. On the other hand, the combination of these two compounds at 1.0 g l^{-1} each (assays 17–19), resulted in a moderate xylose assimilation (∼47%) with formation of ∼29.0 g l^{-1} xylitol.

When the medium was added only with vanillin at 2.0 g l^{-1} (assays 2, 6, 10 and 14) both xylose consumption and xylitol production by *C. guilliermondii* were similar to the reference conditions (without inhibitor). At pH 5.5 the xylose consumption and xylitol production were not influenced by the increase of the cellular concentration from 1.0 to 3.0 g l^{-1} (assays 2 and 10), showing values of 94% and 98% and 59.3 and 55.0 g l^{-1}, respectively. On the other hand, at pH 7.0 (assays 6 and 14) increasing the cell concentration from 1.0 to 3.0 g l^{-1} both xylose consumption and xylitol production were improved from 63% to 79% and from 34.5 to 41.0 g l^{-1}, respectively. The incomplete xylose use observed at pH 7.0 (6 and 14) was not due to the presence of the vanillin, since that xylose use in the reference medium (without this compound) was similar. These results reveal that the xylose-to-xylitol bioconversion by *C. guilliermondii* was mainly affected by pH of the medium. Rodrigues et al. (2003) also observed similar behavior in fermentations of *C. guilliermondii* based on sugar cane bagasse hemicellulosic hydrolysate. According to these authors, the maximum values