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Xylitol production from corn fibre hydrolysates by a two-stage fermentation process☆

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Abstract

Pichia guilliermondii strain NRRL Y-12723 fermented mixtures of xylose and arabinose to form xylitol and arabitol. However, cultures grown on a mixture of glucose, xylose and arabinose, in ratios characteristic of corn fibre hydrolysates, preferentially utilised glucose and only slowly metabolised pentose sugars, with low yields of sugar alcohols. A two-stage, sequential fermentation scheme for the production of xylitol and arabitol from a mixture of sugars was developed. Following glucose consumption, cells were removed from mixed sugar cultures and replaced with cells from cultures grown on xylose alone. In the second fermentation stage, xylose and arabinose were successfully fermented to xylitol and arabitol. Dilute acid hydrolysates of corn fibre were suitable substrates for the two-stage fermentation process, but only after treatment with a mixed-bed deionization resin. © 2000 Elsevier Science Ltd. All rights reserved.

Keywords: Corn fibre hydrolysate; Fermentation; *Pichia guilliermondii*; Xylitol

1. Introduction

Corn fibre is an abundant co-product of wet-milling, primarily composed of the outer seed coat (pericarp) of corn kernels [1]. Currently, corn fibre is combined with other co-products such as steep liquor and/or stillage residues to produce corn gluten feed. Because of the increasing supply and relatively low commercial value of corn fibre, research has been active to find new, value-added uses for this material.

Corn fibre can be hydrolysed (saccharified) to produce a mixture of sugars, including glucose, xylose and arabinose [2]. These sugars are potential substrates for conversion to a variety of value-added products [3]. Xylose can be reduced to form xylitol, valued as a natural sweetener. Xylitol is equivalent to sucrose in sweetness, but will not cause tooth decay and is safe for diabetics [4]. Although xylitol has been conventionally produced by hydrogenation of xylose from wood fibre [5,6], biocatalysis could offer improved conversion efficiencies and simplified product purification [7,8]. While many microorganisms produce xylitol from xylose, the yeast-like fungus *Pichia guilliermondii* (*Candida guilliermondii*, asexual state) has the reported advantage of high conversion efficiencies with negligible production of ethanol [9,10]. In a recent screen of diverse *P. guilliermondii* isolates, strain NRRL Y-12723 was identified as a promising organism for the production of xylitol from xylose [11]. However, xylose utilisation by yeasts is subject to glucose repression [8,12] and strain NRRL Y-12723 produced little xylitol in the presence of glucose. To circumvent this problem, a two-stage fermentation process for production of xylitol from mixed sugars was developed.

☆ Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval of the product to the exclusion of others that may also be suitable.

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2. Methods

2.1. Microorganism and culture conditions

Pichia guilliermondii strain NRRL Y-12723 was obtained from the ARS Culture Collection at the National Center for Agricultural Utilization Research, Peoria, IL. Working stocks were maintained on YM agar (Difco Laboratories, Detroit, MI). Liquid cultures were grown on the basal medium of Meyrial et al. (1991), containing 1.0% (w/v) yeast extract and 0.67% (w/v) yeast nitrogen base (Difco Laboratories), supplemented with purified sugars (Sigma Chemical Co., St. Louis, MO) or corn fibre hydrolysates. All liquid culture media were filter sterilised.

Preinocula were 10 ml cultures in 50 ml Erlenmeyer flasks containing either 2.0% (w/v) glucose or 2.0% (w/v) xylose, incubated for 2 days at 28°C on a rotary shaker at 100 rpm. For mixed-sugar fermentations, parallel sets of duplicate fermentation cultures were inoculated to 5×10^7 cells/ml from glucose-grown preinocula and incubated under identical conditions.

For two-stage fermentations, mixed sugar cultures were incubated for 1 day, until glucose but not xylose was consumed. *P. guilliermondii* cells from the first stage were then removed from cultures by centrifugation for 20 min at approximately $1400 \times g$. Cells from xylose-grown preinocula were similarly harvested, and gently resuspended in the glucose-depleted culture medium. This culture was immediately reincubated for the second fermentation stage. Care was taken to maintain sterile conditions during cell transfers, and culture purity was monitored microscopically and by plating culture samples.

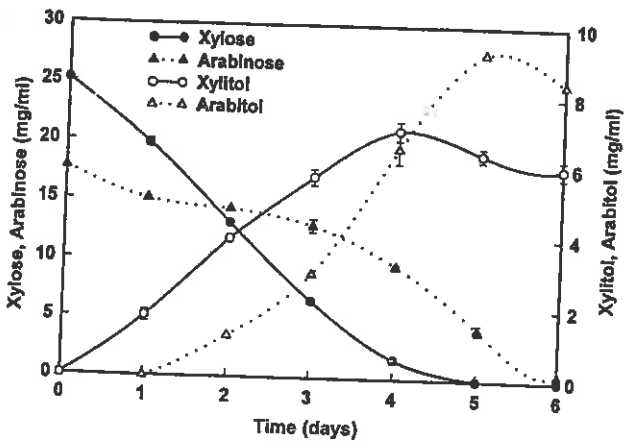

Fig. 1. Fermentation of xylose and arabinose by *P. guilliermondii* strain NRRL Y-12723. Symbols: xylose, ●; arabinose, ▲; xylitol, ○; arabitol △.

2.2. Corn fibre hydrolysis and deionization

Corn fibre hydolysate was prepared as previously described [13]. Briefly, corn fibre was dried at 65°C, ground in a hammer mill, and passed through a 28 mesh screen. Ground corn fibre was mixed with 1.0% (v/v) H_2SO_4 (4.5 ml acid solution/gram solid) and autoclaved for 1 h. After cooling, the resulting liquid fraction was filtered through cheese cloth, neutralised using $Ca(OH)_2$, and then clarified by centrifugation for 10 min at $14\,000 \times g$.

Hydrolysates were diluted 1:2 into 2 × basal medium, either directly or after deionization using a mixed-bed resin (AG 501 X-8 (D), Bio-Rad Laboratories, Hercules, CA). Hydrolysates were treated batchwise (25 g resin/100 ml) until conductivities were reduced from approximately 4500 to 500 μΩ/cm.

2.3. Analytical procedures

Sugars and sugar alcohols were quantitated by high-performance liquid chromatography (HPLC), using an ion moderated partition chromatography column (Supelcogel C611, 300 × 7.8 mm, Supelco, Bellefonte, PA), eluted under isocratic conditions with 0.1 mM sodium hydroxide at 0.5 m/min at 60°C, and detected with a differential refractometer (Thermo Separations Products, San Jose, CA).

3. Results and discussion

3.1. Fermentation of purified sugars

P. guilliermondii strain NRRL Y-12723 was previously identified in a survey of diverse isolates as a strain that efficiently fermented xylitol to xylitol [11]. When grown on a mixture of xylose and arabinose, this strain formed xylitol and arabitol (Fig. 1). Xylose was preferentially fermented before arabinose, with maximal xylitol yields of approximately 28% of initial substrate produced within 4 days.

However, when presented with a mixture of glucose, xylose, and arabinose, in ratios typical of dilute acid hydrolysates of corn fibre, strain NRRL Y-12723 preferentially utilised glucose, and then only slowly metabolised pentose sugars with little accumulation of sugar alcohols (Fig. 2). Consequently, a two-stage fermentation process to produce xylitol and arabitol from mixed-sugar cultures (Fig. 3) was developed. At 24 h, cultures grown on mixed sugars had consumed all available glucose and had not yet significantly metabolised xylose or arabinose. At this point, *P. guilliermondii* cells were removed from the mixed-sugar cultures by centrifugation. At the same time, cells were harvested from 48 h preinocula grown on basal medium

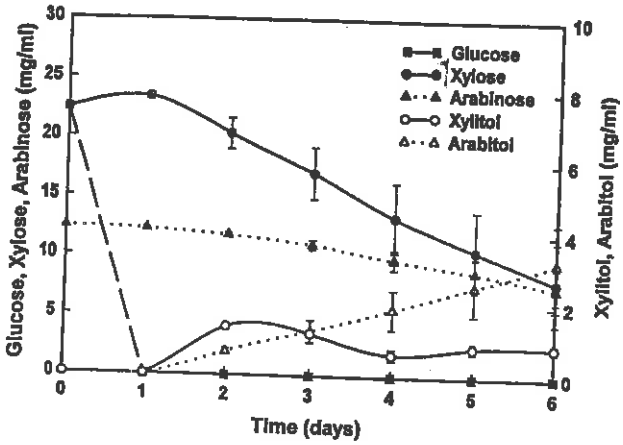

Fig. 2. Fermentation of glucose, xylose, and arabinose by *P. guilliermondii* strain NRRL Y-12723. Symbols: glucose, ■; xylose, ●; arabinose, ▲; xylitol ○; arabitol, △.

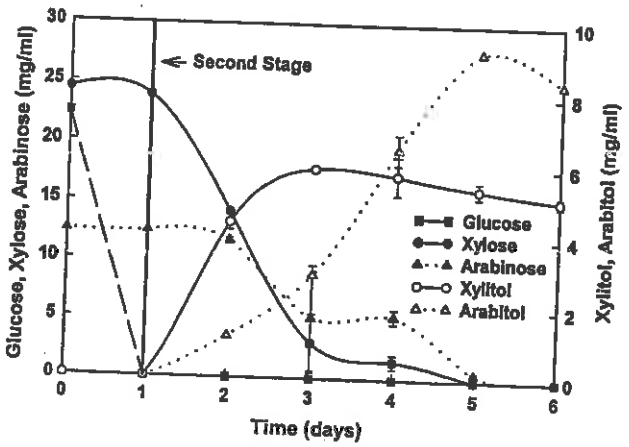

Fig. 3. Two-stage fermentation of glucose, xylose, and arabinose by *P. guilliermondii* strain NRRL Y-12723. Symbols: glucose, ■; xylose, ●; arabinose, ▲; xylitol ○; arabitol, △.

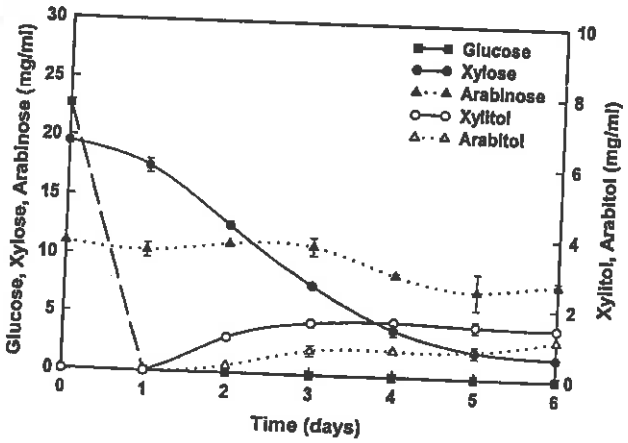

Fig. 4. Fermentation of corn fibre hydrolysate by *P. guilliermondii* strain NRRL Y-12723. Symbols: glucose, ■; xylose, ●; arabinose, ▲; xylitol ○; arabitol, △.

containing 2.0% xylose. These xylose-grown cells were gently resuspended in the glucose-depleted culture supernatant, and cultures were reincubated as before. During the second fermentation stage, xylitol and arabitol were produced at levels comparable to those of cultures that initially contained only xylose and arabinose (Fig. 3). Maximal xylitol yields of approximately 24% of initial xylose were obtained at Day 3, while maximal arabitol yields occurred at Day 5.

3.2. Fermentation of corn fibre hydrolysates

In medium containing diluted corn fibre hydrolysates, *P. guilliermondii* strain NRRL Y-12723 rapidly consumed all available glucose, and then slowly and partially metabolised xylose and arabinose, with little accumulation of xylitol or arabitol (Fig. 4). However, when untreated corn fibre hydrolysates were fermented by the two-stage process, xylitol and arabitol yields were not improved (Fig. 5). In spite of the fact that glucose was rapidly metabolised from this medium, we suspected that the hydrolysates contained substances that inhibited the fermentation of xylose and arabinose. Even mild acid treatments of biomass are known to generate biologically inhibitory compounds such as furfurals, and the neutralisation of acids results in elevated levels of salts [14–16].

Numerous approaches have been tested to remove such fermentation inhibitors or minimise their formation [17–21]. For fermentation by *P. guilliermondii*, we found that corn fibre hydrolysates could be successfully treated with a mixed-bed deionization resin. As expected, deionized corn fibre hydrolysates produced poor yields of xylitol and arabitol in single-stage fermentations (Fig. 6). Two-stage fermentations of deionized hydrolysates produced xylitol yields of approximately 27% of initial substrate, equivalent to yields from two-stage fermentations of purified sugars (Fig. 7).

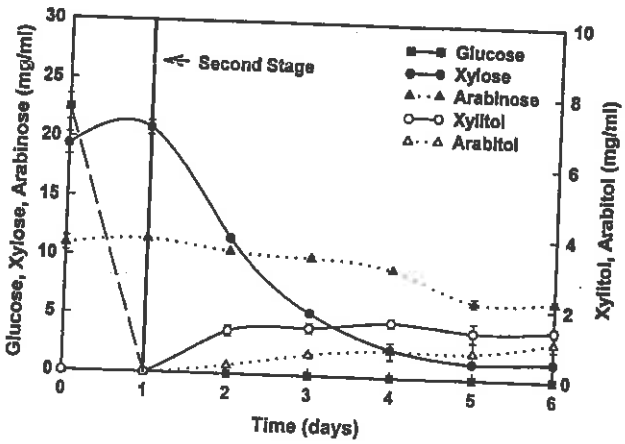

Fig. 5. Two-stage fermentation of corn fibre hydrolysate by *P. guilliermondii* strain NRRL Y-12723. Symbols: glucose, ■; xylose, ●; arabinose, ▲; xylitol ○; arabitol, △.

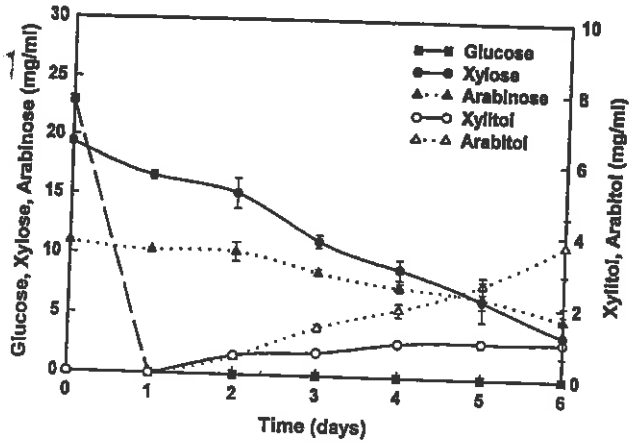

Fig. 6. Fermentation of deionized corn fibre hydrolysate by *P. guilliermondii* strain NRRL Y-12723. Symbols: glucose, ■; xylose, ●; arabinose, ▲; xylitol O; arabitol, Δ.

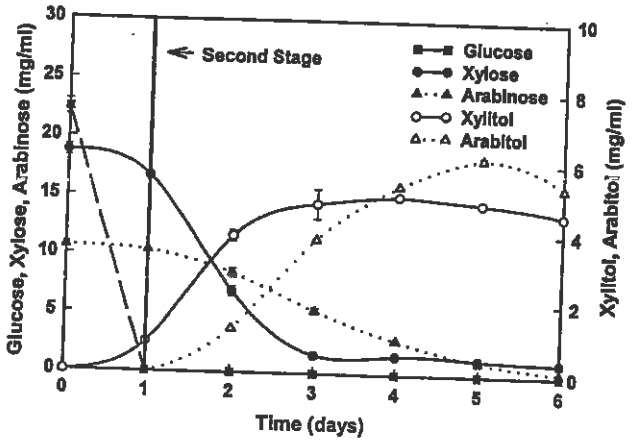

Fig. 7. Two-stage fermentation of deionized corn fibre hydrolysate by *P. guilliermondii* strain NRRL Y- 12723.Symbols: glucose, ■; xylose, ●; arabinose, ▲; xylitol O; arabitol, Δ.

It should be possible to adapt the two-stage fermentation process to utilise a series of bioreactors containing immobilised cells. A primary reactor would contain cells grown on glucose, while a secondary reactor would contain cells grown on xylose. Since xylitol is sequentially produced before arabitol, the process could be regulated to bias product yields in favour of the more commercially valuable xylitol. In theory, an organism other than *P. guilliermondii* could be used to carry out the first stage of the fermentation, perhaps for production of a valuable co-product from glucose. However, the compatibility of this organism with *P. guilliermondii* would have to be confirmed experimentally.

In summary, a two-stage fermentation process was developed to allow the production of xylitol from mixed sugars by *P. guilliermondii* strain NRRL Y-12723. This general strategy might prove useful in a variety of fermentations involving multiple substrates, and particularly in fermentations that suffer from glucose repression.

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References

[1] May JB. Wet milling: process and products. In: Watson SA, Ramstad PK, editors. Corn: Chemistry and Technology. St. Paul, MN: American Association of Cereal Chemists, 1987:377–97.
[2] Bothast RJ, Saha BD. Ethanol production from agricultural biomass substrates. Adv Appl Microbiol 1997;44:261–86.
[3] Leathers TD. Upgrading fuel ethanol coproducts. SIM News 1998;48:210–7.
[4] Pepper T, Olinger PM. Xylitol in sugar-free confections. Food Technol 1988;42:98–106.
[5] Emodi A. Xylitol: its properties and food applications. Food Technol 1978;32:28–32.
[6] Melaja A, Hamalainen L. Process for making xylitol. U.S. Patent 4008285, 1977.
[7] Nigam P, Singh D. Processes for fermentative production of xylitol—a sugar substitute. Proc Biochem 1995;30:117–24.
[8] Saha BC, Bothast RJ. Microbial production of xylitol. In: Saha BC, Woodward J, editors. Fuels and Chemicals from Biomass. Washington, DC: American Chemistry Society, 1997:307–19.
[9] Barbosa MFS, de Medeiros MB, de Mancilha IM, Schneider H, Lee H. Screening of yeasts for production of xylitol from D-xylose and some factors which affect xylitol yield in *Candida guilliermondii*. J Ind Microbiol 1988;3:241–51.
[10] Meyrial V, Delgenes JP, Moletta R, Navarro JM. Xylitol production from D-xylose by *Candida guillermondii*: fermentation behaviour. Biotechnol Lett 1991;13:281–6.
[11] Leathers TD, Gupta SC. Xylitol and riboflavin accumulation in xylose-grown cultures of *Pichia guilliermondii*. Appl Microbiol Biotechnol 1997;47:58–61.
[12] Panchal CJ, Bast L, Russell I, Stewart GG. Repression of xylose utilization by glucose in xylose-fermenting yeasts. Can J Microbiol 1988;34:1316–20.
[13] Dien BS, Hespell RB, Ingram LO, Bothast RJ. Conversion of corn milling fibrous co-products into ethanol by recombinant *E. coli* strains K011 and SL40. World J Microbiol Biotechnol 1997;13:619–25.
[14] Bothast RJ, Dien BS, Hespell RB, Lawton JW. Conversion of corn fiber to ethanol. In: Proceedings of the Third Liquid Fuel Conference. St. Joseph, MI: American Society of Agricultural Engineers, 1996:241–52.
[15] Fanta GF, Abbott TP, Herman AI, Burr RC, Doane WM. Hydrolysis of wheat straw hemicellulose with trifluoroacetic acid. Fermentation of xylose with *Pachysolen tannophilus*. Biotechnol Bioeng 1984;26:1122–5.
[16] Hahn-Hägerdal B, Jeppsson H, Olsson L, Mohagheghi A. An interlaboratory comparison of the performance of ethanol-producing micro-organisms in a xylose-rich acid hydrolysate. Appl Microbiol Biotechnol 1994;41:62–72.
[17] Dien BS, Iten LB, Hespell RB, Bothast RJ. Development and scale-up of a process for conversion of corn fiber into ethanol.

Proc Corn Utilization and Technol Conf, Corn Refiners Assoc, Inc. and National Corn Growers Assoc, 1998:195.

[18] Frazer FR, McCaskey TA. Wood hydrolysate treatments for improved fermentation of wood sugars to 2,3-butanediol. Biomass 1989;18:31–42.

[19] Grohmann K, Bothast RJ. Saccharification of corn fibre by combined treatment with dilute sulphuric acid and enzymes. Proc Biochem 1997;32:405–15.

[20] Moniruzzaman M, Dien BS, Ferrer B, Hespell RB, Dale BE, Ingram LO, Bothast RJ. Ethanol production from AFEX pretreated corn fiber by recombinant bacteria. Biotechnol Lett 1996;18:985–90.

[21] Parajo JC, Dominguez H, Dominguez JM. Charcoal adsorption of wood hydrolysates for improving their fermentability: influence of the operational conditions. Bioresource Technol 1996;57:179–85.