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Hemp Biomass Pretreatment and Fermentation with non-*Saccharomyces* Yeasts: Xylose Valorization to Xylitol

Stefano Raimondi[a,b], Raffaella Ranieri[a], Alan Leonardi[a], Gianluca Ottolina[c], Maddalena Rossi[a,b], Alberto Amaretti[ab]*

[a]Dipartimento di Scienze della Vita, Università di Modena e Reggio Emilia, via Campi 103, 41125 Modena, Italy
[b]Biogest-Siteia, University of Modena and Reggio Emilia, via Amendola 2, 42122 Reggio Emilia, Italy
[c]Istituto di Scienze e Tecnologie Chimiche "Giulio Natta", Consiglio Nazionale delle Ricerche, via Corti 12, 20133 Milan, Italy
alberto.amaretti@unimore.it

Hemp hurds are the main byproduct from hemp fibers supply chain and they could represent valuable feedstock of lignocellulosic biomass for biorefineries. The industrial hemp variety "Carmagnola", is characterized by low amounts of ash and high amount of carbohydrates. Alpha-cellulose (44% w/w), hemicellulose (25%), and lignin (23%) were fractionated using an organosolv pretreatment. The enzymatic hydrolysis of the cellulose fraction yielded up to 60% of glucose, that can easily find application as substrate for industrial fermentations. On the other hand, the black liquor originating from hemicellulose contains mainly xylose and minor amounts of other sugars. In the perspective of finding an application of black liquor, 50 yeasts belonging to 24 ascomycetous species were screened both in aerobiosis and anaerobiosis for the production of ethanol and the sugar alcohols xylitol and arabitol from xylose. *Pichia fermentans* WC 1507, *Wickerhamomyces anomalus* WC 1501, and *Kluyveromyces bacillosporus* WC 1404 were found to consume xylose, yielding xylitol in aerobic conditions. In particular, aerobic flask cultures of *P. fermentans* WC 1507 containing 120 g/L xylose showed the highest xylitol production values, yielding 63.5 g/L xylitol with a $Y_{P/S}$ of 71.5%.
Black liquor, exploited as a carbon source, has been successfully utilized by the three selected yeast strains at a concentration of 20 g/L in the culture medium, resulting in comparable or higher yields of biomass and xylitol compared to the medium containing pure xylose. A higher concentration of black liquor in the culture medium (to reach 120 g/l of xylose) has determined an inhibition of the growth of yeasts indicating the need for treatments for the removal of inhibitors. These preliminary results can be considered promising for the microbial valorization of lignocellulosic hemp feedstock toward the production of xylitol.

1. Introduction

The conversion of the linear fossil-fuel economy into a circular route requires the adoption of industrial strategies capable of enhancing the second-generation feedstocks. In this context, lignocellulosic biomass (LCB) is a promising resource for closing this loop. Around 145 billion tons of LCB residues are produced every year from crops making LCB a widely available and inexpensive raw material that doesn't compete with food production (Haldar et al., 2022). Thanks to its high carbohydrate content, LCB is an important source of fermentable sugars like glucose, arabinose, mannose, and xylose as well as aromatic compounds, and is estimated that it could supply hundreds of molecules of industrial interest, generated by biorefineries. Among these, sugar alcohols such as xylitol and arabitol have been ranked among the top 12 value added chemicals derivable from biomasses and have received increasing scientific attention (Werpy and Peterson 2004; Erickson et al. 2012; Raimondi et al. 2022). Xylitol is a five-carbon sugar alcohol with diabetic-friendly sweetening power, anti-cariogenic properties, and can inhibit microbial growth (Morais et al., 2019), which is why it is widely used in food, dental and pharmaceutical products with a rising world market of 200 billion tons per years that is expected to increase during 2021-2026 (Ravella et al., 2022). Xylitol bioconversion can be realized by fermentative yeasts able to utilize xylose for energy metabolism, and actually the most studied are *Scheffersomyces stipitis*,

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Debaryomyces hansenii, *Kluyveromyces marxianus*, (Quieroz et al., 2022) and various species belonging to the genus *Candida*, e.g. *C. boidinii*, *C. guillermondii*, *C. parapsilosis*, and *C. tropicalis* (Tamburini et al., 2015).
The agricultural waste already used in biorefineries includes stover, wheat, rice, barley straw, sorghum stalks, coconut husk, sugarcane bagasse, and wood (De Bhowmick et al., 2018) but attention has recently increased for industrial hemp (*Cannabis sativa L*.) for both biochemical and bioenergy production (Kreuger et al., 2011; Brar et al., 2022). Its rising market, increasing in both the cultivation area and the industrial application (textile, food, and animal feed, paper production, biofuel, construction, and biodegradable plastics) make it a low-cost and widely available feedstock (Rehman et al., 2021). Nowadays, the principal limit for LCB application is represented by the complex network between its chemical components, which makes it recalcitrant to enzymatic attack and requires energy-costly pre-treatment, to release its valuable products (Liu et al., 2021). During the last decades a lot of physical, and chemical strategies to separate and fractionate its polymeric constituents have been implemented; out of these organosolv pretreatment, compared to other methods, has proved to be a highly efficient solution capable of both high cellulose digestion and removal of lignin solution (Gandolfi et al., 2014). The liquor obtained from this method contains lignin and dissolved monosaccharides from hemicellulose and cellulose (xylose and glucose), that can be recovered for successive utilization (Ferreira et al., 2019; Sun et al., 2018).
In this study we explored the biodiversity of non-*Saccharomyces cerevisiae* strains (Amaretti et al. 2012), in search of candidates that could be exploited for ethanol, arabitol, and xylitol production. *P. fermentans* WC 1507 was selected among 50 screened strain for xylose-to-xylitol bioproduction. The black liquor rich in xylose, obtained by organosolv pretreatment of the industrial hemp variety "Carmagnola" was thus explored as carbon source.

2. Materials and methods

2.1 Strains and culture conditions

Fifty non-*Saccharomyces cerevisiae* yeast strains, belonging to the ascomycetous species *Candida castellii* (n = 1), *Candida freyschussii* (n = 1), *Candida humilis* (n = 1), *Candida maltosa* (n = 1), *Candida sake* (n = 1), *Hansenula jadinii* (n = 1), *Kluyveromyces bacillosporus* (n = 1), *Kluyveromyces lactis* (n = 9), *Kluyveromyces lodderae* (n = 1), *Kluyveromyces marxianus* (n = 8), *Pichia angusta* (n = 1), *Pichia burtonii* (n = 1), *Pichia fermentans* (n = 1), *Pichia guilliermondii* (n = 1), *Saccharomyces boulardii* (n = 1), *Saccharomyces castellii* (n = 1), *Saccharomyces dairenensis* (n = 1), *Saccharomyces exiguus* (n = 2), *Saccharomyces spencerorum* (n = 2), *Torulaspora delbrueckii* (n = 1), *Wickerhamomyces anomalus* (n = 1), and *Zygosaccharomyces rouxii* (n = 12) were obtained from ATCC (Manasses, VA, USA), CBS (Utrecht, the Netherlands), or from our own collection. All the strains were aerobically cultured at 30°C in YPD broth (BD Difco, Sparks, MD, USA).
The strains were screened for the ability to grow on xylose and produce xylitol in MY medium, that contained the following: 20 g/L xylose, 3 g/L yeast extract (BD Difco, Sparks, MD, USA), 2 g/L $(NH_4)_2SO_4$, 3 g/L KH_2PO_4, 1 g/L K_2HPO_4, and 1 g/L $MgSO_4 \cdot 7H_2O$. The same medium containing 20 g/L glucose was utilized as a control. All the chemicals were obtained from Sigma-Aldrich (Steinheim, Germany) unless otherwise stated. Static tubes and shake flasks were utilized to compare anaerobic and aerobic incubation conditions.
For specific experiments, 120 g/L xylose was utilized in the formulation of MY medium or 100 g/L xylose was added at the end of the exponential phase of cultures initiated with 20 g/L.
The conversion yield ($Y_{P/S}$) was calculated as percent ratio between the mass of xylitol produced and xylose consumed.

2.2 Organosolv pretreatment of hemp biomass

Pretreated lignocellulosic hemp biomass was tested as feedstock of fermentable sugars. In particular, black liquor from organosolv pretreatment was prepared according to Gandolfi et al. (2014) and utilized as a raw source of xylose. Chopped hemp hurds, provided by Assocanapa (Carmagnola, Italy), were separated from dust and short fiber, milled, sieved at 0.5 mm, and extracted with methanol in a Soxhlet apparatus. Organosolv pretreatment was carried out in a high-pressure reactor system (BR-300, Berghof, Germany) resuspending extracted hurds in aqueous methanol (65%), containing 3% w/w of sulfuric acid as a catalyst. The reactor was pressurized to 5 bar with N_2 and kept at 165°C, for 90 min. Solid cellulose was separated from the cooled reaction mixture by filtration. To precipitate the dissolved lignin, methanol was removed from the liquid fraction by rotary evaporation. After filtration, the liquid fraction (black liquor) derived from hemicellulose hydrolysis, was freeze-dried and utilized as a carbohydrate source to provide 20 g/L or 120 g/L xylose in black liquor-containing MY medium (hereinafter referred to as MYBL-20 and MYBL-120, respectively).

2.3 Chemical analysis

Xylose, xylitol, arabitol, and ethanol in the supernatants of the cultures were quantified by HPLC with refractive index detector (1200 System, Agilent Technologies, Waldbronn, Germany) and Aminex HPX-87 H ion exclusion column. Isocratic elution was carried out at 60°C with 0.6 ml min^{-1} of 5 mM H_2SO_4 (Raimondi et al., 2014). The analytes were identified by comparison of the retention time with that of a standard solution.
Growth was monitored by measuring the turbidity at 600 nm (OD_{600}).

2.4 Statistical analysis

All values are means of three separate experiments. *t*-test and ANOVA followed by Tukey *post hoc* comparisons were utilized for the comparison of means. Differences were considered statistically significant for $P < 0.05$.

3. Results and discussion

3.1 Screening of yeast strains for growth and xylitol production on xylose

Fifty mesophilic ascomycetous yeasts were screened for the ability to grow in a medium containing xylose as the sole carbon source. All the strains were incapable of fermenting xylose in anaerobiosis and did not yield ethanol, xylitol, and arabitol under this condition. In aerobiosis, most of the strains were not able to grow at a detectable level while *C. freyschussii* ATCC 18737, *C. sake* ATCC 28138, *K. bacillosporus* ATCC 200960, *K. marxianus* WC 1402, *K. marxianus* WC 1405, *K. marxianus* WC 1411, *P. angusta* WC 1502, *P. fermentans* WC 1507, *W. anomalus* WC 1501 grew at some extent in 72 h of incubation (Table 1). For most of these strains, biomass production was generally significantly lower in xylose compared to glucose ($P > 0.05$), consistently with the extent of broth acidification. *K. bacillosporus* ATCC 200960 yielded the highest biomass concentration on xylose, reaching the same turbidity obtained on glucose. None of the yeasts yielded arabitol from xylose, even those strains that have previously been reported to produce arabitol from glycerol (Amaretti et al. 2018). *K. bacillosporus* ATCC 200960, *P. fermentans* WC 1507, and *W. anomalus* WC 1501 were the only strains that exhibited xylose generation and were thus selected for deeper investigation of xylitol production.

*Table 1: Screening of yeast strains for growth and xylitol production in MY medium containing 20 g/L xylose. Values were measured after 72 h of aerobic incubation. * indicates a statistically significant difference in growth, compared to glucose (t-test, n = 3); - indicates xylitol concentration < 0.1 g/L.*

	Glucose		Xylose		
Heading1	OD_{600}	pH	OD_{600}	pH	Xylitol (g/L)
C. freyschussii ATCC 18737	7.30	4.1	2.55 *	5.7	-
C. sake ATCC 28138	2.90	5.4	2.90	5.6	-
K. bacillosporus ATCC 200960	7.35	3.6	7.00	3.8	0.7
K. marxianus WC 1402	6.54	4.8	1.81 *	6.1	-
K. marxianus WC 1405	6.06	4.8	0.50 *	6.2	-
K. marxianus WC 1411	5.31	3.5	2.17 *	6.2	-
P. angusta WC 1502	9.96	3.6	3.20 *	6.1	-
P. fermentans WC 1507	8.57	3.6	5.01 *	5.8	1.4
W. anomalus WC 1501	8.36	3.4	4.66 *	6.5	0.5

3.2 Production of xylitol by selected strains in semi-synthetic medium

Since xylose conversion into xylitol is reported to be affected by osmotic stress or high substrate concentration (Tamburini et al. 2015), *K. bacillosporus* ATCC 200960, *P. fermentans* WC 1507, and *W. anomalus* WC 1501 were cultured with 20 g/L xylose and, at the entrance into stationary phase after 24 h, were given an additional 100 g/L xylose. These conditions were reported to trigger efficient arabitol production by *W. anomalus* WC 1501 (Amaretti et al., 2020; Raimondi et al., 2022) during stationary phase. The pulse had the effect of improving xylitol production by *K. bacillosporus* ATCC 200960 and *P. fermentans* WC 1507, while the effect on *W. anomalus* WC 1501 was minor (Figure 1). After 120 h of incubation, *P. fermentans* WC 1507 exhibited the highest ($P < 0.05$) conversion of xylose into xylitol ($Y_{P/S}$ = 61%), obtained for the consumption of 63 g/L xylose and the generation of 39 g/L xylitol.

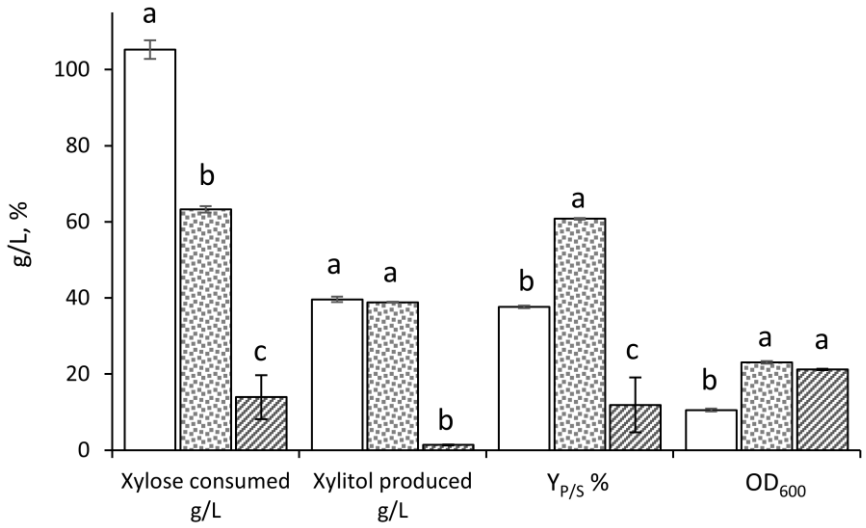

Figure 1: Effect of pulsing 100 g/L xylose to stationary-phase cultures of K. bacillosporus ATCC 200960 (white), P. fermentans WC 1507 (dotted), and W. anomalus WC 1501 (dashed). Xylose consumption, xylitol production, $Y_{P/S,}$ and OD_{600} are reported. Values are means ± SD, n = 3, within each series means with a common letter are not significantly different (ANOVA, with Tukey post hoc, P > 0.05).

Due to the highest $Y_{P/S}$, *P. fermentans* WC 1507 was the candidate strain, selected for further investigation. To investigate whether xylitol generation was growth associated or took place mainly during the stationary phase likewise arabitol production by *W. anomalus* WC 1501, cultures of *P. fermentans* WC 1507 where 120 g/L xylose were provided at the beginning, were compared to those that were enabled to grow with 20 g/L and were given a pulse of 100 g/L in the stationary phase. The cultures that received 120 g/L since the beginning performed significantly better (P < 0.05) than those that received the pulse, in terms of xylose consumed, xylitol generated, and xylose to xylose conversion yield (Figure 2). After 120 h xylose consumption had reached 88.8 g/L and xylose production 63.5 g/L, with a $Y_{P/S}$ of 71.5%. These results point out *P. fermentans* as a promising species in the panorama xylitol producing yeasts, to be utilized in biorefineries valorizing xylose-rich LCB hydrolysates. This species is still poorly investigated for xylose-to-xylitol production, even if it has non-pathogenic, safe, and fast-growing characteristics that make it attractive for industrial applications (Prabhu et al., 2020).

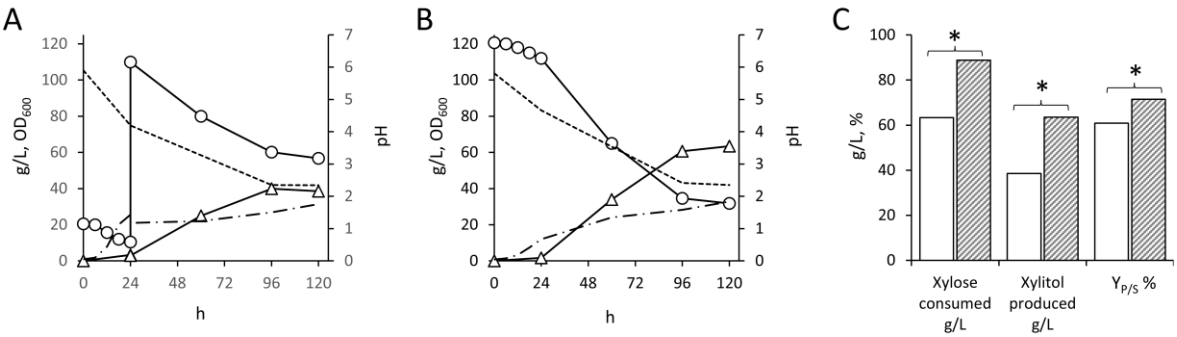

*Figure 2: Time course of xylose (circles), xylitol (triangles), OD600 (dash-dotted line), and pH (dotted line) in cultures of P. fermentans WC 1507 that received a pulse of 100 g/L xylose at the stationary phase (panel A) or that started with 120 g/L xylose (panel B). Xylose consumption, xylitol production, $Y_{P/S,}$ and OD_{600} in cultures that received concentrated xylose in the stationary phase (white) or at the beginning (dashed) are reported in panel C. Values are means, SD < 10%, n = 3, * indicates a significant difference (t-test, P < 0.05).*

3.3 Growth and xylitol production in black liquor

Shake flasks experiments were carried out to investigate the ability of *K. bacillosporus* ATCC 200960, *P. fermentans* WC 1507, and *W. anomalus* WC 1501 to grow and produce xylitol in MYBL-20 and MYBL-120 medium, where lyophilized black liquor was utilized in adequate amount to provide the medium with 20 or 120

g/L xylose, respectively. The three strains grew well in MYBL-20, yielding turbidity values ranging between 9.7 and 11.2 after 72 h of aerobic incubation. In this medium, the strains produced 1.7 to 3.4 g/L xylitol, the highest concentration being generated by *P. fermentans* WC 1507 ($P < 0.05$) (Figure 3). To the best of our knowledge, this is the first xylitol bioproduction report from hemp hurds hydrolysate by the species *P. fermentans*. The three yeast strains did not present any growth in MYBL-120, likely due to the presence of inhibitory substances derived from LCB pretreatment (Wei Kit Chin et al. 2020).

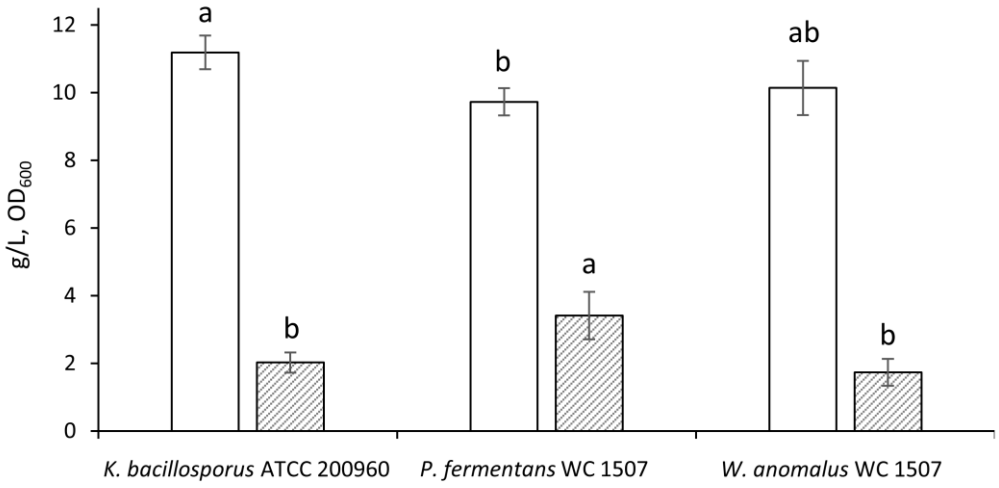

Figure 3: OD_{600} (white bars) and xylitol concentration (dashed bars) observed in cultures of K. bacillosporus ATCC 200960, *P. fermentans* WC 1507, and *W. anomalus* WC 1501, grown aerobically in MYBL-20 medium. *Values are means, SD < 10%, n = 3, means with a common letter are not significantly different (ANOVA, with Tukey post hoc, P > 0.05).*

4. Conclusions

The screening of the yeasts failed to obtain any candidate strain for producing ethanol or arabitol from xylose. Three strains were identified as xylitol producers, with *P. fermentans* WC 1507 presenting the most promising behavior, yielding 63.5 g/L xylose with a $Y_{P/S}$ of 71.5%. Preliminary fermentation experiments of the black liquor from organosolv pretreatment of hemp hurds indicated that, at the highest concentration, the liquor exerted an inhibitory effect on the growth of the yeasts strains. At the lowest concentration, *P. fermentans* WC 1507 grew well and produced xylitol in a comparable amount to the semisynthetic medium containing pure xylose. These promising results could lead to future perspectives in the biotechnological valorization of lignocellulosic hemp feedstock.

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