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Utilization of *Pachysolen tannophilus* and *Pichia kudriavzevii* for the production of xylitol on undetoxified corn cob hydrolysates

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Abstract. Xylitol is a natural polyol with broad applications in the food and pharmaceutical industries. However, its large-scale production through chemical means is still an expensive and not environmentally friendly process. Therefore, great attention has been paid to low-cost and renewable substrates like corn cobs (CC), which can be utilized to improve the economic outlook of xylitol production. In this study, CC were used as a feedstock for xylitol production, with the help of yeasts and filamentous fungi. The results obtained in this study showed that the amount of xylitol produced from CC hydrolysate was similar to the amount of xylitol obtained on xylose substrate. Overall, yeast produced higher amounts of xylitol than filamentous fungi. *Pachysolen tannophilus* had the highest xylitol production at pH 5.0, 72 h fermentation time, substrate concentration 15%, and inoculum size 1.5×10^8 cfu mL^{-1}, while *Pichia kudriavzevii* performed better at pH 5.0, with a 72 h fermentation time, substrate concentration of 20%, and inoculum size of 2.5×10^8 cfu mL^{-1}. When comparing the combined optimal parameters with and without supplementation, supplementation with 1.5% methanol, has increased the xylitol production of *P. tannophilus* and *P. kudriavzevii* by 31% and 18.6%, respectively. These findings demonstrate the robustness of these yeast strains for sustainable and cost-effective xylitol production from CC waste.

Key words: corn cob hydrolysate, *P. tannophilus*, *P. kudriavzevii*, xylose, xylitol production.

INTRODUCTION

Xylitol is a polyol composed of five carbon atoms (Cristobal-Sarramian & Atzmüller, 2018). It can be used for various applications, such as a building block for organic synthesis, as a sugar substitute in the management of diabetes, and as a sweetener in food industries (Mathew et al., 2018; Baptista et al., 2021).

Xylitol can be produced from various lignocellulosic residues, such as wheat straw/bran, rice straw, sugarcane, corncobs, corn stalks, and corn stover (Ribeiro et al. 2016; Venkateswar Rao et al., 2016; Bedő et al., 2021). These feedstocks are commonly composed of three main polymers: hemicellulose (20–40%), cellulose (40–60%), and lignin (10–25%) (Rocha-Meneses, 2019). The conversion of cellulose and hemicellulose into xylitol is commonly done by hydrolysis and hydrogenation processes (Hilpmann et al., 2018).

The hydrogenation process uses hydrogen gas and heterogeneous catalysts to convert xylose into xylitol (Ayubi et al., 2021; Lu et al., 2021). Some of the heterogeneous catalysts commonly employed in this process include nickel, ruthenium, rhodium, platinum, palladium, and copper (Carvalho, 2021). The hydrogenation process occurs at temperatures between 80–130 °C and pressures between 40–70 bar (Hilpmann et al., 2018).

Delgado-Arcaño et al. (2021) investigated the conversion of hemicellulose from corncob into xylitol using hydrolysis-hydrogenation processes. The results obtained in their study show that it is possible to simultaneously hydrolyze and hydrogenate CC with the help of Ru catalysts. The authors concluded that it is possible to make the process cost-effective by decreasing the reaction times and the energy input requirements. Another study by Ribeiro et al. (2017) utilized corncob for the simultaneous conversion of cellulose and xylan. The authors were able to achieve 75% yields of sorbitol and 77% of xylitol in a six hours reaction. The authors concluded that this strategy can efficiently maximize conversion yields. Ahuja et al. (2022) studied the detoxification process of corncob hydrolysates for further xylitol production. For this, activated carbon was used in the detoxification process. The results obtained in this study show that removing 93% of furfurals and 94% of phenolic compounds is possible. The detoxified material can be further converted into xylitol by means of fermentation. The authors obtained 122.47 g L^{-1} of xylitol fermentation, with a selectivity of 95%, and concluded that it is possible to reduce the operational costs of xylitol production by approximately 38%. Although the chemical synthesis of xylitol is a very attractive process, it still has energetic, environmental, and economic limitations mainly due to the complex separation and purification steps that require the utilization of expensive catalysts, high pressures, and temperatures (Queiroz et al., 2022). Therefore, there is a search for alternative biotechnological processes that will make the chemical synthesis of xylitol cheaper and environmentally friendly (Queiroz et al., 2022). As a result, great attention has been paid to the utilization of corn cobs (CC) as a feedstock for xylitol production due to their low-cost, vast abundance, renewability, and high cellulose and hemicellulose content (Mohlala et al., 2016). However, the majority of the studies currently available focus mainly on the conversion of cellulose derivates for xylitol production, and there are limited studies on the conversion of the hemicellulose fraction CC into xylitol (Baptista, 2018).

In this study, robust and efficient microbial strains (yeasts and filamentous fungi) were isolated and analyzed for the conversion of undetoxified CC hydrolysates into xylitol. The optimum operational parameters that produced the highest xylitol concentrations were identified, seeking an improvement of the economic outlook of xylitol production.

MATERIALS AND METHODS

Isolation and identification of microorganisms

Three filamentous fungi (*Rhizopus stolonifer, Aspergillus niger, Penicillium digitatum*) and three yeasts (*Pachysolen tannophilus, Pichia kudriavzevii,* and *Saccharomyces cerevisiae*) were isolated from soil samples and wood shavings and used in the experiments.

The soil samples and wood shavings were obtained from sawdust dump sites located at sawmills in Tanke, Ilorin, Nigeria. The soil samples were diluted with physiological saline, and the suspension was homogenized in a shaker at 150 rpm for 3 h. Aliquots (1 mL) of 10^{-1} to 10^{-3} serial dilutions were inoculated in agar plates containing xylose medium using the spread plate method. The medium had the following composition: xylose 20 g L^{-1}, peptone 15 g L^{-1}, ammonium sulphate 1 g L^{-1}, KH_2PO_4 1 g L^{-1}, $MgSO_4$ 0.5 g L^{-1}, yeast extract 10 g L^{-1}, and agar 20 g L^{-1} (Zhang et al., 2013). The plates were incubated at room temperature for 2 days, and distinct colonies were sub-cultured on fresh plates of the xylose medium until pure cultures of the isolates were obtained and transferred to agar slants of the xylose medium. Identification of the isolates was carried out using molecular techniques as described by Altschul et al. (1997).

Substrate preparation and hydrolysis

Corn cobs collected from a food waste dump site were dried in the sun until the moisture content was reduced to about 10%. The dried CC were crushed to a particle size of about 15 mm. The CC were hydrolyzed with 1% H_2SO_4 at 15% (w/v) solid loading for 60 min at 121 °C. The hydrolysates were then neutralized using 1N NaOH.

Fermentation

The inoculum was prepared by washing off spores of a fully-sporulated (5-day old) culture of each isolated fungus from potato dextrose agar slant using sterile distilled water. Colonies from a 48-hour old culture were suspended in sterile distilled water for the yeast isolates. The spore and cell suspensions were adjusted to a concentration of about 1.0×10^5 spores mL^{-1} or cfu mL^{-1} for fungi and yeast, respectively. Inoculum size was set using the improved Neubauer haemocytometer (Narasimha et al., 2006). The fermentation medium was adjusted according to Srivani & Pydi Setty (2012) method with 15% (w/v) of D-xylose or CC as separate carbon sources in the media. The hydrolyzed corncob was supplemented with nutrients and mineral salts with the following composition: yeast extract (2 g), $MgSO_4.7H_2O$ (0.4 g), KH_2PO_4 (5 g), and $(NH_4)_2SO_4$ (2 g). The initial medium pH was set at 5.0. In the batch fermentation, 2.0×10^8 spores cfu^{-1} per mL of isolated yeasts or fungi were used on 100 mL of each fermentation medium (D-xylose vs. CC hydrolysate) at pH 5. The fermentation was performed in 250 mL Erlenmeyer flasks on a rotary shaker (LH Fermentation, Model Mk V orbital shaker) at 150 rpm for 120 hours. The amount of xylitol produced was measured every 24 h.

Optimization of xylitol production

The following parameters were varied to know the effects on xylitol production: CC hydrolysate concentration (5–25% w/v), pH (3–7), inoculum size

(1.0×10^8–3.0×10^8 cfu mL^{-1}) and fermentation time (24–120 h), using the 2 highest xylitol producers on CC hydrolysates (the constant conditions used are 15% (w/v) CC hydrolysate, pH 5, inoculum size 2.0×10^8 cfu mL^{-1}, temperature 25 °C and fermentation time of 120 h.

Methanol supplementation

The effect of 1.5% (v/v) methanol supplementation on each isolate was monitored using combined optimized parameters. (El-Batal & Khalaf, 2004). The initial pH was maintained at pH 5, and the flasks were incubated with the spore suspensions of the isolated organisms. Batch fermentation was performed at 25 °C with an agitation speed of 150 rpm for 168 h. Samples were assayed for xylitol content at 24 h intervals.

Xylitol assay

Fermentation samples were centrifuged at 150 rpm for 30 min at 25 °C, and the supernatant was analyzed for xylitol content by spectrophotometric measurement at 412 nm (Searchtech 752N UV-VIS) as described by Sánchez (1998). A 0.5 mL volume of 0.5 M formic acid was added to 1 mL of the sample in a glass tube. Then, 1 mL of 5 mM sodium periodate was added to the solution and vortexed. After vortexing, the glass tube was exposed to room temperature for 15 seconds, after which a 1mL solution comprising 0.1 M acetylacetone, 2 M ammonium acetate, and 0.02 M sodium thiosulfate was added. The tube was sealed and boiled in water for 2 minutes. Next, it was cooled under running water, and the absorbance was measured at 412 nm. The amount of xylitol was calculated from a xylitol standard curve, which was obtained by conducting the assay under similar conditions using standard concentrations of xylitol (99% purity).

Statistical analysis

Statistical significance was measured using ordinary one-way analysis of variance (ANOVA), while Duncan's multiple comparisons test established multiple comparisons between means. The statistical analysis was performed in the software IBM SPSS and SigmaPlot for Windows version 10.0 (SysStatSoftwares Inc.). All the data are expressed as means of triplicates ± SEM, and values of $p < 0.05$ were considered significant.

RESULTS AND DISCUSSION

Screening of isolates for xylitol production on xylose and CC hydrolysate

Fig. 1 shows the production of xylitol on xylose-containing media using isolated organisms. As it can be seen from the figure, the utilization of filamentous fungi gives lower xylitol concentrations than when yeasts are utilized. For instance, *A. niger P. digitatum* and *R. stolonifera* had a relatively low xylitol production, with their concentrations being 0.07–0.79 g L^{-1}, 0.23–0.80 g L^{-1}, and 0.17–1.57 g L^{-1}, respectively. These results are similar to the findings reported by Sampaio et al. (2003). In their experiments, *Aspergillus* and *Penicillium* sp. yielded small amounts of xylitol in xylose-containing media. In addition, Kang et al. (2016) showed that only trace quantities of xylitol were produced during fermentation with *Aspergillus* sp. A rationale for the low accumulation of xylitol by filamentous fungi could be a result of the discrepancy between the oxygen requirement that advances xylitol accumulation and the oxygen

required for fungal growth. Most fungi are obligate aerobes, while xylitol production and accumulation favour microaerobic or reduced conditions.

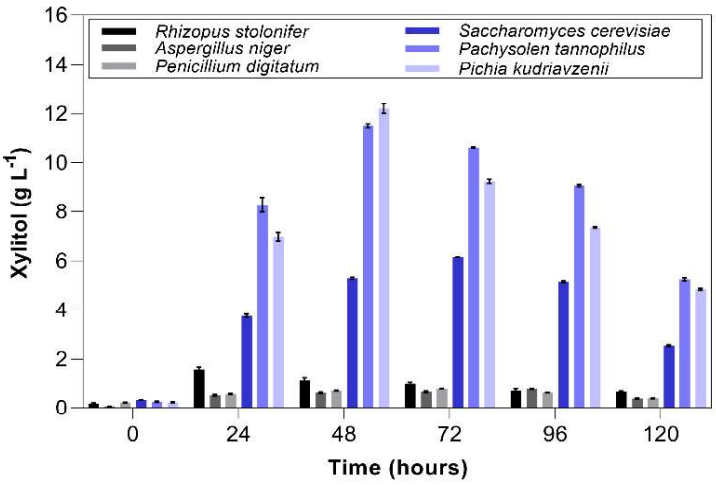

Figure 1. Production of xylitol using isolated organisms by submerged fermentation on xylose-containing media.

On the other hand, the three different types of yeasts utilized in this study had more appealing results, being 0.34–6.15 g L^{-1} for *S. cerevisiae,* 0.26–11.50 g L^{-1} for *P. tannophilus,* and 0.23–12.21 g L^{-1} for *P. kudriavzenii*. *R. stolonifera* achieved its highest xylitol production at 24h, while *A. niger* reached its highest concentrations after the 96 h reaction. *P. digitatum* and *S. cerevisiae* reached their highest xylitol production at 72 h, and *P. tannophilus* and *P. kudriavzenii* at 48 h of reaction.

The yeasts *P. tannophilus* and *P. kudriavzevii* were selected as the best xylitol-producing strains. Despite growing on undetoxified hydrolysate, the yeasts utilized in this study produced xylitol concentrations higher than those reported in previous studies (Gong et al., 1981; Barbosa et al., 1988; Dahiya et al., 1991; Vandeska et al., 1995), which confirms their relatively high tolerance for inhibitors present in the hydrolysate. *P. tannophilus* has been shown to tolerate up to 2 g L^{-1} of furfural in a xylose broth (Yang et al., 2012).

Fig. 2 shows xylitol production by submerged fermentation using isolated organisms on CC hydrolysate. Similar to Fig. 1, filamentous fungi had lower xylitol concentrations than when yeasts were utilized. For instance, *R. stolonifera*, *A. niger*, and *P. digita*tum had xylitol concentrations between 0.55–0.82 g L^{-1}, 0.58–1.23 g L^{-1}, and 0.35–1.71 g L^{-1}, respectively. When *S. cerevisiae* was used as yeast in the fermentation process, the xylitol concentrations varied between 0.71–5.82 g L^{-1}, while *P. tannophilus* produced 0.38–12.15 g L^{-1}L, and *P. kudriavzenii* 0.44–12.02 g L^{-1}. As shown in Fig. 2, *P. tannophilus* and *P. kudriavzenii* had similar amounts of xylitol on both xylose-containing media and CC hydrolysate. Their xylitol concentrations were significantly higher ($p < 0.05$) than that of the filamentous fungi *A. niger* and *P. digitatum.*

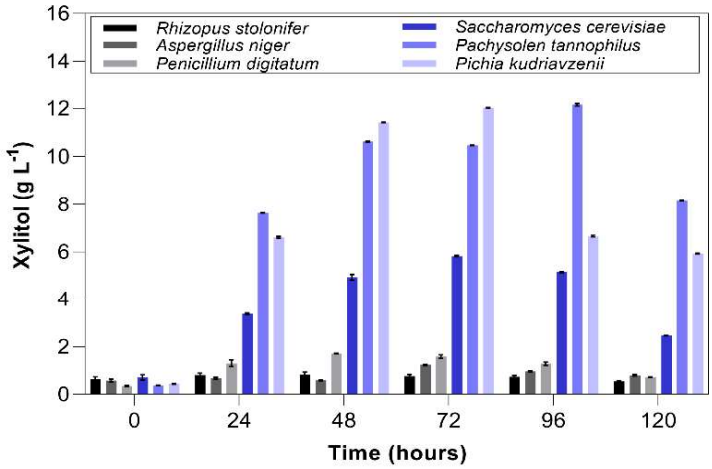

Figure 2. Production of xylitol by submerged fermentation using isolated organisms on CC hydrolysate.

Optimization of xylitol production on CC hydrolysate

The optimization experiments were performed under the following constant parameters: pH 5, inoculum size 2.0×10^{8} cfu mL^{-1}, and 15% (w/v) CC hydrolysate.

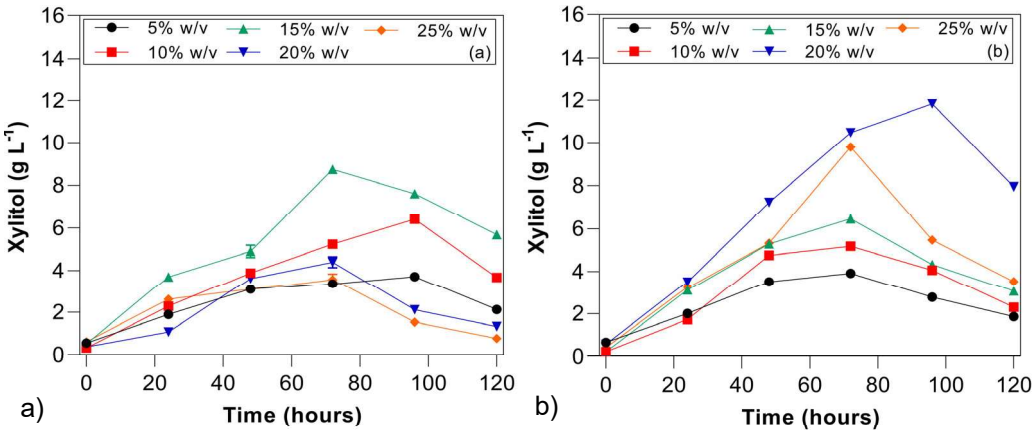

Figure 3. Effect of varying substrate concentration on xylitol production by (a) *P. tannophilus* and (b) *P. kudriavzevii* using CC hydrolysates.

Fig. 3 shows the effect of CC concentration on xylitol production using *P. tannophilus* and *P. kudriavzevii*. As can be seen from the figure, *P. tannophilus* had the highest xylitol production (8.75 g L^{-1}) at a substrate concentration of 15%, while *P. kudriavzevii* achieved its highest xylitol concentrations (11.83 g L^{-1}) at a substrate concentration of 20% w/v. Both organisms had a large drop in the xylitol production when substrate concentrations higher than 15% w/v and 20% w/v were used. These results can be caused by substrate inhibition since research has shown that substrate inhibition affects xylitol productivity by limiting the specific growth rate of the yeasts. This inhibition depends on the type of yeast and the complex nature of hemicellulose,

and the multiple compounds present upon sugar breakdown, including aliphatic or phenolic acids, furaldehydes, and other weak acids. It is known that many of these compounds inhibit the growth of the microorganisms during conversion to xylitol (Queiroz et al., 2022).

The influence of pH on the production of xylitol using *P. tannophilus* and *P. kudriavzevii* on CC hydrolysate is shown in Fig. 4. As can be seen from the figure, the maximum xylitol concentration was recorded at pH 5.0 for both *P. tannophilus* and *P. kudriavzevii* showing xylitol amounts of 8.84 g L^{-1} and 11.74 g L^{-1}, respectively. The initial pH of the fermentation medium seemed to be an influential factor in the growth of the isolates, their physiological activities, and the compounding of xylitol in the fermentation medium. From 24 h of fermentation, *P. tannophilus* and *P. kudriavzevii* have shown a significant increase ($p < 0.05$) in the xylitol concentration produced at pH 5.0. These observations are supported by Kresnowati et al. (2016), which reported that the optimum initial pH of cultivation is highly yeast dependent.

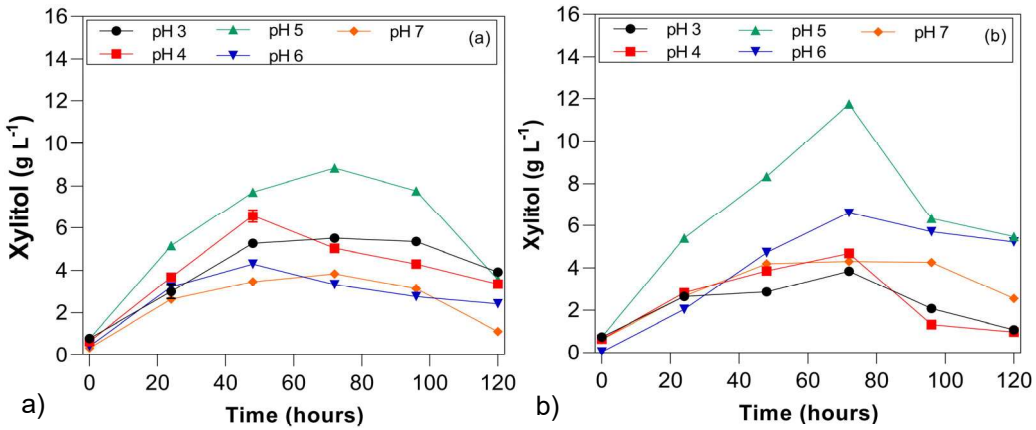

Figure 4. Effect of varying pH on xylitol production by (a) *P. tannophilus* and (b) *P. kudriavzevii* using CC hydrolysates.

The influence of the inoculum size on xylitol concentrations of *P. tannophilus* and *P. kudriavzevii* is shown in Fig. 5. As it can be seen from the figure, *P. tannophilus* had the highest xylitol concentration (12.82 g L^{-1}) when an inoculum size of 1.5×10^8 cfu mL^{-1} was used. At the same time, *P. kudriavzevii* achieved its higher value with an inoculum size of 2.5×10^8 cfu mL^{-1}. Compared to 1.0×10^8 cfu mL^{-1}, *P. tannophilus* had a spike in xylitol concentration at 1.5×10^8 cfu mL^{-1}, which is dissimilar to the steady increase in xylitol production seen in *P. kudriavzevii* as the inoculum size increased. Although observations reported in *P. kudriavzevii* align with some works that report an increase in inoculum concentration to a linear rise in xylitol production, Manjarres-Pinzón et al. (2021) found that xylitol is a metabolite directly associated with cell density. Kresnowati et al. (2016) also reported that xylitol concentration increased, and biomass growth and concentration reduced markedly over an initial concentration range of 2×10^7 cells mL^{-1} to 6×10^7 cells mL^{-1}. When yeast cell concentration was hiked to 6×10^7 cells mL^{-1}, 0.102 g L^{-1} xylitol was derived from D-xylose. This characteristic was also replicated

with *Debaryomyces hansenii* (Kresnowati et al., 2016). Therefore, further studies are required in order to identify the optimum inoculum size for each microorganism.

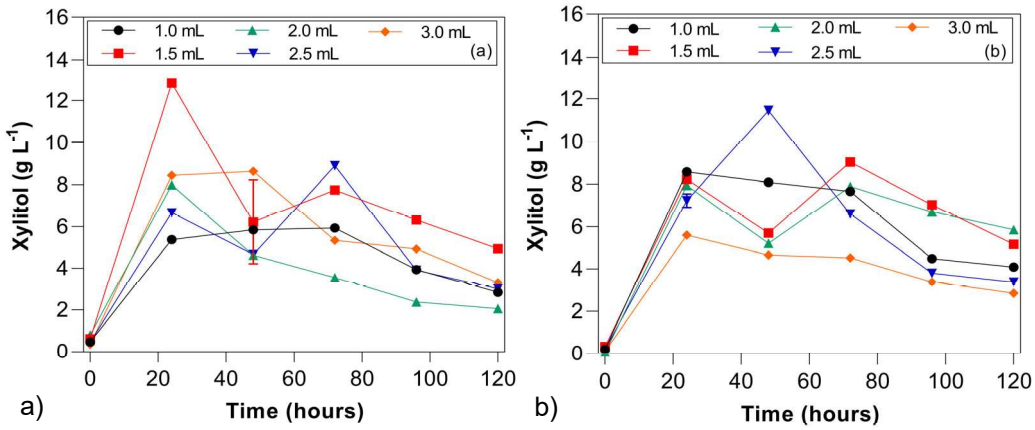

Figure 5. Effect of the inoculum size on xylitol production by (a) *P. tannophilus* and (b) *P. kudriavzevii* using CC hydrolysates.

The effect of fermentation time on xylitol concentrations is reported in Fig. 6. As it can be seen from the figure, there was a very high xylitol production at 96 h and 72 h by *P. tannophilus* and *P. kudriavzevii*, respectively. These results align with the findings of Jeevan et al. (2011). The authors reported that *Pichia* sp. had the highest xylitol accumulation at 72 h in synthetic and corncob hemicellulosic media. Interestingly, the media used by Jeevan et al. (2011) was detoxified using pH adjustment and charcoal adsorption before fermentation, unlike what was used in our study. Similar studies were reported by Narisetty et al., 2021., where *P. fermentans* was used in undetoxified sugarcane bagasse hydrolysate media for xylitol production. Under these conditions, the authors achieved a maximum xylitol production after 192 h of fermentation. They concluded that the rate of xylitol production by yeasts is influenced by inhibitors and the high glucose to xylose ratio in the hydrolysate.

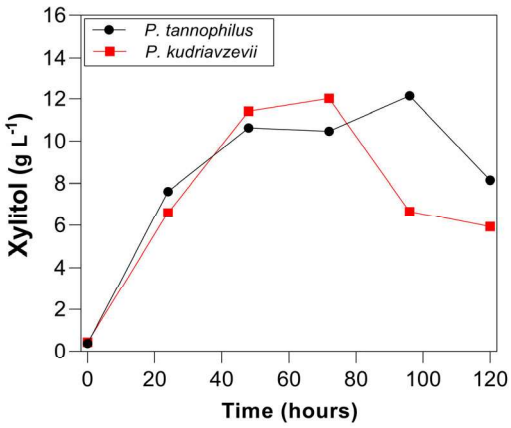

Figure 6. Effect of varying fermentation time on the production of xylitol on CC hydrolysates.

Fig. 7 shows the xylitol production by *P. tannophilus* and *P. kudriavzevii* on CC hydrolysates supplemented with 1.5%v/v methanol. As seen from the figure, without methanol supplementation, *P. tannophilus had a* xylitol production of 9.07 g L^{-1}. However, when supplemented with 1.5% methanol under combined optimal parameters, the xylitol concentrations increased to 11.9 g L^{-1}, a 31.0% increase. On the other hand,

P. kudriavzevii had a xylitol concentration of 11.6 g L^{-1} in non-supplemented conditions and 13.7 g L^{-1} with methanol supplementation, a 18.6% increase.

Overall, the xylitol production in pre-optimization experiments was higher than combined optimal conditions. However, the addition of methanol highly improved xylitol concentrations in both yeasts. El-Batal & Khalaf (2004) observed that all concentrations (0.5 to 10%) of methanol aaded to the fermentation medium resulted in increased xylitol production by *Candida tropicalis* compared to the control medium which had no methanol supplementation. This increase in concentration with supplementation can be due to the oxidation of methanol providing NADH, which is one of the co-factors that drive xylitol accumulation to the medium (Boontham et al., 2014). Xylitol concentrations increased due to the optimization experiments, and *P. kudriavzevii* was more influenced by optimized factors than *P. tannophilus*. These results suggest that the starting parameters utilized in the fermentation process were more conducive for the growth and xylitol production of *P. tannophilus* than *P. kudriavzevii.*

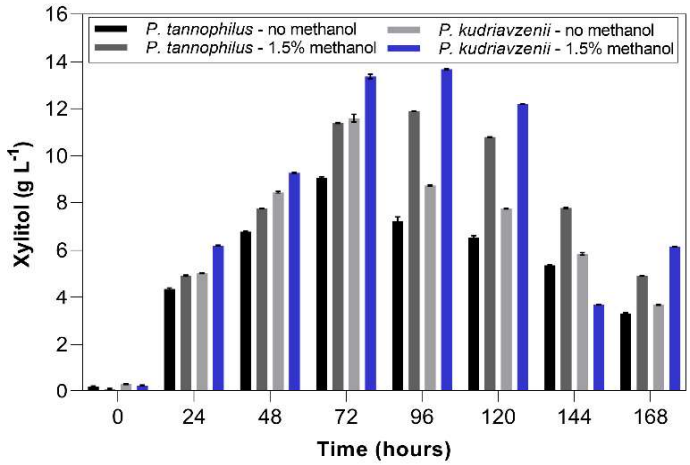

Figure 7. Xylitol production by *P. tannophilus* and *P. kudriavzevii* on CC hydrolysates supplemented with 1.5%v/v methanol.

CONCLUSIONS

In this study, undetoxified CC hydrolysates were used for xylitol production with the help of isolated strains of yeasts and fungi. In screening experiments, the isolated wild-type yeasts and filamentous fungal strains produced similar amounts of xylitol in xylose-containing and CC hydrolysate media. The yeasts *P. tannophilus* and *P. kudriavzevii* produced significantly higher amounts of xylitol than the filamentous fungi, demonstrating the robustness of these yeasts.

The optimal fermentation conditions for *P. tannophilus* and *P. Kudriavzevii* were reported at pH 5.0, 72 h fermentation time, substrate concentration of 15% and 20%. The optimum inoculum size for *P. Tannophilus* was 1.5×10^8 cfu mL^{-1}, and for *P. kudriavzevii* 2.5×10^8 cfu mL^{-1}. Under these conditions, when supplemented with

1.5% methanol, *P. tannophilus* and *P. kudriavzevii* enhanced their xylitol concentration by 31.0% and 18.6%, respectively.

These results suggest that removing the substrate detoxification step can help achieve sustainable and cost-effective pathways for xylitol production. Another interesting implication of this study is that it demonstrates dual prospects of the overall process since using agricultural waste can significantly reduce the environmental impacts caused by the utilization of specifically grown feedstocks.

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