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Evaluation of Fermentative Xylitol Production Potential of Adapted Strains of *Meyerozyma caribbica* and *Candida tropicalis* from Rice Straw Hemicellulosic Hydrolysate

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Abstract: Dilute acid hydrolysis of lignocellulosic biomass generates inhibitors in the hydrolysate which hamper yeast metabolism and the fermentation process. Therefore, understanding the effect of these compounds on the performance of microorganisms becomes essential to achieve improved product yields. In this study, the effect of acetic acid, furfural, and hydroxymethylfurfural was evaluated on yeast growth and fermentation efficiency. Various parameters for the pretreatment of rice straw, such as an acid catalyst, and its concentration and residence time, were optimized for the maximum liberation of sugars in the hydrolysate. Further, the yeast strains *Candida tropicalis* and *Meyerozyma caribbica* were adapted for the tolerance of inhibitors at higher concentrations. A comparative analysis was carried out using un-adapted and adapted strains of *Candida tropicalis* and *Meyerozyma caribbica* for xylitol production. The findings of this study revealed that sulfuric acid (1.25% *v*/*v*) at 121 °C for 30 min can efficiently convert rice straw xylan to xylose, with the release of 16.07 g/L xylose in the hydrolysate. Further, the adaptation results showed an increase of 76.42% and 69.33% in xylose assimilation by *C. tropicalis* and *M. caribbica*, respectively. The xylitol production with the adapted *C. tropicalis* was increased by 7.54% to 28.03 g/L xylitol. However, the xylitol production with the adapted *M. caribbica* was increased by 8.33%, yielding 26.02 g/L xylitol in the non-detoxified hydrolysate when compared to the un-adapted strains. Repeated batch fermentation was carried out for seven batches, and xylitol was found to be efficiently produced by the yeasts during five successive batches without any significant loss in the xylitol yield. Moreover, the results suggest that *M. caribbica* is a promising microorganism for the transformation of rice straw-derived xylose to xylitol.

Keywords: xylitol; rice straw; shake flask fermentation; yeast adaptation; dilute acid hydrolysis; fermentation inhibitors

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1. Introduction

Lignocellulosic biomass is abundantly available as low-cost, renewable, and sustainable energy source rich in carbohydrates (cellulose and hemicellulose) and non-carbohydrate fractions. In the present global scenario, tremendous efforts have been made to efficiently utilize the carbohydrate fraction of biomass to foster the bioeconomy. The bioeconomy, as a broad spectrum, offers a wide range of opportunities for the conversion of biomass to value-added compounds, such as biofuels, biochemicals, and bioenergy [1]. Among various forms of lignocellulosic biomass, rice is the second-largest crop grown in India, with a production rate of 103 million tonnes. The global paddy production is escalating significantly with the increase in demand and population. The rising demand for rice is a cause of concern, because the rice harvesting process generates a significant amount of waste in the form of straws and husks [2]. Rice straw is mainly composed of cellulose (35%), hemicellulose (18%), and lignin (15%) [2,3]. These macromolecules can be converted to valuable biomolecules such as ethanol, xylitol, furfural, phenolic, etc. [3,4].

Xylitol, a naturally occurring pentitol, is classified as one of the most versatile platform chemicals that can be obtained from biomass. It is used as a crucial ingredient in numerous products manufactured by the food, pharmaceutical, and odontological industries, due to its low glycemic index, insulin-independent metabolism, and anti-cariogenic nature [5]. The demand for xylitol has steadily risen over the years, and its global market share is projected to expand from an estimated value of USD 1190.12 million in 2021 to USD 1475.87 million by the 2030s, at a CAGR of about 6% [6]. Commercially, the demand for xylitol is fulfilled by the chemical hydrogenation of xylose at high pressure and temperature in the presence of chemical catalysts such as Ni, Ru, Pt, and Pl. However, this process is eco-unfriendly, energy-intensive, and expensive, as it requires toxic catalysts, extreme reaction conditions, and a series of complicated purification procedures to separate the xylitol from other by-products formed during the production process [7,8]. The biotechnological route of xylitol production using microorganisms, such as yeasts and bacteria, serves as a promising alternative to the chemical hydrogenation process due to several advantages, such as their environment-friendly nature, low energy requirement, high product yield, and relatively easier process [5,9].

Among different microorganisms, yeasts such as *Candida* and *Debaromyces* are regarded as efficient xylitol producers, due to their high xylose assimilation rates and xylitol productivity [10]. *Candida tropicalis* has been an extensively investigated yeast for xylitol production due to its ability to utilize pentoses, high tolerance to inhibitors, and propensity to thrive in all kinds of biomass-derived hemicellulosic hydrolysates [1–5,11]. Moreover, *Meyerozyma caribbica*, a mesophilic yeast that is part of the same family as *Candida*, i.e., Debaromycetaceae, is gaining popularity in biorefineries, due to its capability to utilize a variety of carbon sources from lignocellulosic biomass [12,13]. According to recent studies, it is reported to be an efficient pentose-fermenting yeast, with xylitol as the main product of pentose metabolism [14,15].

One of the key aspects of xylitol production via the biotechnological route is the pretreatment of biomass for the solubilization of hemicellulosic sugars. Acid hydrolysis is frequently used for the pretreatment of biomass because of its low cost, high reaction rates, and ability to recover approximately 70–95% of hemicellulosic sugars [16]. However, the process also generates inhibitory compounds, such as acetic acid, furfural, and 5-hydroxymethylfurfural, which adversely affect the growth of microorganisms and thereby reduce the overall fermentation performance [17,18]. Several approaches have been proposed in the literature for mitigating the toxicity of these inhibitors on microorganisms, such as the adaptation of microbial strains, entrapment of microbial cells, and detoxification of hydrolysates by activated charcoal, ion-exchange resins, and over-liming [8,19–21]. The adaptation of microorganisms has been described as an efficient method for increasing the natural tolerance of microorganisms by pre-exposing them to non-lethal concentrations of inhibitors. As compared to un-adapted strains, the use of adapted strains can increase fermentation yields and productivity, even in high concentrations of inhibitors [22]. The implementation of adaptation strategies has been reported in previous studies for ethanol production [23,24].

Keeping the above points in view, the present study was aimed at assessing the efficacy of a minimally explored yeast, *M. caribbica*, in comparison to the most widely used yeast, *C. tropicalis*, for conversion of rice straw-derived xylose to xylitol. The effect of inhibitors on xylitol production was studied in the presence of acetic acid, furfural, and hydroxymethyl furfural. Furthermore, in order to enhance the natural tolerance of yeasts towards inhibitory compounds in the hydrolysate, an adaptation strategy was employed. Xylitol production by both un-adapted and adapted strains of *M. caribbica* and *C. tropicalis* was carried out.

2. Materials and Methods

2.1. Chemicals and Biomass

All the chemicals and media used in the present study were of analytical or commercial grade and procured from Sigma-Aldrich, Co., Denmark, and HIMEDIA laboratories,

Mumbai, India unless specified otherwise. Rice straw (RS) biomass was collected from local fields adjoining the Center of Innovative and Applied Bioprocessing, Mohali, India. The biomass was shredded with a shredder, followed by oven-drying at 70 °C for 48 h. The compositional analysis of the dried RS used was the same as suggested in a previous study [25].

2.2. Microorganisms Used

Two in-house isolated strains, including *Candida tropicalis* OK165575 [25] and *Meyerozyma caribbica* MZ057612 [26], were used to evaluate their efficiency for xylitol production from D-xylose, as well as xylose derived from the RS hemicellulosic hydrolysate. The yeast strains were maintained on yeast extract 10 g/L, peptone 20 g/L, and xylose 20 g/L (YPX) agar plates and stored at 4 °C.

2.3. Optimization of Dilute Acid Pre-Treatment of RS

The acid hydrolysis of the RS was carried out to solubilize the maximum concentration of fermentable sugars in the hydrolysate under different parameters, such as acid catalysts (HCl, H_2SO_4 HNO_3, CH_3COOH, H_3BO_3, and H_3PO_4), acid concentration (0.5%, 1%, 1.25%, 1.5%, and 2%), and residence time (15 to 60 min), in an autoclave. First, the shredded RS biomass was mixed with an acidic solution in 1:10 (solid-to-liquid ratio) at room temperature. The slurry prepared was then introduced to the reaction vessel and pre-treatment was carried out at a temperature of 121 °C and contact time of 30 min. The biomass was also treated with hot water, without adding the reagent, with the same conditions of temperature and time, to serve as the control set. The solid and liquid fractions were separated by centrifugation at 8000 rpm for 15 min. Further, the supernatant was neutralized and analyzed for the presence of sugars (glucose, xylose, and arabinose) and inhibitory compounds (acetic acid, furfural, and hydroxymethylfurfural) using HPLC.

2.4. Large-Scale Pre-Treatment of RS

The best-chosen parameters obtained after optimization were used to carry out the final hydrolysis. One kilogram of RS was mixed with an acidic solution and autoclaved at the optimized process conditions. The vessel containing the hydrolyzed biomass was cooled immediately to terminate undesirable reactions. Subsequently, the solid and liquid fractions were separated using a muslin cloth, followed by vacuum filtration with Whatman filter paper no. 1 (pore size = 11 microns). The filtrate obtained was analyzed by HPLC to determine the content of reducing sugar and fermentation inhibitors. The hydrolysate was stored at 4 °C for further use.

2.5. Preparation of RS-Derived Fermentation Medium

The RS hydrolysate was separated into two different fractions for the preparation of detoxified and non-detoxified hydrolysates. The detoxified hydrolysate was prepared by using activated charcoal. The activated charcoal 2.0% (*w*/*v*) was added to the hydrolysate and incubated at 30 °C, 200 rpm, for 1 h. The activated charcoal was removed by vacuum filtration using Whatman filter paper no. 1. Further, the pH of the hydrolysate was adjusted to 5.5 using calcium oxide. However, the non-detoxified hydrolysate medium of pH 5.5 was obtained by using CaO. The neutralized hydrolysates were filtered using vacuum filtration, and the clear filtrates obtained were then analyzed for the estimation of fermentable sugars and inhibitors. The hydrolysates were concentrated using a vacuum evaporator at 70 °C to reach a xylose concentration > 50 g/L, an appropriate concentration for efficient conversion by the isolates into xylitol, as optimized [1–5,25].

2.6. Inoculum Preparation

The inocula of the experimental yeasts were prepared by aseptically transferring a loopful of cells from YPX agar plates to 20 mL of YPX broth medium in 100 mL conical flasks. The flasks were then incubated at 30 °C and 150 rpm for 24 h in a rotary shaker.

2.7. Effect of Toxic Inhibitors on Fermentation Efficiency of C. tropicalis and M. caribbica

The ability of both yeasts to tolerate inhibitory compounds during xylitol fermentation was evaluated. The fermentation medium was composed of ammonium sulphate 1 g/L, yeast extract 1 g/L, magnesium sulphate heptahydrate 0.08 g/L, disodium orthophosphate 0.2 g/L, potassium dihydrogen phosphate 0.2 g/L, glucose 6 g/L, and xylose 50 g/L. Both the yeasts were inoculated in the fermentation medium containing the aforementioned components and synthetic inhibitory compounds, such as furfural (1–5 g/L), HMF (1–5 g/L), and acetic acid (2–10 g/L), which were present in the acidic hydrolysate as a result of degradation. The acetic acid was added to the medium at different pH values (4.5, 5.5, and 6.5) to assess its effect on the microorganism in different conditions. All the fermentation experiments were carried out in 250 mL Erlenmeyer flasks containing 100 mL of medium, followed by incubation at 30 °C, 150 rpm for 72 h in a rotary shaker. The samples were withdrawn periodically to estimate the xylose consumption and xylitol production.

2.8. Adaptive Evolution of Yeasts in RS Hemicellulosic Hydrolysate

The ability to produce xylitol by adapted yeasts was further assessed in hydrolysate containing biomass-derived xylose. The fermentation experiments were performed in 500 mL Erlenmeyer flasks with 200 mL non-detoxified and detoxified RS hydrolysate, supplemented with the same medium components used in the previous experiments. The cultures were incubated at 30 °C, 150 rpm for 24 h in a rotary shaker. The yeast cells, which were separated by centrifugation at 8000 rpm for 15 min, were transferred to a fresh fermentation medium. The samples were withdrawn periodically after every 24 h to estimate the xylitol production and xylose consumption.

2.9. Evaluation of Fermentation Potential of Un-Adapted and Adapted Yeasts

The ability of un-adapted and adapted strains of *C. tropicalis* and *M. caribbica* to convert commercial xylose and RS-derived xylose to xylitol was evaluated by carrying out fermentation in 500 mL Erlenmeyer flasks with 200 mL of medium. The pH of the medium was set at 5.5 by using 1 mol·L^{-1} HCl or 5 mol·L^{-1} NaOH, followed by sterilization at 121 °C for 15 min. The fermentation media were inoculated with 5% (v/v) yeast cultures and incubated at 30 °C, 150 rpm for 120 h in a rotary shaker. Samples were withdrawn at regular time intervals to estimate the xylose consumption and xylitol production.

2.10. Repeated Batch Fermentation

The usage of yeast cells without preparing fresh inoculum was evaluated by transferring 5% (v/v) broth from each preceding fermentation batch to the successive batches of fermentation. The test was repeated for up to 7 batches of fermentation, and samples were withdrawn periodically to estimate the concentrations of xylose and xylitol. Each batch of fermentation lasted about 72 h.

2.11. Analytical Methods

The moisture content of the biomass at various stages of pre-treatment was analyzed by an MA 35 moisture analyzer. The reducing sugars, inhibitory compounds, and xylitol were estimated by HPLC (Agilent Technologies, 1260 Infinity), fitted with a reverse-phase BIORAD Aminex HPX-87H column (300 × 7.8 mm), with 5 mM sulfuric acid as the mobile phase, a 0.55 mL/min flow rate, and 60 °C column temperature.

2.12. Statistical Analysis

All the experiments were repeated at least three times, and the data were analyzed by using GraphPad Prism software version 5. The error bar was calculated from the mean of three replicates. The significant differences were calculated by two-way ANOVA using Bonferroni post-tests at $p < 0.0001$.

3. Results and Discussion

3.1. Effect of Inhibitors on Xylitol Production

The optimum process parameters for RS pretreatment to extract the maximum concentration of xylose from the RS biomass were 1.25% (*v*/*v*) H_2SO_4 concentration, 121 °C hydrolysis temperature, and 30 min residence time with a solid-to-liquid ratio of 1:10 (g/mL), as observed from Figure 1.

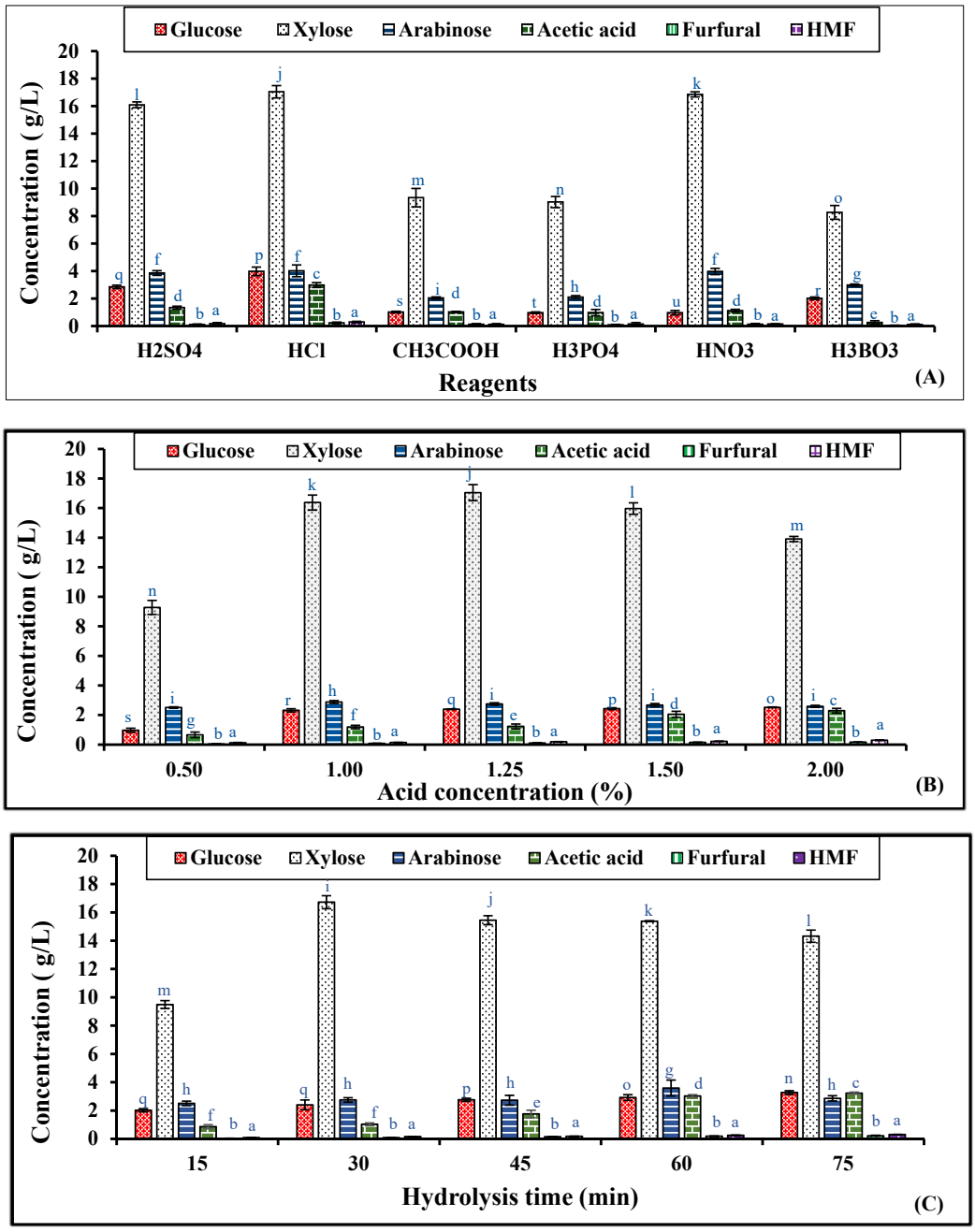

Figure 1. Optimization of RS hydrolysis by varying different (**A**) reagents, (**B**) sulfuric acid concentrations (0.5 to 2.0%), and (**C**) residence times of rice straw hydrolysis by 1.25% sulfuric acid (15 to 75 min). Significant differences were calculated by two-way ANOVA using Bonferroni post-tests and are indicated by small letters. At different hydrolysis conditions, the bars with different lowercase letters represent significant differences. Bars with the same letters are not significantly different, according to $p < 0.0001$.

The pretreatment of the lignocellulosic biomass generated inhibitory compounds such as acetic acid, furfural, and hydroxymethyl furfural (Table 1). These inhibitors influence the efficiency of the fermentation process performed by using a microbial platform [27]. Also, such byproducts have an adverse effect on the growth of microbes, making them inefficient for utilizing sugars, ultimately resulting in a poor yield of products. Therefore, the selection of microbes with a natural tolerance to inhibitory compounds is generally recommended to increase fermentation yields. Hence, the tolerance to different by-products, such as HMF, acetic acid, and furfural, of the isolated yeasts, *C. tropicalis* and *M. caribbica*, was evaluated. The efficiency of fermentation by measuring the xylitol yield in the presence of these inhibitors was estimated by withdrawing a sample every 24 h during the fermentation.

Table 1. Chemical composition of detoxified and non-detoxified rice straw hydrolysate obtained by sulfuric acid treatment.

Concentration (g/L)	Raw Hydrolysate	Detoxified Hydrolysate	Concentrated Non-detoxified Hydrolysate	Concentrated Detoxified Hydrolysate
Glucose	2.4 ± 0.24	1.97 ± 0.05	6.98 ± 0.03	6.30 ± 0.14
Xylose	16.72 ± 1.04	15.97 ± 0.98	49.16 ± 0.16	52.15 ± 1.12
Arabinose	2.75 ± 0.45	2.70 ± 0.02	8.08 ± 0.12	8.14 ± 0.04
Acetic acid	1.03 ± 0.17	0.44 ± 0.03	3.04 ± 0.06	1.35 ± 0.02
Furfural	0.08 ± 0.03	-	-	-
HMF	0.16 ± 0.02	-	0.44 ± 0.04	-

The key enzyme involved in the production of xylitol from xylose during the fermentation process involving yeasts is xylose reductase (XR). During xylitol production, XR requires a continuous supply of NADPH. The maintenance of the NADPH level in the yeast for xylitol production by XR is mainly determined by an efficient recycling system, including formate dehydrogenase and glucose dehydrogenase [5,10,12,15]. Hence, the involvement of such enzymes is very crucial for xylitol production with yeasts. The robustness of such enzymes in the yeast, along with the appropriate conditions and biomass types, decides the xylitol production.

3.1.1. Effect of Acetic Acid on Xylitol Production

Acetic acid is a compound formed in the hydrolysate as a result of the breakdown of the hemi-acetyl linkage. Acetic acid is present in higher concentrations compared to HMF and furfural during biomass hydrolysis [28]. An acetic acid accumulation above the threshold levels causes a drop in the intracellular pH, as well as in the fermentation broth. These high levels of acetic acid are supposed to slow the growth rate of microbes, resulting in incomplete cycles of fermentation. Therefore, the tolerance of yeasts towards acetic acid was evaluated in a synthetic medium containing variable concentrations of acetic acid (2–10 g/L) at different pH values (4.5, 5.5, and 6.5). The effect of the variable pH values of the medium was analyzed to see the reported decrease in the toxic undissociated form of acetic acid with an increase in pH. The pKa of acetic acid is 4.75, and, therefore, at lower pH, protonated acetic acid is predominant, and, at higher pH, deprotonated acetic acid is predominant [29]. The results of the present study revealed that the toxic nature of acetic acid was alleviated with an increase in pH from 4.5 to 6.5. The highest xylitol yield and xylose assimilation (99%) were observed at pH 5.5 with both yeast strains. The xylose consumption was not affected by acetic acid at 2–10 g/L concentration. However, it was observed from the results that the xylitol yield started declining with an increase in the acetic acid concentration from 2 to 10 g/L. This decrease in the xylitol yield might be due to the diversion of xylose flux in the yeast to energy generation [19]. Inhibitors such as acetic acid might be affecting the activity of xylose reductase (XR). The acetic acid concentration (2 g/L) at pH 5.5 decreased the xylitol production rate to 4.18% and 4.90% in comparison to the control medium (without acetic acid) during fermentation by

C. tropicalis and *M. caribbica*, respectively (Table 2). Further, it was observed that the xylitol production was the maximum at pH 5.5 compared to pH 4.5 and 6.5, which might be due to the decrease in toxicity of acetic acid, or pH 5.5 might be the optimum pH for the yeast metabolism and activity of xylose reductase (XR) enzyme. Moreover, it was observed that the fermentation time was increased at pH 6.5. This increase in incubation time might be responsible for salt build-up by neutralization, or an unfavorable pH for XR enzyme activity. Both *C. tropicalis* and *M. caribbica* strains were able to tolerate acetic acid up to the concentration of 10 g/L at pH 5.5 and 6.5, with significantly higher xylitol yields in comparison to the yields obtained at pH 4.5. It was observed that the acetic acid at a 10 g/L concentration and at pH 4.5 turns out to be toxic for both the yeasts, as observed in terms of poor growth and xylitol yields. However, at pH 5.5, the yeast *C. tropicalis* was able to tolerate 10 g/L acetic acid with 29.76 g/L xylitol titer, while *M. caribbica* exhibited low xylitol titer (12.04 g/L), due to the diversion of the xylose flux to energy generation. Similarly, in another study, a high cell density of *Candida sojae* JCM 1644 was observed in a medium supplemented with 3% acetic acid, whereas the cell density was decreased when the concentration of acetic acid was increased to 5%, indicating the sensitivity of the strain to a high acetic acid concentration [30]. In the present study, the xylose consumption and xylitol production were not found to be affected when the acetic acid concentration was 2 g/L and the pH was 4.5. However, when the concentration of acetic acid was increased further, the xylitol production was decreased significantly at pH 4.5 during fermentation by both yeasts (Table 2). Similar observations have also been reported during fermentation by using *C. tropicalis* and *C. gulliermondii* [31,32].

Table 2. Effect of acetic acid concentration in rice straw hydrolysate on xylitol production at different pH values by *Candida tropicalis* and *Meyerozyma caribbica*.

Fermentation Parameters			Xylitol Concentration (g/L)		Xylitol Yield (g/g)	
Acetic Acid Concentration (g/L)	**pH**	**Time (h)**	***Candida tropicalis***	***Meyerozyme caribbica***	***Candida tropicalis***	***Meyerozyma caribbica***
Control	4.5	48	33.26 ± 0.11^{Da}	33.92 ± 0.23^{Na}	0.67	0.65
	5.5	48	35.12 ± 0.79^{Aa}	34.88 ± 0.12^{Ma}	0.70	0.69
	6.5	48	34.23 ± 0.13^{Ca}	34.02 ± 0.28^{Na}	0.68	0.66
2.17 ± 0.27	4.5	48	31.51 ± 0.13^{Ga}	31.24 ± 0.016^{Sa}	0.63	0.62
	5.5	48	33.65 ± 0.01^{Da}	32.13 ± 0.02^{Qb}	0.67	0.64
	6.5	48	32.07 ± 0.04^{Da}	31.89 ± 0.03^{Ra}	0.64	0.63
3.95 ± 0.06	4.5	72	26.49 ± 0.017^{Ka}	24.45 ± 0.015^{Vb}	0.53	0.49
	5.5	48	34.02 ± 0.48^{Da}	33.07 ± 0.87^{Oa}	0.68	0.66
	6.5	48	34.50 ± 0.20^{Ba}	32.23 ± 0.007^{Pb}	0.69	0.65
6.02 ± 0.02	4.5	72	29.00 ± 0.16^{Ja}	22.05 ± 0.021^{Wb}	0.58	0.47
	5.5	72	31.58 ± 0.70^{Fa}	27.04 ± 0.013^{Ub}	0.63	0.60
	6.5	72	32.48 ± 0.36^{Ea}	28.12 ± 0.047^{Tb}	0.65	0.60
10 ± 0.11	4.5	-	6.57 ± 0.28^{La}	2.36 ± 0.016^{Zb}	0.13	0.13
	5.5	72	29.76 ± 0.028^{Ha}	12.04 ± 0.043^{Yb}	0.59	0.501
	6.5	72	29.24 ± 0.16^{Ia}	13.97 ± 0.007^{Xb}	0.59	0.55

Significant differences were calculated by two-way ANOVA using Bonferroni post-tests and are indicated by small and capital letters. For variable concentrations of acetic acid, different uppercase letters in a column represent a significant difference, while lowercase letters represent significant differences in a row for xylitol production by the two yeasts. Different letters denote significant a difference between the two, and the same letters denote no significant difference between the two at $p < 0.0001$.

3.1.2. Effect of Furfural and Hydroxymethylfurfural on Xylitol Production

It has been reported in previous studies that furfural and HMF break down the DNA and inhibit the protein and RNA synthesis mechanism of the microorganisms, thereby affecting their growth and fermentation efficiency [33,34]. Therefore, the effect of furfural and HMF on xylitol production was also evaluated by varying the concentrations of furfural

(1–4 g/L) and HMF (1–4 g/L) in the fermentation medium. To study the synergistic effect of both inhibitors on the yeasts, the medium was supplemented with 2 g/L acetic acid, along with furfural at different concentrations. The maximum xylitol production was observed at 1 g/L furfural by both *C. tropicalis* and *M. caribbica*, which was decreased upon further increasing the furfural concentration. When the concentration of furfural was increased beyond 1 g/L to 4 g/L, a gradual decrease in xylitol yield (16.28%) was observed during fermentation by *C. tropicalis*, whereas, in the case of *M. caribbica*, a 23.13% decrease in xylitol yield was observed (Figure 2a). Moreover, the growth of the yeasts was not completely inhibited at a 4 g/L furfural concentration, which might be due to the capability of the yeasts to reduce the inhibitory effect of furfural by converting it to less-toxic compounds, such as furoic acid or furfuryl alcohol [35]. Similarly, 22.64 g/L of xylitol titre and 0.44 g/g of xylitol yield were reported after the adaptive evolution of *Geotrichum* sp. in sugarcane bagasse hydrolysate. *Geotrichum* sp. was able to tolerate 6 g/L of furfural, along with glucose and xylose as the carbon source [36]. In another study, a furfural concentration of 500 m g/L was reported for the highest inhibition of cell growth of *Pichia stiptis NCIM* 3497 and poor xylitol yields [29].

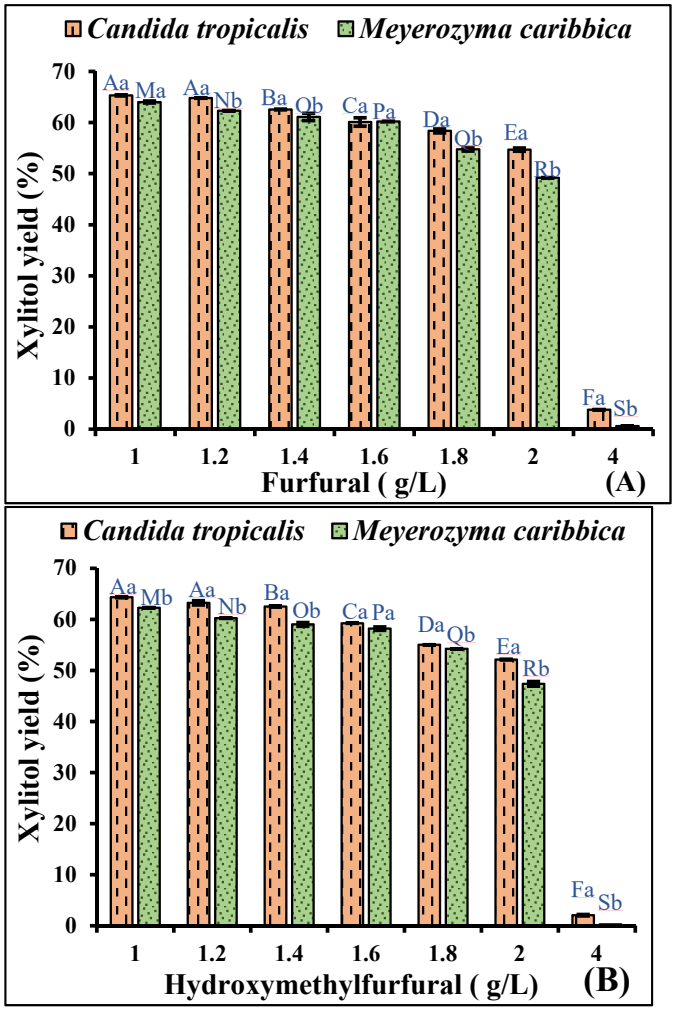

Figure 2. Effect of inhibitory compounds (**A**) furfural, (**B**) hydroxymethylfurfural (HMF) on xylitol fermentation efficiency of *C. tropicalis* and *M. caribbica* in rice straw hydrolysate. Significant differences were calculated by two-way ANOVA using Bonferroni post-tests and are indicated by small and capital letters. In different concentrations of furfural and HMF, bars with different uppercase letters are significantly different, while bars with lowercase letters represent a significant difference in xylitol production by the two yeasts. Different letters denote a significant difference between the two, and the same letters on the bar denote no significant difference between the two at $p < 0.0001$.

Further, fermentation by both yeasts in the medium containing acetic acid at 2 g/L, furfural at 1 g/L, and HMF at variable concentrations (1–4 g/L) was carried out to evaluate the synergistic effect of these three inhibitors on the process of fermentation. A noteworthy effect of HMF was seen on both *C. tropicalis* and *M. caribbica*, with a gradual decrease in the xylitol yield. When the concentration of HMF was increased from 1 to 2 g/L, the xylitol yield by *C. tropicalis* was decreased to 18.95%, whereas a decrease of 23.90% was seen in the xylitol production efficiency of *M. caribbica*. A further decline in xylitol yield was seen during fermentation by both yeasts when the medium was supplemented with 4 g/L HMF, and, ultimately, a complete inhibition of microbial growth in the medium was observed (Figure 2b). Similarly, the synergistic effects of low, medium, and high concentrations of HMF, furfural, and acetic acid were analyzed on xylose consumption and xylitol production by *Pichia stiptis* NCIM 3497. The authors have observed 11.5% and 50% reductions in xylitol yield in the presence of the lower and moderate concentrations of inhibitors, respectively, in comparison to the control. However, 66.67% and 67.57% reductions in the xylitol yield were observed in the presence of a higher concentration of furfural, HMF, and acetic acid at 48 and 72 h of fermentation, respectively [29]. Similarly, a high concentration of HMF, furfural, and acetic acid, above 3 g/L, was reported to negatively affect xylitol production by *Rhodotorula mucilaginosa* PTD3 I [37].

3.2. Adaptation of Yeasts in RS Hydrolysate

The increase in the concentration of inhibitors led to a decrease in the xylitol yield, which could be due to the slow sugar assimilation by the yeasts in the presence of inhibitors [38]. Therefore, the cells were allowed to adapt in the non-detoxified RS hydrolysate to increase the consumption of sugars in the presence of inhibitors. Both the yeasts, *C. tropicalis* and *M. caribbica*, were inoculated in the non-detoxified hydrolysate comprising xylose and glucose, along with certain inhibitory compounds (Table 1). The cells were transferred from the previous batch to the next successive batch every 24 h, and a significant increase in xylose assimilation was observed in each cycle. The lowest xylose consumption rate was 1.40 g/L/h and 1.50 g/L/h in the first cycle (I), and the highest was 2.47 g/L/h and 2.54 g/L/h in the last cycle (V) of fermentation by *M. caribbica* (Figure 3a) and *C. tropicalis* (Figure 3b), respectively. Similarly, the rate of xylose consumption has been observed to increase during fermentation in sugarcane bagasse hemicellulosic hydrolysate with adapted cells in comparison to un-adapted *Candida guilliermondii* yeast cells [1].

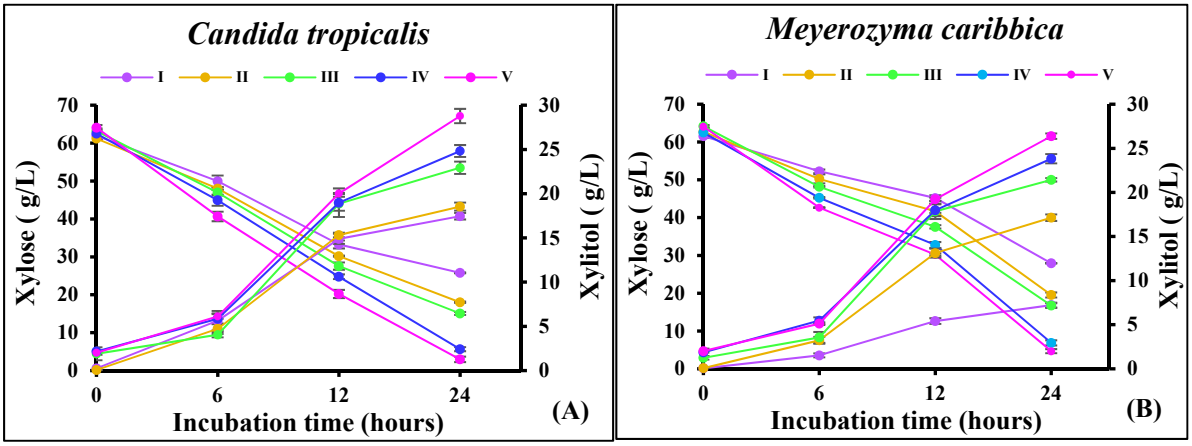

Figure 3. Xylose assimilation and xylitol production profiles in repeated batch fermentation by (**A**) *C. tropicalis*, (**B**) *M. caribbica* in rice straw hydrolysate medium. First cycle (I), second cycle (II), third cycle (III), fourth cycle (IV), and fifth cycle (V).

3.3. Comparative Assessment of Fermentation in RS Hydrolysate with Un-Adapted and Adapted Yeasts

Fermentative tests were carried out by shake flask fermentation using both adapted and un-adapted strains of *C. tropicalis* and *M. caribbica*. A high concentration of xylitol 28.03 g/L, with a 0.57 g/g xylitol yield and productivity of 0.29 g/L/h, was observed during fermentation by the adapted strain of *C. tropicalis* in non-detoxified hydrolysate compared to the un-adapted strain (26.32 g/L xylitol; 0.53 g/g xylitol yield; 0.21 g/L/h xylitol productivity). The xylitol yield increased by 7.54% and 3.38% during fermentation by adapted *C. tropicalis* in non-detoxified and detoxified RS hydrolysate, respectively (Figure 4a,b). However, the maximum xylitol concentration of 26.02 g/L, with 0.52 g/g of xylitol yield and productivity of 0.21 g/L/h, was observed during fermentation by the adapted strain of *M. caribbica* in the non-detoxified hydrolysate, in comparison to the un-adapted strain (23.38 g/L xylitol; 0.48 g/g xylitol yield; 0.19 g/L/h xylitol productivity). There were 8.33% and 7.40% increases in xylitol yield with the adapted strain of *M. caribbica* during fermentation in the non-detoxified and detoxified RS hydrolysates, respectively (Figure 4c,d). In a similar study, a low xylitol concentration of 17.33 g/L and 0.44 g/g xylitol yield was reported from steam-pretreated acid-catalyzed sugarcane bagasse hydrolysate using *M. caribbica* JA9 [39], while a 0.54 g/g xylitol yield was obtained from detoxified corn cob hydrolysate after 120 h [40]. The xylitol yield from the corn hydrolysate was lower than the xylitol yield of the present study. In a study on the exploration of five different yeasts for xylitol production from sugarcane bagasse hydrolysate, *Meyerozyma caribbica* could utilize only 89% of the xylose, and residual sugar was present at the end of fermentation, whereas a complete utilization of xylose was observed during fermentation by *C. tropicalis*. Contrarily, in the present study, both the yeasts were highly competitive and completely utilized the xylose by the end of the fermentation. The higher xylitol yield in the present study could be due to the RS hydrolysate containing a lower concentration of glucose, and therefore the alcohol dehydrogenase activity was not expressed, favoring high xylitol productivity [40].

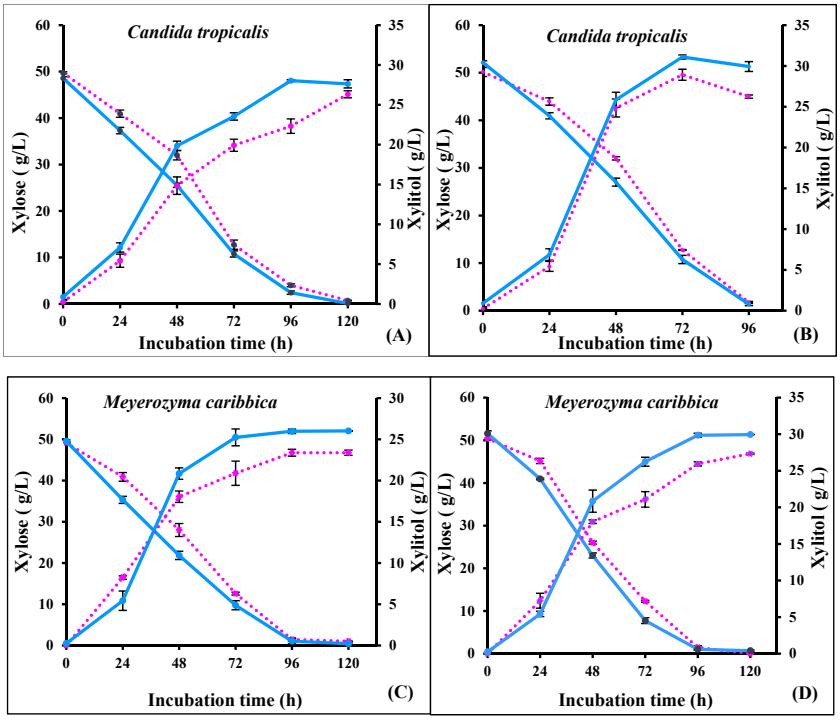

Figure 4. Fermentation profile of un-adapted (dotted line) and adapted (solid line) strains of *C. tropicalis* in (**A**) non-detoxified rice straw hydrolysate, and (**B**) detoxified rice straw hydrolysate; and *M. caribbica* in (**C**) non-detoxified rice straw hydrolysate, and (**D**) detoxified rice straw hydrolysate.

A comparative analysis of the performance of the yeasts for xylitol production on rice straw and other biomasses is presented in Table 3. The analysis suggests the higher potential of the identified yeasts for xylitol production, at least from rice straw biomass.

Table 3. Comparison of xylitol production by different strains.

Microorganism	Raw Material/Method of Detoxification	Initial Xylose (g/L)	Xylitol Titre (g/L)	Xylitol Yield (g/g)	Xylitol Productivity (g/L·h)	References
C. tropicalis	Rice straw/Activated charcoal and neutralization	52.13	31.1	0.61	0.54	Present study
M. caribbica	Rice straw/Activated charcoal and neutralization	51.57	29.95	0.58	0.42	Present study
C. tropicalis	Rice straw/non-detoxified	49.74	28.03	0.57	0.29	Present study
M. caribbica	Rice straw/non-detoxified	49.12	26.02	0.52	0.21	Present study
C. tropicalis MTCC 6192	Rice straw	45	25.83	0.60	0.26	[35]
C. tropicalis	Rice straw/Charcoal and ion exchange resins	46.2	26.5	0.58	0.53	[20]
C. tropicalis BCRC 20520	Wood chip/Ca $(OH)_2$ neutralized and activated charcoal	60	32.5	0.54	0.73	[41]
M. caribbica	Sugarcane bagasse/non-detoxified	40	11.37	0.54	0.10	[39]
M. caribbica	Sugarcane trash/non detoxified	35.2	6.49	-	-	[42]
M. caribbica	D-xylose	40	21.56	0.539	0.29	[43]

3.4. Repeated Batch Fermentation in Non-Detoxified and Detoxified Hydrolysate

Repeated batch fermentation was carried out to omit the need for fresh seed culture preparation. The fermentation was carried out by inoculating 5% (v/v) broth from the preceding batch to the successive batch for up to seven cycles in both non-detoxified and detoxified RS hydrolysates. The results of the repeated batch fermentation showed that the cells can be used for up to five batches, with each batch lasting for 72 h, without any significant effect on the efficiency of the yeast for xylitol production. During fermentation in non-detoxified media, significant declines of 24.62% and 25.97% were seen in the xylitol yield after the fifth to seventh cycles of fermentation by *C. tropicalis* and *M. caribbica*, respectively (Figure 5a). However, in the detoxified hydrolysate, declines of 21.79% and 29.68% in xylitol efficiency were observed after the fifth to seventh cycles of fermentation by *C. tropicalis* and *M. caribbica*, respectively (Figure 5b). Similarly, D-lactic acid has been produced by *Sporolactobacillus* sp. using repeated fermentation for up to four batches, with each batch lasting for 48 h. Consistent lactic acid production was observed in the first three batches, and a slight decrease of 3.29% was observed in the fourth batch of fermentation [44]. Also, repeated fed-batch fermentation was conducted for xylitol production by using *C. magnoliae* for three cycles in a synthetic D-xylose-based medium. The researchers observed a constant xylitol yield of 0.727 g/g, 0.719 g/g, and 0.720 g/g in the first, second, and third batches of fermentation, respectively [45]. The simultaneous production of poly-3-hydroxybutyrate, xylitol, and xylonic acid was reported with xylose-rich sugar mixtures by a wild strain of *Burkholderia sacchari* [46].

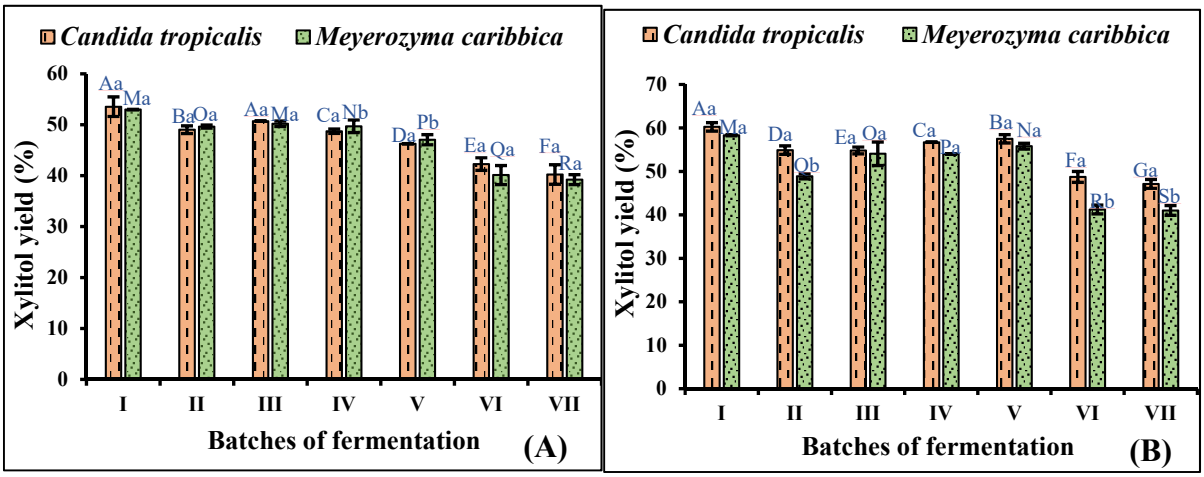

Figure 5. Repeated batch fermentation of adapted strains of *C. tropicalis* and *M. caribbica* in (**A**) non-detoxified rice straw hydrolysate, and (**B**) detoxified rice straw hydrolysate. Significant differences were calculated by two-way ANOVA using Bonferroni post-tests and are indicated by small and capital letters. In different batches, bars with different uppercase letters are significantly different, while bars with different lowercase letters represent significant differences in xylitol production by the two yeasts. Different letters denote significant difference between the two, and the same letter on the bar denotes no significant difference between the two at $p < 0.0001$.

4. Conclusions

The presence of inhibitors in biomass hydrolysates has been observed to be a major barrier, hindering the production of xylitol by microbial fermentation. Understanding the effect of such inhibitors on yeast growth is an important step to overcoming this difficulty during fermentation. The present study was performed to elucidate the fermentative performance of *C. tropicalis* and *M. caribbica* for xylitol production in rice straw (RS) biomass hydrolysate, with the formation of toxic inhibitors during the process. A high concentration of inhibitors was found to severely inhibit the growth of yeast, resulting in poor xylitol yields. In order to enhance the xylitol yield, an adaptation strategy was employed to increase the natural tolerance of yeasts in the presence of a high concentration of inhibitors. Hence, adapted strains of *C. tropicalis* and *M. caribbica* were developed, documenting the better tolerance to inhibitors when compared to the un-adapted strain. The rate of xylose assimilation after adaptation was increased to 76.42% and 69.33% in *C. tropicalis* and *M. caribbica*, respectively. Moreover, repeated batch fermentation studies were carried out to omit the need for seed culture preparation, and it was found that the culture can be used for up to five batches of fermentation. Further, the findings of the present study indicate that the *M. caribbica* strain is a promising yeast, which could be used for xylitol production from a low-cost substrate. Importantly, the high xylitol yield with *M. caribbica* was close to the yield obtained from biomass hydrolysate by using *C. tropicalis*, even without employing optimized process conditions for xylitol fermentation.

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