Sustainable 2G Ethanol Production From Switchgrass Biomass via Co-Fermentation of Pentoses and Hexoses Using Novel Wild Yeasts

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Abstract

The production of second generation (2G) ethanol remains an interesting proposition for the implementation of sustainable and net carbon-neutral energy systems. 2G makes use of renewable lignocellulosic feedstocks, generating fermentable sugars that are converted to ethanol or other bio-based products. To be economically viable, 2G biorefineries must make use of all processing streams, including the less desirable C5 sugar stream. In this work, a strategy of sequential acid and alkaline pretreatment of the lignocellulosic feedstock switchgrass for improvement of fermentable sugar yield, and the subsequent utilization of wild yeasts for co-fermentation of its C5-C6 sugar streams are presented. Hemicellulose-enriched hydrolysates, obtained by dilute acid pretreatment of switchgrass, were fermented by a newly-isolated wild *Scheffersomyces parashehatae* strain–UFMG-HM-60.1b; corresponding ethanol yield (Y_{PS}) and volumetric productivity (Q_P) were 0.19 g/g and 0.16 g/L h, respectively. Afterwards, the remaining switchgrass cellulignin fraction was subjected to optimized alkaline delignification at 152 °C for 30 min. The delignified solid fraction was subjected to contiguous enzymatic saccharification and fermentation, releasing a C6 sugar stream in which *Saccharomyces cerevisiae* 174 strain displayed a productivity of 0.46 g/g (Y_{PS}) and 0.70 g/L h (Q_P), whereas the *S. parashehatae* UFMG-HM-60.1b presented Y_{PS} and Q_P of 0.29 g/g and 0.38 g/L h, respectively. Upon combining the conversion of hemicellulose (37%) and cellulose-derived sugars (57%), the *S. parashehatae* strain provided higher yield (94%) than the generic *S. cerevisiae* (90%). Henceforth, our integrated pretreatment and co-fermentation process provides a pathway for maximum utilization of the switchgrass carbohydrates for 2G ethanol production.

1. Introduction

Bioethanol production is an economical and environment-friendly alternative to fossil fuels as well as contribute towards energy independence. Also, bioethanol production can offer socio-economic advantages, such as increase in farming income for developing countries and reduction of greenhouse gas emissions [1]. Bioethanol has long established itself as the world's main biofuel source and the global bioethanol market is valued at \$ 34 billion in 2020, which is expected to grow at a compound annual growth rate of 14% and reach \$ 65 billion by 2025 [2]. In 2019, the world bioethanol production was about 29 billion gallons. USA leads the bioethanol production, followed by Brazil, at 16 and 9 billion gallons, respectively [3].

Bioethanol can be obtained from different raw materials including starches, lignocellulose, and algae, accordingly named as first (1G), second (2G) and third-generation (3G) biofuels. Lignocellulosic biomass does not compete with food production [4] and ethanol production from lignocellulosic feedstocks, such as switchgrass, would increase the productivity per hectare of marginal lands, contributing to the reduction of cultivated area bar[5]. Despite these advantages, large-scale implementation of 2G ethanol production is still under development and is faced with multiple challenges in achieving economic viability. Uninterrupted feedstock supply is one such challenge and will most likely be solved through a year-round feedstock switching strategy between corn stover, miscanthus, and switchgrass [6]. The

perennial, warm-season C4 grass, switchgrass (*Panicum virgatum* L.), is native to North America [7] and presents important benefits for bioethanol production, such as the potential for carbon sequestration, nutrient recovery from runoff water, soil remediation, provision of habitats for grassland birds and higher production potential in USA [8]. Hence, development of strategies for feasible utilization of switchgrass will benefit the 2G biofuel industry.

The major components of switchgrass are cellulose (32–34%), hemicellulose (26–27%) and lignin (17–18%), on a dry weight basis [9]. Pretreatments are usually applied to reduce biomass recalcitrance and to increase the hydrolytic efficiency of cellulose and hemicellulose polymeric fractions into fermentable sugars (i.e., glucose, xylose, galactose and arabinose). Lignin is recovered from the pretreatment hydrolysates and commonly used as a fuel source in biorefineries. Successively, the resulting sugars are fermented to ethanol, which is then distilled for fuel purposes [8, 10].

Among the different biomass pretreatment techniques, acid and alkaline hydrolysis are more promising because acid pretreatment hydrolyzes the hemicellulose fraction, whereas alkaline pretreatment induces delignification [11]; thereby these two methods promote systematic fractionation of lignocellulosic biomass. However, the process efficiency of some previously reported pretreatment strategies did not meet the required cost-benefits, making it difficult to implement on a large scale. Therefore, a biomass pretreatment strategy that economically increases the fermentable sugar yield will be of remarkable importance.

Aside from an efficient pretreatment process, the concomitant fermentation of five and six carbon sugars (C5 and C6) is an important strategy to improve the commercial viability of 2G ethanol [12, 13]. In this context, the physiological characteristics expected in an ideal microorganism are broad spectrum of substrate uptake, ability to withstand high sugar and alcohol concentration, resilience in lower pH, resistance to inhibitory compounds, and minimal by-products formation [14]. Although *Saccharomyces cerevisiae* is largely employed in industrial alcohol fermentations, it lacks the ability of assimilating C5 sugars. New yeast strains capable of metabolizing C5 sugars are under development by using genetic engineering tools [15–17]. On the other hand, there have been very few reports on wild strains that can assimilate both C5 and C6 sugars and synthesize ethanol with robust process efficiencies and scale-up properties [18]. Recently, a novel Brazilian wild strain, classified as *Scheffersomyces parashehatae* UFMG-HM-60.1b, was isolated from the tropical fauna and was shown to have the potential for metabolizing xylose. We are reporting the fermentation of switchgrass sugars using this novel wild yeast strain for the first time in this study.

In this study, we have compared the fermentation of switchgrass sugars by *S. parashehatae* and *S. cerevisiae*. Switchgrass biomass was initially processed with dilute acid pretreatment for separating the cellulignin and hemicellulose fractions. Subsequently, the cellulignin fraction was subjected to alkaline pretreatment for delignifying the pretreated solids and to enhance cellulose conversion. To maximize C6 sugar yields, a 2² full factorial optimization was carried out by varying the time and temperature of alkaline pretreatment. Ethanol production from *S. cerevisiae* 174 and the wild strain *S. parashehatae*

UFMG-HM-60.1b was evaluated using the switchgrass enzymatic hydrolysates. In a second approach, ethanol production from switchgrass hemicellulosic hydrolysates was evaluated solely using the wild strain. This work was performed with the impetus of maximizing switchgrass sugar utilization, which would eventually increase biorefinery profitability.

2. Materials And Methods

2.1 Biomass

Switchgrass biomass (*P. virgatum*) was supplied by the Department of Biological & Agricultural Engineering at the University of Arkansas, in Fayetteville, USA. The switchgrass biomass composed of leaves and stalk was dried and ground in a Wiley mini-blade mill such that the biomass particle size was reduced below 20-mesh (0.84 mm).

2.2 Microorganisms

S. cerevisiae 174 was obtained from the culture collection of Department of Biotechnology in Lorena School of Engineering- SP-Brazil, while *S. parashehatae* UFMG-HM 60.1b was kindly provided by the culture collection of Federal University of Minas Gerais, in Belo Horizonte, Brazil. Stock cultures of both *S. cerevisiae* and *S. parashehatae* were prepared by transferring a loopful of strains from the slant into 125 mL Erlenmeyer flasks containing 50 mL of medium (pH 5.5) composed of glucose (30 g/L), yeast extract (10 g/L) and peptone (20 g/L). The flasks were incubated in a rotatory shaker (Innova 4000 Incubator Shaker, New Brunswick Scientific, Enfield, CT, USA) at 30°C for 24 h, at 100 rpm for *S. cerevisiae* and 200 rpm for *S. parashehatae*. After growing, both yeasts were separated via centrifugation at $2,777 \cdot g$ for 10 min at room temperature, washed with sterilized water, re-suspended and used for inoculation in the fermentation processes.

2.3 Dilute acid pretreatment

The flowchart of biomass fractionation methodology is presented in Fig. 1. Milled switchgrass biomass and 1% (w/v) sulfuric acid solution were mixed in a 1 L Parr 4525 reactor (Moline, IL, USA) at a 10% solid content. Mixture in the reactor was pretreated at 140°C, for 40 min and 144 rpm. Pretreatment time was measured after the reactor reached the desired temperature and the process was stopped by circulating cold water through the cooling coils. Pretreated slurry was separated into liquid and solid fractions using a Buchner funnel lined with a Whatman #1 filter paper. A total of 10 runs were performed and the liquid prehydrolyzates from all the runs were pooled and concentrated under reduced pressure using a Savant SpeedVac Concentrator SPD 1010 (Thermo Scientific, NC, USA). The final volume of hemicellulosic hydrolysate was 400 mL.

The concentrated acid prehydrolyzate was detoxified according to the adapted method of Alves et al., (1998) [19] in order to reduce the concentration of inhibitor compounds generated during the dilute acid pretreatment. In short, the detoxification process included: (i) increasing the pH from 0.5 to 7 with NaOH solution; (ii) reducing the pH to 5.5 with phosphoric acid and addition of activated charcoal at 2.5% (w/v);

(iii) and finally, incubation at 30°C for 1 h and 200 rpm. After detoxification, the hydrolysate was filtered under vacuum in order to separate the liquid fraction and autoclaved at 0.5 atm (110°C) for 20 min before being used in the fermentation process.

2.4 Alkaline pretreatment of cellulignin and enzymatic hydrolysis

Cellulignin obtained after dilute acid hydrolysis was pretreated by alkaline solution in a 1 L digester (B03 1L, *PHD Equipamentos para Laboratórios* Piracicaba-SP, Brazil). Sodium hydroxide solution (1% w/v) was used as a catalyst at a solid-to-liquid ratio of 1:10 at different time and temperature conditions. To investigate the influence of these variables on the pretreatment process, the main effects and their interactions were studied following a full factorial design (2^2) with three replicates at center points as described in Table 1. The response variable was glucose yield obtained from enzymatic saccharification (as described in the next section). The solid material (cellulose) after alkaline pretreatment was recovered by filtration using muslin cloth. The recovered solid residue was washed with running tap water until neutral pH and dried at 45°C.

The enzymatic hydrolysis of sequentially pretreated switchgrass was performed in a 125 mL Erlenmeyer flask containing 3 g (dry wt.) of pretreated solids and 40 mL of citrate buffer (50 mM, pH 4.8). The substrate soaked in citrate buffer was supplemented with cellulase enzyme cocktail obtained from Dyadic International, Inc (Cellulase CP CONC) (20 FPU/g of the dry substrate) and surfactant (Tween 20 at 0.1 g/g). Enzymatic hydrolysis was performed at 50°C and 150 rpm in a shaking incubator (Innova 4000, New Brunswick Scientific, CT, USA) for 48 h. Samples were collected at different time intervals, centrifuged, and analyzed to determine the sugars released. Glucose concentration after enzymatic hydrolysis step was used to calculate the digestibility (% of cellulose converted per gram of biomass), by using a correction factor of 0.9 according to the equation proposed by Lu et al. (2012)[20].

2.5 Fermentation of switchgrass enzymatic hydrolysate

S. cerevisiae and the wild *S. parashehatae*, were used for the fermentation of switchgrass enzymatic hydrolysate. For *S. cerevisiae* the enzymatic hydrolysate was supplemented with yeast extract (1 g/L), peptone (1 g/L), diammonium hydrogen phosphate (1 g/L), dipotassium hydrogen phosphate (1 g/L), magnesium sulfate and manganese sulfate (each at 0.5 g/L) and adjusted to pH 5.5. Fermentation assays were carried out in 125 mL Erlenmeyer flasks containing 50 mL of supplemented hydrolysate medium and 0.5 g/L of yeast suspension. The experiments were performed in the shaking incubator at 30°C, 150 rpm for 96 h. In another set of fermentation, *S. parashehatae* inoculated enzymatic hydrolysate was supplemented with ammonia sulfate (5 g/L), malt extract (3 g/L) and yeast extract (3 g/L). Fermentation assays were carried out in a similar fashion at 30°C and 200 rpm for 96 h. In both experiments, samples were collected periodically to determine the sugars consumption and production of ethanol.

2.6 Fermentation of switchgrass hemicellulosic hydrolysate

Switchgrass hemicellulosic hydrolysate was diluted with sterilized water to achieve xylose concentration of 30 g/L and then was supplemented with ammonium sulfate (5 g/L), malt extract (3 g/L) and yeast extract (3 g/L). Fermentation assays were carried out in 125 mL Erlenmeyer flasks containing 50 mL of supplemented hemicellulosic hydrolysate medium and 0.5 g/L of *S. parashehatae* cell suspension. The experiments were performed in the shaking incubator shaker at 30°C and 200 rpm for 96 h. In both experiments, samples were collected periodically to determine the sugars consumption and production of ethanol as well as yeast biomass.

2.7 Analytical methods

Glucose, xylose and ethanol concentrations were determined using a Shimadzu LC- 10AD (Kyoto, Japan) high performance liquid chromatography system, coupled to a refractive index detector, and equipped with a Aminex HPX-87H analytical column (300 × 7.8 mm; Bio-Rad, Hercules, CA, USA). Sulfuric acid at 0.01 N was used as eluent at a flow rate of 0.6 mL/min, column temperature of 45°C, and injection volume of 20 µL.

Scanning electron microscopy (SEM) analysis of samples was performed as previously defined by Kristensen *et al.* [21]. The X-ray diffraction (XRD) analysis was performed by using a Seifert ISO-Debyeflex 3003 (Germany) instrument. The crystallinity was analyzed by regulating the diffractometer at 40 kV, 30 mA; radiation wavelength was Cu K α ($\lambda = 1.54 \text{ \AA}$). A 2θ range of 10 to 50 degrees was used with a step size of 0.05° for scanning all samples. The crystallinity index (Crl) was calculated by the empirical method described by Park *et al.* and Segal *et al.*[22, 23].

Ethanol yield (Y_{PS}) and volumetric productivity (Q_P) were considered as response variables for analysis of significance and were calculated at the time corresponding to a consumption of more than 90% of fermentable sugars. Y_{PS} (g/g) is defined as the ratio between ethanol production (g/L) and sugar consumption (g/L), whereas Q_P (g/L h) is the ratio between ethanol production (g/L) and fermentation time (h).

3. Results And Discussion

3.1 Effect of sequential pretreatments on chemical composition

Physico-chemical characterization of switchgrass biomass *in natura* was conducted to examine the carbohydrate availability, where Fig. 2 illustrates switchgrass components. Cellulose and hemicellulose accounted for roughly 70% (w/w) of switchgrass biomass with a relatively low lignin content at about 18% (w/w). Switchgrass was pretreated with dilute acid, generating hemicellulosic hydrolysates; the remaining cellulignin fraction was subjected to the optimized alkaline pretreatment to induce delignification. After dilute acid pretreatment (Fig. 2), cellulose and lignin content of switchgrass increased by 25% and 105%, respectively, while the hemicellulose content decreased by 67%. These

results are aligned with previous reports by Samuel *et al.* [24], where under similar severity of dilute acid pretreatment, switchgrass cellulose and lignin content increased by 20% and 50%, respectively, while the hemicellulose content decreased by 73%. Dilute acid hydrolysis breaks down glycosidic bonds, releases acetyl groups and depolymerizes hemicellulose [25], resulting in xylan solubilization, which in turn increases biomass cellulose and lignin content.

The switchgrass cellulignin obtained from dilute acid pretreatment was subsequently subjected to alkali pretreatment and the response was roughly a 1.2-fold increase in cellulose content, with simultaneous reduction of lignin and hemicellulose quantities (Fig. 2). Introduction of alkali solution, serving as an agent for delignification, resulted in the disruption of structural linkages and reduction of degree of polymerization of lignin [26]. The two-stage pretreatment was demonstrated to be effective, with only 5% (w/w) of lignin leftover in the pretreated solids. Although there was a reduction in hemicellulose content, the loss of fermentable sugars was minimal compared to the total benefits. Several strategies could be implemented to achieve similar results, however, chemically-mediated biomass pretreatments represent low-cost methods, which are promising for industrial implementation [27].

With respect to the characterization of hemicellulosic hydrolysate, xylose concentration was roughly three-fold higher than that of glucose at 148 g/L and 57 g/L, respectively. These results are consistent with the data obtained from the characterization of retained solids in Fig. 2. Small concentrations of furfural (1.3 g/L) and 5-hydroxymethylfurfural (1.3 g/L) were also detected in the hemicellulosic hydrolysate. Among other by-products, acetic acid (13 g/L) presented the highest concentration, which is in agreement with the structural properties of biomass.

3.2 Effect of sequential pretreatment and saccharification on biomass structure

Switchgrass fibers *in natura* and after dilute acid and alkaline pretreatments as well as enzymatic hydrolysis were subjected to SEM (Fig. 3) and X-ray diffraction analyses (Fig. 4).

Through SEM analysis, it was determined that the natural integrity of original biomass was intact and unmodified where fibers were aligned and pores undetectable, implying that cellulose is likely coated and protected by hemicellulose and lignin (Fig. 3A). After acid hydrolysis (Fig. 3B), evident disruption of the surface layer was observed, owing to the depolymerization of hemicellulose and to some extent the damage resulting from pressure and temperature. However, the deeper layers and the fiber core were determined to be undisturbed. By contrast, after subsequent alkaline pretreatment (Fig. 3C), there was a clear rupture of fibers and a disruption of compactness, which was visualized by the voids and disorganization of the surface, attributed to lignin removal. Results presented in Fig. 3 shows that the dilute acid followed by alkaline pretreatment strategy was effective for breaching the tightly woven switchgrass cell walls, as compared to solely dilute acid pretreatment.

The results obtained from X-ray diffraction (Fig. 4) demonstrated that, partial removal of lignin and hemicellulose led to increase in degree of crystallinity of pretreated biomass. Switchgrass *in natura*

presented a CrI of 48%, whereas after dilute acid and dilute acid plus alkaline pretreatments the CrI increased to 51% and 55%, respectively. It is well known that, structurally, hemicellulose and lignin can be classified as heteropolymers with a hyperbranched structure [28, 29]. Therefore, they possibly comprised the amorphous moiety of the complex lignocellulosic molecular arrangement. When sequential pretreatments are employed, removal of lignin and most of hemicellulose leads to the reduction of amorphous constituents and increases the overall crystallinity. Similar results have been reported for sugarcane bagasse, where sequential pretreatments led to successive increase in CrI [30, 31]. On the other hand, after enzymatic hydrolysis the CrI of leftover solid residues decreased to 37.5% which is to be expected after biologically catalyzed depolymerization of crystalline cellulose moieties of switchgrass.

3.3 Optimization of alkaline pretreatment of switchgrass

In this work, the variables for the center point of 2^2 full factorial design were defined at 121 °C, related to a pressure of 1 atm at static conditions, and 0.5 h. These conditions were chosen based on previous reports for lignocellulosic biomass pretreatments [32–34]. Pretreatment at 90 °C was defined as a non-pressurized low-level condition, while the maximum was set as 152 °C. The variable time was defined as 0.5 h and 1.5 h for the low and high levels, respectively. The results of the full factorial design considering the two process variables *i.e.*, time and temperature, are shown in Table 2.

The variability in glucose yield was approximately 12%, between the lowest treatment level at 0.5 h, 90 °C, and the most severe treatment condition at 152 °C for 1.5 h. Statistical analysis of the results was performed using glucose yield as the responsive variable. The statistical significance of the studied variables was evaluated and presented using the Pareto chart (Fig. 5) and analysis of variance (Table 3) for the first order model of investigated variables.

As shown in Fig. 5 and Table 3, neither the pretreatment time nor interaction between time and temperature had a significant effect on glucose yield at 95% confidence level. On the other hand, pretreatment temperature had a positive effect on glucose yield, at 95% confidence level, indicating that higher temperatures are suitable to obtain higher glucose yields during the saccharification step. However, temperatures higher than 152 °C were not considered, since process viability would be compromised due to high energy costs. As shown in Fig. 6, optimization of alkaline pretreatment was centered on a linear model with $R^2 = 0.97$ without curvature ($p < 0.05$). The model for glucose yield, as a function of pretreatment time and temperature, is represented by Eq. (1).

$$\text{Glucose Yield} = 26.46 + 7.31 \times \text{time} + 0.21 \times \text{temperature} - 0.04 (\text{time} \times \text{temperature}) \quad (1)$$

Taking the positive effect of pretreatment temperature into account, it can be hypothesized that this variable enhances the mechanism of action of alkaline pretreatment via breaking down lignin, separating the structural bonds between lignin and carbohydrates and thereby reducing the degree of polymerization, increasing the surface area of biomass and finally, facilitating enzyme access to cellulose (Zheng et al., 2018;35 Sarkar et al., 2012;36 Rezende et al., 2011)37 [35–37]. Since time was not a significant factor affecting enzymatic saccharification, the low-level setting (0.5 h) was selected for

optimized conditions, aiming for operating cost reductions. Thus, the final selected conditions for alkaline pretreatment of switchgrass cellulignin were 0.5 h and 152 °C.

3.4 Ethanol production from hemicellulosic and enzymatic hydrolysates

The liquid hydrolysate generated after enzymatic saccharification was employed as carbon feedstock for ethanol production by the benchmark *S. cerevisiae* strain, establishing parameters for comparison of fermentation performance with the wild strain, *S. parashehatae*. The fermentation yield and kinetics of sugar consumption of *S. cerevisiae* are displayed in Fig. 7; the initial glucose concentration of 26 g/L was depleted within 10 h, while ethanol concentration reached 12 g/L within 17 h. The yield factor (Y_{PS}) resulting from this process was 0.46 g ethanol/ g of enzymatic hydrolysate, while the productivity rate was 0.70 g/L h. The reported theoretical maximum yield for glucose or xylose is 0.51 g/ g; therefore, *S. cerevisiae* achieved a conversion rate of 90%, justifying its current use in the bioethanol industry [38].

Unfortunately, *S. cerevisiae* cannot metabolize xylose, thus no changes occurred in xylose concentrations during the course of fermentation (Fig. 7). No lag phase was detected, indicating that the presence of xylose did not repress the anabolic pathway or glucose catabolism to any extent. Early investigations have documented a noticeable catabolic repression of an engineered *S. cerevisiae* strain whenever cultivated in the presence of xylose [39]. Furthermore, Dien *et al.* [40], investigating the conversion of pretreated switchgrass biomass into ethanol with *S. cerevisiae* D5A as control and engineered pentose metabolizing *S. cerevisiae* YRH400 strain, reported a 11.4% increase in ethanol production using the modified strain. Interestingly, *S. cerevisiae* YRH400 did not completely exhaust the xylose concentrations as 6.3 g/L of residual xylose was detected in the media at the end of the cycle. Reported ethanol production rate of *S. cerevisiae* D5A strain is in accordance with the current study at 12.5 g/L. Similarly, recent work reported by Knudsen and Ronnow (2020) [41], utilizing a modified *S. cerevisiae* strain capable of assimilating both C5 and C6 sugars, reported 90% conversion rates.

The search for new microorganisms capable of metabolizing pentoses through the pentose phosphate pathway (PPP) is still open. The novel-wild strain *S. parashehatae* UFMG-HM-60.1b was isolated from the Brazilian fauna and was previously identified by the “Yeast Ecology and Biotechnology Laboratory at the Federal University of Minas Gerais – MG-Brazil” to be capable of synthesizing ethanol from xylose sugars. The results about this yeast for 2G ethanol production and the results of fermentation of switchgrass enzymatic hydrolysates are displayed in Fig. 8.

During *S. parashehatae* fermentation, glucose concentrations decreased from 29 to 0 g/L within 24 h. However, due to the ease of hexose assimilation in the first 18 h, xylose which is normally metabolized through the PPP pathway was inhibited and catabolically repressed [42]. Organisms with natural pentose fermentation capabilities have the tendency to assimilate glucose readily over any other available carbon sources [43]. As expected, after glucose exhaustion from the switchgrass enzymatic hydrolysate, xylose concentrations declined sharply to 0.5 g/L after 24 h. Ethanol production reached its peak at 9 g/L within

24 h, corresponding to Y_{PS} and Q_P of 0.29 g/ g (glucose + xylose) and 0.38 g/L h, respectively. In comparison to the fermentation performance by the wild *S. cerevisiae* strain, *S. parashehatae* showed lower efficiency despite its ability to assimilate C5 sugars. These results are similar to the conversion performance of *Pichia stipitis* NRRL Y-7124 which was reported to have an ethanol yield and productivity of 0.33 g/ g and 0.34 g/L h, respectively, for a medium containing both pentoses and hexoses [44].

The low ethanol yield determined for *S. parashehatae* could be attributed to the fact that ethanol was metabolized during the final stage of fermentation, likely due to the overaccumulation of oxidized cofactor NAD^+ . Therefore, the assumption that ethanol was being consumed to regenerate acetaldehyde (catalyzed by alcohol-dehydrogenase) and the cofactor NADH is plausible. Moreover, concomitant to the decrease in ethanol concentration, the deceleration of xylose concentration did not cease supporting the fact that yeast metabolism showed flexibility under redox stress. Indeed, this behavior to consume ethanol in situations of scarcity of carbohydrates has already been reported by other authors with other strains [30].

Fermentation of switchgrass hemicellulosic hydrolysates were carried out using the wild *S. parashehatae* strain with results showed in Fig. 9. Initial xylose concentration was 23 g/L, while that of glucose was 9 g/L. As denoted previously, despite the high concentration of xylose, glucose was easier to assimilate due to lower enzymatic and transport requirements. However, distinct behavior was observed in this case regarding the concomitant metabolism of C5 and C6 sugars. Glucose was rapidly consumed at a higher rate in the beginning, but did not trigger an acute catabolic repression of xylose as reflected in the continuous assimilation at a relatively lower rate (Fig. 9). Ethanol concentration peaked at roughly 6 g/L within 38 h of fermentation, wherein glucose and xylose were fully depleted at approximately 25 and 35 h, respectively. The calculated ethanol yield and productivity corresponded to 0.19 g/ g (glucose + xylose) and 0.16 g/L h, respectively. Interestingly, after xylose depletion, ethanol started to be metabolized as a substrate for cell survival and a concentration lower than 1 g/L was found to be relegated for xylitol production (data not shown). As a result, it was assumed that xylose was mainly directed towards ethanol synthesis. Mesa *et al.* [45], reported ethanol production from xylose-rich hydrolysates by utilizing *P. stipitis*, with a maximum yield of 4.9 g/L after 24 h. Their course of fermentation showed great resemblance to the current study and similarly observed low levels of xylitol production throughout the process.

It is well known that assimilation of xylose contributes to the consumption of NAD^+ and accumulation of NADH, by the action of NAD^+ dependent xylitol dehydrogenase, which mediates the conversion of xylitol into D-xylulose and enters the PPP as a "shunt" [46]. This overaccumulation of reduced cofactor NADH may stimulate ethanol production, regeneration of NAD^+ and subsequently alleviate $NAD^+/NADH$ cofactor imbalance. By the acknowledgement of this mechanism, inexhaustible efforts have been applied on the task of constructing microbial strains capable of efficiently assimilating different sugar-types simultaneously. Genetic engineering tools allowed the aggregation of exogenous genes in recombinant microorganisms, especially *S. cerevisiae*, to overcome this challenge [47]. The involvement of natural wild strains with the ability to metabolize pentoses play a crucial role in this trend, by providing the genes as

well as metabolomic insights. In this context, the strain exploited in this work may also be categorized as a competitive alternative for consolidating ethanol production in biorefineries due to low xylitol accumulation and minimal catabolite repression in addition to the ability to consume both C5 and C6 sugars.

The novel, wild Brazilian *S. parashehatae* strain has potential for further research to assess the optimal conditions for ethanol fermentation. Wild xylose-consuming yeast strains that do not require genetic modifications could be more profitable and cost-effective. The bottleneck of modern biorefineries lies in the search for "readily" efficient microorganisms that can harness lignocellulosic materials as a whole for the synthesis of a variety of bio-based products [48]. Within that context, it will be beneficial to investigate the ample metabolic pathways that reside in the newly isolated yeast strain. Also, combining the current tailored fermentation processes with the emerging metabolomics technology will make high productivity of biorefineries achievable and reproducible.

4. Conclusion

Our current research focuses on switchgrass sugars fractionation and fermentation using a novel wild yeast strain to aid in the demand for robust, sustainable and profitable biorefineries. This was achieved through a sequential pretreatment process that maximized the recovery of fermentable C5 and C6 sugars from switchgrass biomass and then by using a co-fermenting wild yeast strain for ethanol bioconversion. Physico-chemical characterization of switchgrass biomass highlighted the importance of sequential acid and alkaline pretreatments for bringing about significant damage to the cell wall structure and for satisfactory improvement in enzymatic saccharification yields. Moreover, with process optimization the newly-isolated *S. parashehatae* UFMG-HM-60.1b strain could provide improved xylose and glucose conversion rates. One of the bottlenecks of industrial-scale 2G ethanol production is the lack of efficient and readily available microorganisms capable of metabolizing both C5 and C6 fractions. Henceforth, our investigation provides ample insights about pretreatment dynamics and broadens the possible microbial alternatives to ferment lignocellulosic materials.

Declarations

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Availability of data and materials

Data available on request from the authors: The data that support the findings of this study are available from the corresponding author, on request.

Authors 'contributions

D.J.C², K.R² and A.D.³ provided the biomass, conducted the acid pretreatment and the switchgrass cellulignin and hemicellulosic obtaining. F.A.F.A¹., T.M.R¹, and L.PB¹. performed alkaline pretreatment, enzymatic hydrolysis, fermentation and biomass characterization. C.A.R.⁴ indicated and provided the feasible wild yeast. J.C.S¹, D.J.C² and S.S.S¹ supervised all work. All authors contributed to the data analysis and manuscript writing.

Ethics approval and consent to participate

Not applicable for this study.

Consent for publication

Informed consent was obtained from all individual participants included in the study.

Competing interests

The authors declare that they have no conflict of interest.

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Tables

Due to technical limitations, table 1, 2, 3 is only available as a download in the Supplemental Files section.

Figures

Figure 5

Pareto chart for glucose yield (%), according to the statistical analysis of 22 full factorial design carried out to evaluate the influence of time and temperature on the alkaline pretreatment of switchgrass cellulose-lignin

Supplementary Files

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