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Integrating high solids enzymatic hydrolysis and co-culture fermentation to improve ethanol production from deep eutectic solvent pretreated rice straw

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ABSTRACT

Rice straw is an abundant agricultural residue with significant potential for sustainable ethanol production. However, the major bottlenecks in converting rice straw into ethanol are biomass recalcitrance, high enzyme costs, and inefficient utilisation of mixed sugars. In this study, an integrated process combining microwave-assisted ChCl:glycerol pretreatment, optimised high-solids enzymatic hydrolysis at low enzyme loading, and co-culture fermentation was developed to enhance ethanol production from rice straw. High-solids enzymatic hydrolysis conditions were optimised by applying central composite design and response surface methodology. At a solids loading of 9.89%, the highest total sugar (TS) yield of 94.92% was obtained compared to a TS of 43.29% at 30% solids loading. The optimal hydrolysis conditions of 17% (w/v) solids loading, 3 FPU/g cellulose enzyme loading, and 75 h hydrolysis time were predicted by the quadratic model and validated, resulting in 75.7% TS yield. Fermentation of the resulting hydrolysates demonstrated that co-culture fermentation outperformed mono- and sequential cultures, achieving a maximum ethanol concentration of 41.1 g/L, with corresponding yields and volumetric productivity of 0.46 g/g and 1.71 g/L.h, respectively. In comparison, co-culture fermentation of hydrolysates derived from 1% H_2SO_4 pretreatment resulted in lower ethanol yield (0.35 g/g) and productivity (0.67 g/L.h). Thus, the ability to attain high ethanol titre and yield at reduced enzyme dosage and high solids loading highlights the effectiveness of microwave-assisted deep eutectic solvent pretreatment and co-culture fermentation using *Saccharomyces cerevisiae* and *Candida tropicalis*. This integrated strategy provides an innovative approach to advancing lignocellulosic bioethanol production from agricultural residues.

1. Introduction

The growing concerns over climate change and the rapidly declining fossil fuel resources due to increasing global energy demand have intensified interest in sustainable and renewable biofuels [1]. Ethanol stands out among available renewable liquid fuels because of its compatibility with existing energy infrastructure, without requiring major modifications to vehicles (e.g., E5-10 blends), pipelines, or storage facilities [2]. However, industrial-scale production of ethanol from first-generation feedstocks (e.g., sweet corn, sugarcane, maize, soybeans, etc.) could negatively impact food security and land use [3,4]. Thus, there is a need to develop efficient second-generation ethanol processes based on non-food lignocellulosic biomass (agricultural residues, wood, and energy crops) [3,5]. Rice straw is one of the most abundant agricultural residues, with an estimated annual global production of around 731 million tons, and Asia alone accounts for about 50% of that output [6,7]. Rice straw is composed of 32-39% cellulose, 20-36% hemicellulose, 14-22% lignin and 10-17% ash [8–10]. On the other hand, the presence of silica in lignocellulosic biomass has been reported to decreased conversion of carbohydrates into monomeric

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sugars during enzymatic hydrolysis [11].

Despite its high cellulose and hemicellulose content, rice straw remains vastly underutilised and is often improperly disposed of or burned in open fields, a practise that has been banned in major agricultural regions due to its severe environmental consequences [12]. These characteristics make rice straw an attractive and renewable feedstock for ethanol production [13]. However, pretreatment is a prerequisite for deconstructing the recalcitrant lignocellulosic structure of rice straw enabling enhanced enzymatic hydrolysis and release of fermentable sugars [14,15]. Conventional pretreatment methods typically require high energy inputs, substantial chemical consumption, and the formation of inhibitors, which negatively affect downstream processing [16]. On the other hand, pretreatment methods using deep eutectic solvents (DESs) offer a better alternative due to their low toxicity, biodegradability, and cost-effectiveness [17–19]. Microwave assisted-ChCl: glycerol pretreatment has been applied to rice straw leading to high delignification with minimal loss of cellulose/hemicellulose components [20]. The use of microwave heating enables rapid and uniform heating, thereby reducing pretreatment time and improving delignification efficiency [21]. However, to fully realise the industrial-scale potential of microwave pretreatment, future research is required to optimise pretreatment process parameters, improve unit production, and reduce production costs [22,23].

However, despite advances in pretreatment technologies, enzymatic hydrolysis represents a major cost bottleneck in ethanol production from lignocellulosic biomass, primarily due to the high enzyme dosages required to achieve acceptable sugar yields for industrial-scale fermentation [23]. Another key challenge associated with ethanol production from rice straw is the efficient fermentation of mixed sugars resulting from enzymatic hydrolysis, as the hydrolysates contain both hexoses and pentoses [24]. Thus, the application of high-solids enzymatic hydrolysis is essential to increase sugar concentrations and improve process economics. This also presents additional challenges, such as poor mass transfer, high viscosity, and end-product inhibition [25]. Therefore, integrated processes that synergistically combine effective pretreatment with tailored enzymatic hydrolysis conditions can significantly enhance cellulose conversion efficiency while reducing enzyme costs [26]. To this end, design of experiments (DoE) using Central Composite Design (CCD) and Response Surface Methodology (RSM) is a valuable tool widely employed to optimise process conditions and achieve high product yields [27,28].

Saccharomyces cerevisiae is the most widely used yeast for large-scale ethanol production because of its high ethanol productivity under optimal conditions [29], however, it cannot utilise pentose sugars [30]. In contrast, *Candida tropicalis* can utilise pentoses such as xylose, although it generally yields lower ethanol when used alone [31]. Co-culture fermentation has emerged as a promising strategy to enable simultaneous utilisation of mixed sugars, improve ethanol yield, and enhance overall process efficiency [32–34]. Despite the potential of co-culture fermentation, the performance of *S. cerevisiae–C. tropicalis* co-culture systems using enzymatic hydrolysates derived from DES-pretreated rice straw remains poorly understood. In this study, an integrated process is developed to improve bioethanol production from rice straw by optimising high-solids enzymatic hydrolysis at low enzyme loading followed by co-culture fermentation using *S. cerevisiae* and *C. tropicalis*. Efficiencies of mono-culture, co-culture, and sequential culture fermentation of hydrolysates of ChCl:glycerol pretreated rice straw were evaluated. In addition, hydrolysates obtained from rice straw pretreated by a conventional method (1% H_2SO_4, 121°C, 30 min, autoclave) were treated to co-culture fermentation using *S. cerevisiae* and *C. tropicalis* and benchmarked against ChCl:glycerol pretreated rice straw. Thus, the findings from this study are expected to provide insights into cost-effective process integration strategies for advancing sustainable lignocellulosic bioethanol production.

2. Materials and methods

2.1. Materials

The untreated RS consists of cellulose (41.8%), hemicellulose (24.9%), lignin (17.0%), ash (15.0%) and others (1.3%) [20]. Untreated RS (≤0.5 mm of particle size) was pretreated with ChCl:glycerol (molar ratio of 1:2) under microwave irradiation (10% solids loading, 140°C, 5 min) following the method previously reported [20]. The composition pretreated rice straw was determined using a method of National Renewable Energy Laboratory (Sluiter et al., 2012 [35] and consisted of glucan (57.6%), xylan (19.3%), lignin (8.2%) and ash (13.9%).

All the chemicals employed were analytical grade and employed as received without any further purification. *S. cerevisiae* (DSM 1334) and *C. tropicalis* (DSM 7524) were purchased from The Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures GmbH (Braunschweig, Germany) and stored at −80°C in cryotubes containing sterilised 20% glycerol solution.

2.2. Enzymatic hydrolysis

Enzymatic hydrolysis of DES pretreated RS was performed using Cellic® CTec3 HS with enzyme activity of 106 filter paper units (FPU)/mL determined using the method described by Ghose [36]. 500 mg of sample (10% w/v) was loaded into a 15 mL screw-capped glass tube containing 5 mL sodium citrate buffer (50 mM, pH 4.8) and enzyme loading of 6 FPU/g cellulose as previously reported [20]. Sodium azide (0.025 g/L) was added to the mixture to prevent contamination and microbial growth. Afterwards, the tube was vortexed to allow proper mixing of the enzyme with the sample (substrate). The hydrolysis was conducted in an orbital incubator (Incu-Shake MAXI, SciQuip Ltd, Rotherham, UK) at 50°C for 72 h and shaking at 180 rpm. After 72 h, the sample was deactivated by a heating block (100°C) for 10 min. Later, the sample was removed and allowed to cool down to room temperature, and the supernatant was collected for sugar analysis using HPLC under the conditions described in 'Analytical method section'.

2.3. Central composite design (CCD) and response surface methodology (RSM)

Central composite design (CCD) was employed by using the Design of Experiments (OriginPro 2025 SR0 software). RSM consists of statistical and mathematical techniques vital for modelling and analysing problems in which the response is determined by numerous interacting variables. The main objective of the response surface is to attain optimised response variables [27,37]. Thus, the effect of three variables (solids loading (X_1, % w/v); enzyme loading (X_2, FPU g^{-1} cellulose); and time (X_3, h)) was evaluated on the response (total sugar yield (% g/g). Three variables for CCD, consisting of six central points and five levels for each variable, i.e., –α, −1, 0, +1, +α, were selected for the optimization, and a total of 20 experimental runs were generated. The range and levels of independent variables and coded values are presented in Table 1. The independent variables and their ranges were defined based on previous studies [15].

Experiments were conducted towards the construction of a mathematical model. The TS yield (Y) was determined using the following polynomial equation (Eq. (1)) [8]:

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{ii}^{k} \sum_{i,j} \beta_{ij} X_i X_j \quad \text{(Eq. 1)}$$

where Y is the response variable; X_i, X_j, and X_k are the corresponding actual values of the variables; β_0 is the regression coefficient of the fitted response at the centre point of the design; β_i is the linear term coefficient, β_{ii} is the regression coefficient for quadratic effects and β_{ij} and β_{ji} are the interaction terms coefficients. Subsequently, non-significant terms were removed from the equation using the backward

Table 1
Central composite design and experimental results of enzymatic hydrolysis ChCl:glycerol pretreated rice straw.

Run order	Coded variables			Actual variables			Total sugar yield (%)	
	Solids loading (X_1)	Enzyme loading (X_2)	Time (X_3)	Solids loading (X_1)	Enzyme loading (X_2)	Time (X_3)	Experimental	Predicted
1	0	0	0	22.5	10	80	60.82 ± 5.26	60.76
2	0	0	0	22.5	10	80	59.21 ± 3.44	60.76
3	0	0	+1.67	22.5	10	147.3	61.28 ± 1.90	64.32
4	0	+1.67	0	22.5	18.4	80	68.95 ± 1.09	70.36
5	−1.67	0	0	9.9	10	80	94.92 ± 1.15	96.15
6	0	0	0	22.5	10	80	59.57 ± 4.36	60.76
7	−1	−1	1	15	5	120	85.30 ± 2.15	83.68
8	0	0	0	22.5	10	80	60.78 ± 2.54	60.76
9	−1	1	−1	15	15	40	82.51 ± 4.85	82.25
10	1	1	1	30	15	120	53.83 ± 2.88	52.05
11	0	0	0	22.5	10	80	62.56 ± 1.16	60.76
12	+1.67	0	0	35.1	10	80	40.97 ± 3.74	40.61
13	0	−1.67	0	22.5	1.6	80	62.92 ± 1.50	62.38
14	0	0	0	22.5	10	80	61.79 ± 2.73	60.76
15	−1	−1	−1	15	5	40	75.57 ± 2.70	76.72
16	−1	1	1	15	15	120	87.76 ± 3.70	85.90
17	0	0	−1.67	22.5	10	12.7	60.43 ± 2.47	58.26
18	1	−1	1	30	5	120	48.45 ± 4.39	48.08
19	1	−1	−1	30	5	40	43.29 ± 2.63	44.53
20	1	1	−1	30	15	40	50.80 ± 5.71	51.80

elimination methodology, followed by the addition of higher degree factors to the polynomial equation 27.

These variables were selected according to the preliminary experimental results as the critical parameters nominated as X_1, X_2, and X_3. The correlation coefficient (R^2), was employed to scrutinise the fit of the quality of the model (i.e. polynomial). Analysis of Variance (ANOVA) was employed to evaluate both the model fit and the significance of its parameters [8,38].

2.4. Fermentation of enzymatic hydrolysates of ChCl:glycerol pretreated rice straw

S. cerevisiae and *C. tropicalis* initially stored at −80°C were reactivated in medium containing (g/L): glucose, 30.0; yeast extract, 3.0; peptone, 5.0; agar, 20.0 at pH 6.0 ± 0.2 and temperature 30°C. Starter culture was developed by growing cells at 30°C and 200 rpm for 12 h in a culture medium containing (g/L): glucose, 30.0; yeast extract, 3.0; peptone, 5.0; pH 6.0 ± 0.2. The fermentation of enzymatic hydrolysates from Section 2.2 was carried out in a 150 mL Erlenmeyer flask in an orbital incubator (30°C, 200 rpm, 24 h) with a working volume of 50 mL supplemented with 4 g/L of yeast extract, 2 g/L of $(NH_4)_2SO_4$, 0.5 g/L of $MgSO_4.7H_2O$ and 1 g/L of KH_2PO_4, adjusted to pH 6.0. The synthetic defined medium consisted of glucose (68.0 g) and xylose (28.0 g), supplemented as for enzymatic hydrolysates. Before inoculation, the starter culture was centrifuged at 13,000 × g for 2 min at 4°C. Nutrient supplementation before fermentation was necessary because the lignocellulosic hydrolysates lack sufficient essential growth factors such as nitrogen, vitamins, and minerals required for yeast cells growth [39].

The resulting pellet was washed once with 10 mL of sterile 0.85% NaCl (w/v) solution and recentrifuged under the same conditions. The pellet was resuspended in the sterile 0.85% NaCl (w/v) solution to an OD_{600} of 1.0. Afterwards, 400 μL of starter culture was inoculated into 50 mL of enzymatic hydrolysate in a 150 mL Erlenmeyer flask. In the case of co-culture, 200 μL each of starter culture (OD_{600} 1.0) of *S. cerevisiae* and *C. tropicalis* was used, while for sequential culture, 200 μL starter culture of *S. cerevisiae* was used initially, followed by 200 μL starter culture of *C. tropicalis* after 9 h of incubation. 1.0 mL of sample was collected every 3 h, supernatant and biomass were separated by centrifugation and stored 4°C. Later supernatant was used for sugar and ethanol analysis, while the biomass was used for cell dry weight measurement.

For comparison, co-culture fermentation was carried out on the hydrolysate obtained following enzymatic hydrolysis of rice straw pretreated with 1% H_2SO_4 in an autoclave at 120°C for 30 min as described earlier.

2.5. Analytical methods

Sugar (glucose and xylose) and ethanol analyses were carried out using a High-Performance Liquid Chromatography (HPLC) system (1260 Infinity) equipped with a Hiplex H column (Agilent) and a Refractive Index Detector. The HPLC conditions were as follows: 5 mM H_2SO_4 was the Mobile phase, with a flow rate of 0.6 mL/min; the column temperature was 25°C, and the detector temperature was 35°C. Before injection, a 1 mL aliquot of culture was centrifuged at 3000× *g* for 5 min, and the supernatant was filtered through a 0.22 μm nylon syringe filter.

2.6. Determination of hydrolysis and fermentation parameters

Total sugars (glucose and xylose) yield (%) was calculated using Equation (2).

$$Y_{S/B} = \frac{S_f - S_i}{Biomass} \times 100 \quad \text{(Eq. 2)}$$

Where S_f is the final sugar (glucose and xylose) concentration (g/L), S_i is the initial sugar (glucose and xylose) concentration (g/L), Biomass is the initial amount of glucan and xylan content in the pretreated RS added to the buffer, as expressed in g/L.

Ethanol yield (g/g) was calculated using Equation (3).

$$Y_{E/B} = \frac{EtOH_f - EtOH_i}{S_i - S_r} \quad \text{(Eq. 3)}$$

Where $EtOH_f$ is the final ethanol concentration (g/L), $EtOH_i$ is the initial concentration (g/L), S_i is the initial sugar (glucose and xylose) concentration (g/L), S_r is the residual sugar (glucose and xylose) concentration (g/L).

Volumetric productivity (g/L.h) was calculated using Equation (4).

$$Q_P = \frac{EtOH_f}{t} \quad \text{(Eq. 4)}$$

Where $EtOH_f$ is the final ethanol concentration (g/L), t is the fermentation time (h)

2.7. Statistical analyses

Plotting of figures were performed using Origin Pro V2025. All experiments were conducted at least in duplicate, and the results were presented as mean ± standard error of the mean. Statistical analysis of data was performed using SPSS Statistics (IBM SPSS Statistics, Version 29.0., USA). Data were subjected to a one-way ANOVA with the Duncan test and the probability value of $p < 0.05$ was considered significant.

3. Results and discussion

3.1. Enzymatic hydrolysis

High-solids enzymatic hydrolysis of DES-pretreated rice straw (RS) was performed using the Design of Experiments. Total sugars (TS) consist of glucose and xylose in the hydrolysate after enzymatic hydrolysis. The effects of solids loading (X_1), enzyme loading (X_2), and hydrolysis time (X_3) on the yield of TS from DES-pretreated RS were investigated by CCD with RSM (Table 1). The close agreement between experimental and predicted responses validates the response surface model across the studied domain. Solids loading exhibited the strongest effect on the yield of TS, with the highest TS yield of 94.92% obtained with 9.89% of solids loading. High TS yields (>80%) were maintained at 15% solids loading across multiple combinations of enzyme loadings and time. On the contrary, solids loading beyond 30% resulted in significant decreases in TS yield (<54%), irrespective of higher enzyme doses and prolonged hydrolysis time.

This reduction mirrors the "high-solids effect," where reduced free water, elevated viscosity and mass transfer constraints limit enzyme-substrate interactions and sugar release at high solids loadings. This effect could be attributed to mass transfer limitations owing to high viscosity and inadequate mixing especially above 20% solids loading [40]. Enzyme loading positively influenced TS yield at low-to-moderate solids loading, with increases from 5 to 15 FPU/g cellulose of enzyme loading enhancing sugar release. Interestingly, at an enzyme loading of 1.59 FPU/g cellulose, TS yield obtained was comparable to the centre points, thereby underscoring the enhanced digestibility imparted by ChCl:glycerol pretreatment, consistent with recent reports of DES-facilitated cellulose accessibility [41]. TS yields were significantly affected by hydrolysis time at lower solids loading, compared to high solids loading indicating that prolonging hydrolysis time was not sufficient in overcoming "the high solids effects''. Strong interactions between solids loading, enzyme loading, and time were evident, an indication that high TS yields required low-to-moderate solids together with adequate enzymes and time.

3.1.1. Model development and statistical analysis

The experimental results presented in Table 1 were analysed by analysis of variance (ANOVA) and the fitted equation model was obtained as shown in Table 2. The model was highly significant ($p < 0.0001$), indicating that the selected variables adequately explained the observed variability in TS yield [42]. High coefficient of determination R^2 (0.99) and adjusted R^2 (0.98) were obtained indicating excellent fit between predicted and experimental values, while the high predicted R^2 (0.94) confirms strong predictive capability of the model. The insignificant lack-of-fit test ($p = 0.083$) indicates that the quadratic model sufficiently described the experimental data within the range investigated.

The linear terms (X_1, X_2 and X_3) of all the variables tested were significant ($p < 0.01$), indicating that all three variables exerted significant individual effects on enzymatic hydrolysis efficiency and yield of TS. The quadratic terms of solid loading (X_1^2) and enzyme loading (X_2^2) were significant ($p < 0.01$), while the quadratic term of hydrolysis time (X_3^2) was insignificant ($p = 0.73$). The significant terms (X_1^2 and X_2^2) reveal strong curvature effects associated with solids loading and enzyme loading. However, the insignificant ($p > 0.05$) interaction effects amongst the variables tested indicated the dominance of main effects particularly solids loading on the hydrolysis efficiency and yield of TS.

Table 2
ANOVA for the quadratic model.[b].

Source	DF	SS	MS	F-Value	Prob > F
Model	9	4005.831	445.092	112.88	<0.0001
X_1	1	3723.668	3723.668	944.2685	<0.0001
X_2	1	76.97402	76.97402	19.5195	0.0013
X_3	1	44.30271	44.30271	11.23453	0.00734
X_1^2	1	104.5394	104.5394	26.50968	<0.001
X_2^2	1	56.62702	56.62702	14.35979	0.00355
X_3^2	1	0.50597	0.50597	0.12831	0.72764
X_1X_2	1	1.51433	1.51433	0.38401	0.54932
X_1X_3	1	5.76218	5.76218	1.46121	0.25454
X_2X_3	1	5.47039	5.47039	1.38721	0.26615
Error	10	39.43443	3.94344		
Lack of fit	5	31.27737	6.25547	3.83439	0.08326
Pure Error	5	8.15706	1.63141		
Total	19	4045.265			

[b]SS-sum of squares, MS-mean square, DF-degree of freedom, $R^2 = 0.99$,Adj. $R^2 = 0.98$, Pred. $R^2 = 0.94$.

The application of multiple regression analysis on the experimental data yielded a regression equation (Eq. (5)) in which predicted response for TS yield could be obtained by means of a second-order polynomial equation.

$$Y = 60.76 - 16.51\boldsymbol{X_1} + 2.37\boldsymbol{X_2} + 1.80\boldsymbol{X_3} + 2.69\boldsymbol{X_1^2} + 1.98\boldsymbol{X_2^2} + 0.19X_3^2 + 0.44X_1X_2 - 0.85X_1X_3 - 0.83X_2X_3 \quad \text{(Eq. 5)}$$

This equation includes all terms regardless of their significances. The significant terms are highlighted in bold. The coefficient sign (±) defines the direction of the relationship between each of variables and the response. The positive sign indicates that as the value of one variable changes, the response changes in the same direction, while for the negative sign, the response operates in the opposite direction. The absolute coefficients measure the strength of the relationship [37].

3.1.2. Three-dimensional (3-D) response surface plots

The 3-D response surface plots (Fig. 1) further illustrate the combined effects of process variables. The interaction between solids loading and enzyme loading (Fig. 1a) shows that high TS yields are achieved only at low solids loading, regardless of enzyme dosage, confirming the dominant limiting role of solids

loading. Similarly, the interaction between solids loading and hydrolysis time (Fig. 1b) demonstrates that extending hydrolysis time cannot compensate for the negative effects of high solids loading. In contrast, the surface response plot depicting enzyme loading and hydrolysis time (Fig. 1c) reveals a relatively broad optimum region, indicating that moderate enzyme dosage combined with sufficient hydrolysis time is adequate to achieve high TS yields, provided solids loading remains within a favourable range (<15% solids) [25].

Overall, the statistical and response surface analyses demonstrate that solids loading is the dominant parameter governing enzymatic hydrolysis efficiency of ChCl:glycerol pretreated rice straw. On the other hand, while enzyme loading and hydrolysis time significantly influence TS yield, their effects are strongly constrained by high solids loading. Therefore, the high TS yields obtained at high solids loading highlight the effectiveness of ChCl:glycerol pretreatment in enhancing biomass digestibility.

3.1.3. Model validation

The quadratic model identified 17% (w/v) of solids loading (X_1), 3 FPU/g cellulose of enzyme loading (X_2), and 75 h of hydrolysis time (X_3)

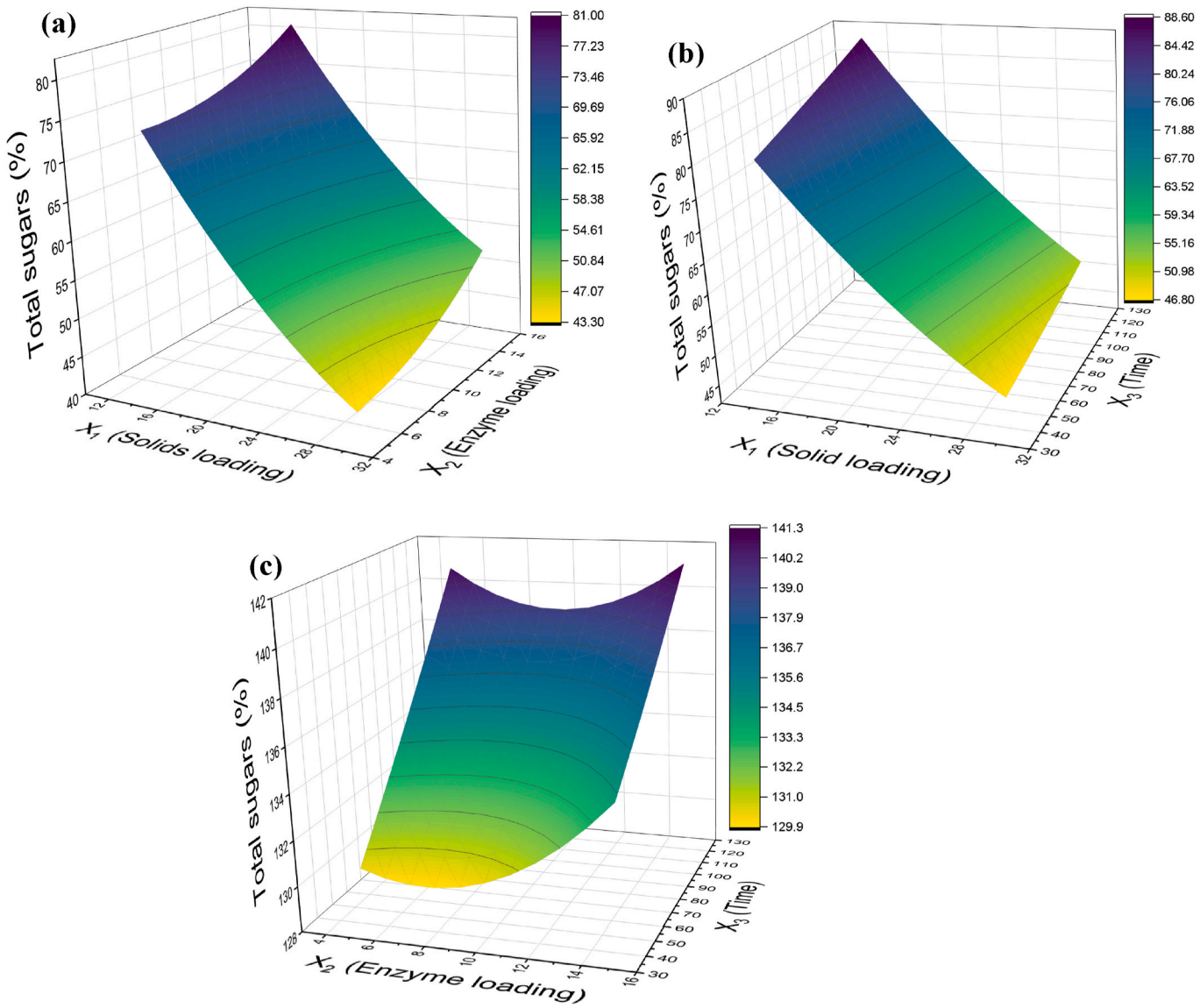

Fig. 1. Response surface curves for enzymatic hydrolysis of ChCl:glycerol pretreated rice straw (a-c) showing interactions between (a) X_1-Solids loading and X_2-Enzyme loading, (b) X_1-Solids loading and X_3-Time, (c) X_2-Enzyme loading and X_3-Time.

as the optimal conditions for enzymatic hydrolysis of DES pretreated rice straw (Table 3). Under these conditions, the measured TS and predicted yield were 75.71 ± 1.83% and 80.67%, respectively, with a standard deviation of 2.45%, indicating that the measured TS and predicted TS yields were in reasonable agreement based on the fitted quadratic model. The predicted optimal conditions were evaluated experimentally in triplicate. TS yield of 77% was reported after enzymatic hydrolysis of DES-pretreated rice straw under un-optimised conditions (6 FPU/g cellulose enzyme loading, 10% solids loading, 72 h of hydrolysis time) [20], compared to 76% of TS yield obtained in the present study at lower enzyme loading (3 FPU/g cellulose) and high solids loading (17%).

Table 3
Optimal values of the independent variables, experimental, and predicted yield of total sugar.

Independent variables			TS yield (%)	
Solids (X_1) (%, w/v)	Enzyme loading (X_2) (FPU/g cellulose)	Time (X_3)(h)	Measured	Predicted
17.0	3.0	75	75.71 ± 1.83	80.67

3.2. Fermentation

Enzymatic hydrolysate of DES-pretreated RS mainly consists of glucose and xylose that yeasts can metabolise to produce ethanol. However, conventional yeast (*S. cerevisiae*) can efficiently metabolise glucose but lacks the ability to utilise xylose for ethanol production [43]. Another yeast, *C. tropicalis*, can utilise both glucose and xylose for ethanol production, but less efficiently than *S. cerevisiae*. Thus, fermentation under different culture conditions (monoculture, co-culture, and sequential culture) was carried out on the enzymatic hydrolysate to compare their efficiencies on ethanol production (Figs. 2–4). Fermentation of the synthetic defined medium was performed with the same culture conditions as described above for comparison.

3.2.1. Mono-culture fermentation of enzymatic hydrolysates

To differentiate intrinsic metabolic characteristics from lignocellulosic hydrolysate-induced inhibitory effects, mono-cultures of *S. cerevisiae* and *C. tropicalis* were performed on both enzymatic hydrolysate and synthetic defined medium containing glucose and xylose as the sole carbon source. Fig. 2 shows the fermentation profiles of mono-

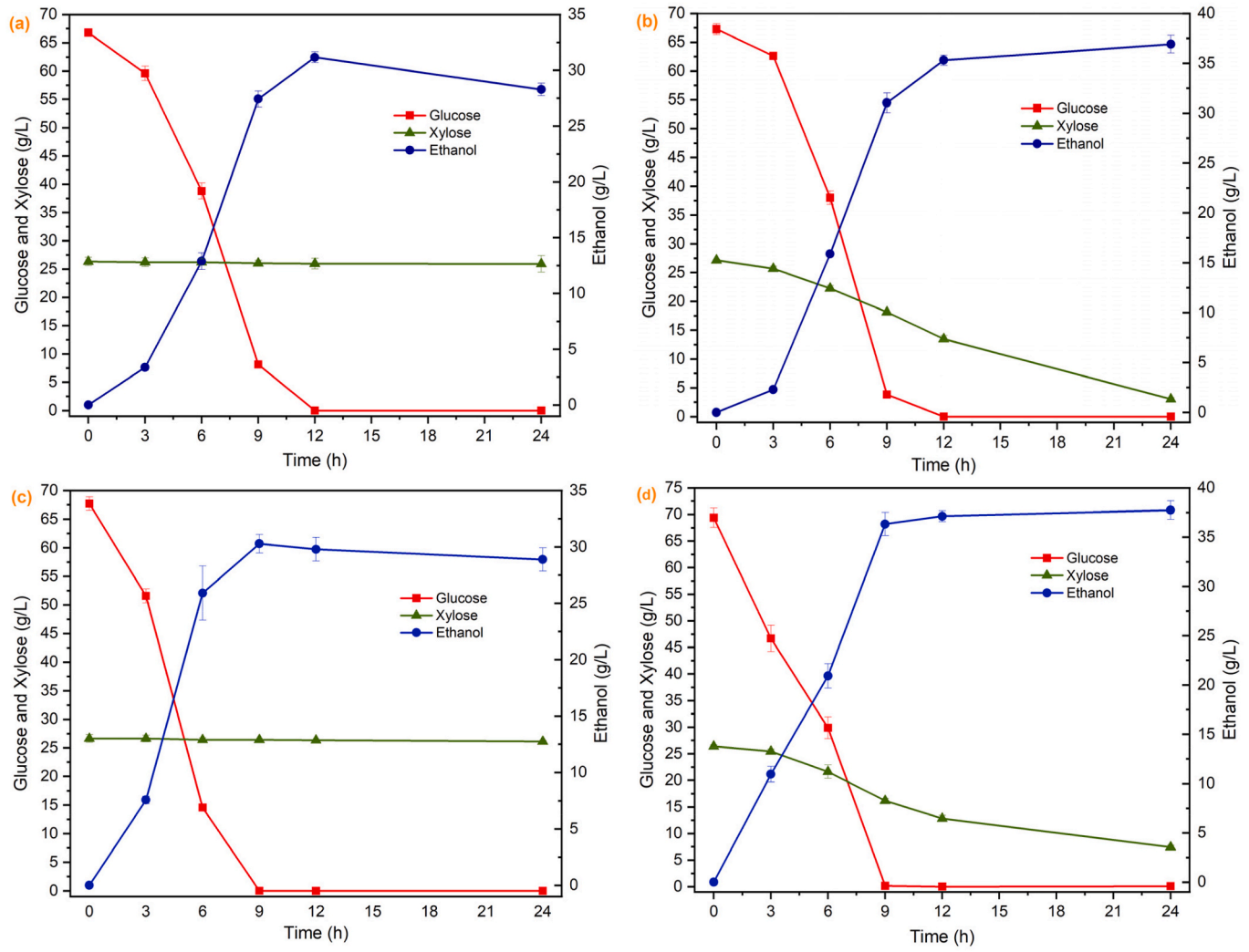

Fig. 2. Mono-culture fermentation: (a) *S. cerevisiae* on hydrolysate of ChCl:glycerol pretreated RS, (b) *C. tropicalis* on hydrolysate of ChCl:glycerol pretreated RS, (c) *S. cerevisiae* on Synthetic sugars, (d) *C. tropicalis* on Synthetic defined media.

cultures of *S. cerevisiae* and *C. tropicalis* on the enzymatic hydrolysate of pretreated RS and synthetic defined medium containing glucose and xylose as the primary fermentable sugars. In the mono-culture of *S. cerevisiae* (Fig. 2a), glucose was rapidly consumed and became completely depleted within 12 h, whereas xylose remained relatively unchanged at around 26 g/L after 24 h. Ethanol level increased steadily, reaching a maximum level of 31.2 g/L after 12 h, corresponding to an ethanol yield of 0.46 g/g. Extending the fermentation time to 24 h resulted in slight decreases in ethanol level (28.3 g/L) and yield (0.42 g/g) with productivity of 1.18 g/L.h (Table 4).

The decreases in ethanol concentration after 24 h could be attributed to its evaporation or metabolism by cells as an alternative carbon source in the absence of glucose [44]. The sugar consumption pattern in mono-culture of *S. cerevisiae* reflects strong glucose catabolite repression - a well-documented phenomenon in *S. cerevisiae* under mixed-sugar fermentation [45,46]. Other authors reported ethanol yield of 0.45 g/g from glucose fermentation in lignocellulosic hydrolysates [47]. and similar trends were reported for glucose-dominated fermentations where pentose metabolism is poorly regulated or energetically unfavourable [48]. On the other hand, *C. tropicalis* demonstrated enhanced fermentation performance on enzymatic hydrolysate (Fig. 2b), primarily due to its ability to simultaneously utilise both glucose and xylose [49]. Although glucose was preferentially consumed, xylose concentration became depleted from 27 g/L to 3.0 g/L over the 24 h of fermentation, indicating a superior capacity for xylose utilisation. Consequently, a maximum ethanol level of 36.9 g/L was achieved after 24 h, corresponding to an ethanol yield of 0.40 g/g and 1.54 g/L.h of productivity. Other authors reported 0.40 g/g of ethanol yield from mixed sugar fermentation using *Scheffersomyces stipitis* [50].

As shown in Fig. 2c and d, slightly higher ethanol yields (0.38-0.50 g/g) were obtained from the synthetic medium than from the enzymatic hydrolysate (0.33-0.47 g/g), indicating that the enzymatic hydrolysate did not inhibit fermenting cells [51]. Across both enzymatic hydrolysate and synthetic medium, *C. tropicalis* consistently outperformed *S. cerevisiae* in terms of xylose utilisation and final ethanol concentration. However, the ethanol yields in mono-cultures of *C. tropicalis* were lower than those of *S. cerevisiae*. Notwithstanding this, *C. tropicalis* has shown potential for industrial lignocellulosic ethanol production, where the efficient conversion of mixed sugars remains a significant challenge [52–54].

3.2.2. *Co-culture fermentation of enzymatic hydrolysates*

Fig. 3 shows the co-culture fermentation profiles of *S. cerevisiae* and *C. tropicalis* on enzymatic hydrolysates of pretreated RS. Glucose was depleted within 9 h of fermentation, while xylose decreased from 26.3 g/L to 3.2 g/L after 24 h (Fig. 3a). Ethanol concentration increased steadily during the course of fermentation reaching a maximum level of 41.1 g/L and a yield of 0.46 g/g with productivity of 1.71 g/L.h after 24

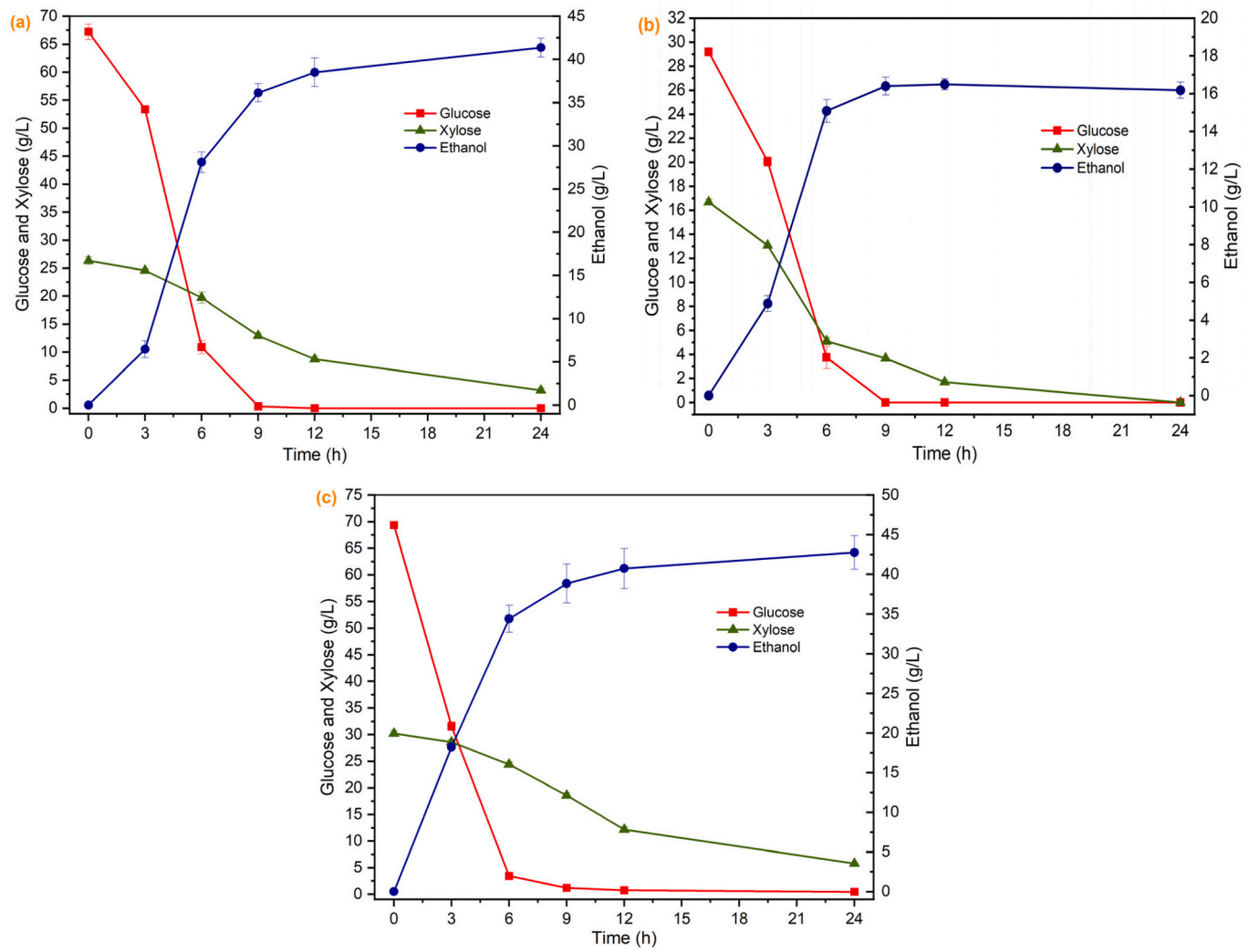

Fig. 3. Co-culture fermentation: (a) Enzymatic hydrolysate of ChCl:glycerol pretreated RS, (b) Enzymatic hydrolysate of H_2SO_4 pretreated RS, (c) Synthetic defined media.

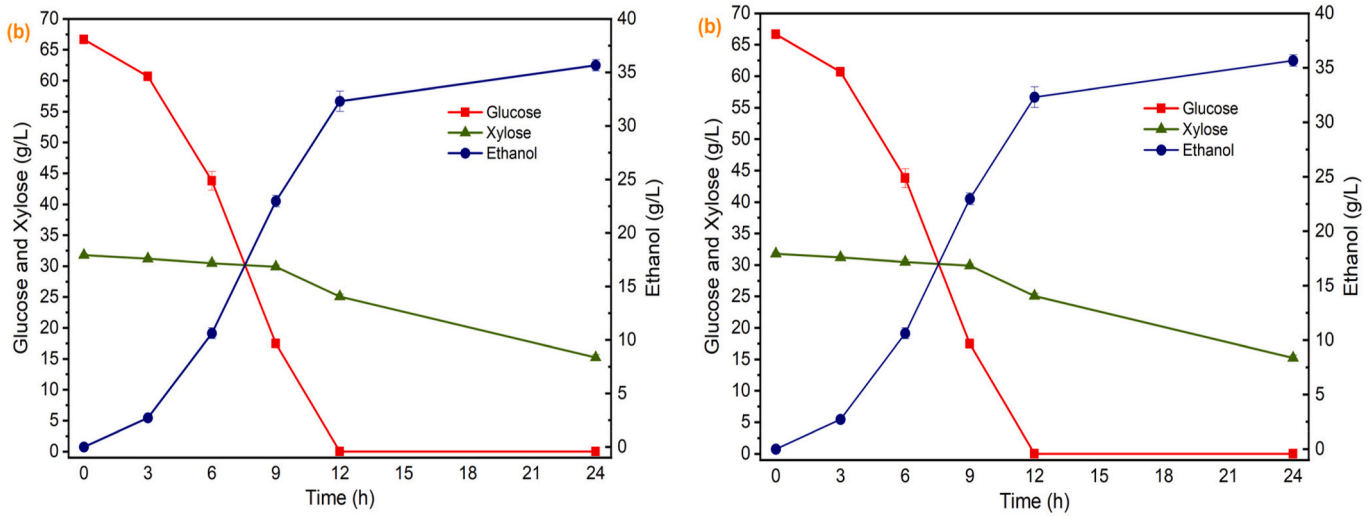

Fig. 4. Sequential fermentation: (a) Enzymatic hydrolysate of ChCl:glycerol pretreated RS, (b) Synthetic defined media.

h. An ethanol titre of 41.1 g/L obtained from the co-culture fermentation is promising for second-generation (2G) ethanol production, although 50 g/L is considered the benchmark [55,56]. A similar fermentation profile was obtained for hydrolysates of 1% H_2SO_4 pretreated RS with

Table 4
Fermentation efficiencies of various cultivation strategies on enzymatic hydrolysate (EH) of ChCl:glycerol pretreated rice straw (RS) and synthetic define medium.

Hydrolysate type and fermentation mode	Ethanol yield (g/g)	Ethanol productivity (g/L.h)
Co-culture (*S. cerevisiae* & *C. tropicalis*) on EH of DES pretreated RS	0.45 ± 0.01	1.71 ± 0.00
Co-culture (*S. cerevisiae* & *C. tropicalis*) on EH of H_2SO_4 pretreated RS	0.35 ± 0.00	0.67 ± 0.01
Co-culture (*S. cerevisiae* & *C. tropicalis*) on synthetic defined medium	0.46 ± 0.01	1.78 ± 0.04
Mono-culture (*S. cerevisiae)* on EH of DES pretreated RS	0.42 ± 0.00	1.18 ± 0.01
Mono-culture (*C. tropicalis*) on EH of DES pretreated RS	0.40 ± 0.01	1.54 ± 0.02
Mono-culture (*S. cerevisiae*) on synthetic defined medium	0.41 ± 0.01	1.14 ± 0.00
Mono-culture (*C. tropicalis*) on synthetic defined medium	0.43 ± 0.01	1.57 ± 0.00
Sequential (*S. cerevisiae* + *C. tropicalis*) on EH of DES pretreated RS	0.42 ± 0.01	1.51 ± 0.02
Sequential (*S. cerevisiae* + *C. tropicalis*) on synthetic defined medium	0.43 ± 0.00	1.49 ± 0.02

16.2 g/L ethanol and a yield of 0.35 g/g with productivity of 0.67 g/L.h after 24 h from an initial TS concentration of 45.9 g/L. However, lower ethanol yield and productivity were obtained from hydrolysates of 1% H_2SO_4-pretreated RS, which might be attributed to the inhibitory effect of the hydrolysate on fermenting cells. Thus, corroborating the findings by other authors using conventional methods for lignocellulosic biomass pretreatment [57].

Furthermore, the slower initial glucose utilisation rate observed in enzymatic hydrolysates compared to co-culture on synthetic defined medium (Fig. 3b), indicates an initial adaptation of fermenting cells to the hydrolysate conditions [58]. The co-fermentation of sugars in the hydrolysates by *S. cerevisiae* and *C. tropicalis* shows that co-culture fermentation of lignocellulosic hydrolysates can result in competitive final ethanol concentrations due to enhanced xylose utilisation under reduced glucose repression [32].

Other authors reported 25 g/L of ethanol and a yield of 0.44 g/g from acid pretreated sugarcane bagasse hydrolysate co-fermented by recombinant *S. cerevisiae* and recombinant *E. coli* [32], while Zhang et al. [59] reported 32.6 g/L of ethanol and 0.42 g/g of yield from alkaline peroxide pretreated wheat straw after co-culture with recombinant *S. cerevisiae* and recombinant *P. Pastoris.* Thus, the 41.1 g/L ethanol and yield of 0.46 g/g after 24 h obtained in the present study indicate superior performance of co-culture fermentation using *S. cerevisiae* and *C. tropicalis.* In addition, the high ethanol titre (41.1 g/L) can potentially support a second-generation (2G) ethanol production from RS [55].

3.2.3. Sequential fermentation of enzymatic hydrolysates

Sequential cultivation involves inoculating the first yeast (*S. cerevisiae*) into the enzymatic hydrolysate and allowing it to grow for a specified period before inoculating the second yeast (*C. tropicalis*) into the medium. The sequential cultivation of *S. cerevisiae* and *C. tropicalis* on enzymatic hydrolysates and synthetic defined medium is shown in Fig. 4. In Fig. 4a, glucose was rapidly consumed by *S. cerevisiae*, and became almost depleted within 9 h of fermentation, while xylose concentration remained relatively unchanged at 25.4 g/L with corresponding ethanol concentration and yield of 25.4 g/L and 0.45 g/g, respectively. The rapid glucose assimilation rate and its depletion within 9 h indicates that *S. cerevisiae* naturally preferred glycolytic metabolism [60].

However, following the inoculation of *C. tropicalis* after 9 h, xylose concentration decreased steadily from 25.4 g/L (9 h) to 5.8 g/L after 24 h, while glucose was depleted after 24 h. The ethanol level further increased to 36.1 g/L after 24 h, whereas ethanol yield dropped to 0.41 g/g from a maximum level of 0.45 g/g at 9 h. However, despite the high xylose depletion rate, only a slight increase in ethanol levels occurred after 12 h (21.2 g/L), with a corresponding drop in ethanol yield (0.35 g/g). Further increases in fermentation time to 24 h resulted in lower ethanol levels (19.5 g/L) and a lower ethanol yield (0.29 g/g), with a productivity of 1.51 g/L.h. The ethanol yield declined from 0.43 g/g at 9 h to 0.29 g/g at 24 h, despite improved xylose consumption by *C. tropicalis*, indicating substantial carbon diversion toward biomass synthesis and cellular maintenance. Similar trends were observed in synthetic defined medium (Fig. 4b); however, the ethanol yield from synthetic medium (0.42 g/g) was higher than that from enzymatic hydrolysates (0.35 g/g) at 12 h. This behaviour is consistent with the typically lower ethanol yield reported for native xylose-fermenting yeasts, particularly in complex lignocellulosic hydrolysates [61].

3.3. Comparison with other process strategies for lignocellulosic ethanol production

The ethanol production process in the present study was compared with other reported lignocellulosic ethanol production processes, as shown in Table 5. The present process employed a low enzyme loading (3.0 FPU/g cellulose) and high solids loading (17% w/v), which is lower than those reported in the literature (15-25 FPU/g solids). In addition to reduced enzyme input, co-culture fermentation (*S. cerevisiae* and *C. tropicalis*) achieved ethanol concentrations of 41.4 g/L, exceeding ethanol of 25 g/L using sugarcane bagasse [32] and ethanol of 32.6 g/L using wheat straw [51]. A higher ethanol concentration (45.0 g/L) was reported by Saini [62] using sugarcane bagasse; however, the process employed substantially higher enzyme loading (25 FPU/g solids) and longer fermentation time (72 h). Furthermore, an ethanol yield of 0.46 g/g was achieved in co-culture fermentation in the present study, which is higher than the ethanol yields of 0.42-0.44 g/g reported in the literature (Table 5). However, techno-economic analyses are needed to evaluate this potential benefit. Overall, the ability to achieve high ethanol titre and yield at significantly reduced enzyme dosage highlights the effectiveness of the microwave-assisted ChCl:glycerol pretreatment in alleviating biomass recalcitrance and improving process efficiency.

4. Conclusions

Application of CCD and RSM in enzymatic hydrolysis resulted in a high TS yield (~76%) at a high-solids loading (17% cellulose) and a low enzyme dosage (3.0 FPU/g cellulose) from ChCl:glycerol pretreated rice straw. Co-culture fermentation of the enzymatic hydrolysate led to a final ethanol titre of 41.1 g/L after 24 h, with a notably high ethanol yield (0.46 g/g) and volumetric productivity (1.71 g/L.h). Thus, compares favourably with similar co-culture systems, which achieved ~48.6 g/L ethanol but lower yield (0.45 g/g) and productivity (~1.58 g/L.h). Therefore, the present study, integrating high-solids enzymatic hydrolysis and a co-culture fermentation strategy for lignocellulosic ethanol production, can support a low-cost, scalable biorefinery concept.

CRediT authorship contribution statement

Longinus Ifeanyi Igbojionu: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Yujie Mao:** Writing – review & editing, Visualization, Validation, Methodology. **Eleanor Binner:** Supervision, Conceptualization. **Alan D. Goddard:** Supervision, Conceptualization. **Alfred Fernandez- Castane:** Writing – review & editing, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Funding acquisition, Conceptualization.

Table 5
A comparison of ethanol production with other reported studies.

Pretreatment	Feedstock	Enzymatic hydrolysis	Fermentation condition	Fermentation efficiency	References
ChCl:glycerol (1:2), microwave (5 min, 120°C)	Rice straw	Batch, 17% (w/v) solids, 3 FPU/g cellulose, 75 h	SHF, Co-culture (*S. cerevisiae* and *C. tropicalis*), 30°C, 24 h.	Ethanol: 41.1 g/L, Ethanol yield: 0.46 g/g	This study
1% H_2SO_4, autoclave (30 min, 120°C)	Rice straw	Batch, 10% (w/v) solids, 10 FPU/g cellulose, 75 h	SHF, Co-culture (*S. cerevisiae* and *C. tropicalis*), 30°C, 24 h.	Ethanol: 16.2 g/L, Ethanol yield: 0.345 g/g	This study
Sequential acid-alkali pretreatment (30 min, 120°C)	Sugarcane bagasse	Batch, 15% solids (w/v), 25 FPU/g dry solids, 72h	SHF, Mono-culture (*K. marxianus* JKH5 C60), 42°C, 72 h.	Ethanol: 45.0 g/L, Ethanol yield: 0.44 g/g	[54]
Acid pretreatment (5 min, 190°C)	Sugarcane bagasse	Batch, 10% (w/w), 15 FPU/g substrate, 48 h	SSCF, recombinant *E. coli* and *S. cerevisiae*, 37°C, 48 h.	Ethanol: 25 g.L-1, Ethanol yield: 0.44 g/g	[32]
Alkaline peroxide pretreatment (35°C, 60 min)	Wheat straw	-	SSCF, recombinant *S. cerevisiae* and recombinant *P. Pastoris.*	Ethanol 32.6 g/L, Ethanol yield 0.42 g/g	[59]
Ionic liquid [TEA][HSO4] coupled with ultrasound irradiation (130°C, 30 min)	Wheat straw	Batch, 5% solids,28 FPU/g of biomass, 48 h	SHF, mono-culture (recombinant *S. cerevisiae* PTCC 5052), 48 h.	Ethanol:42.0 g/L, Ethanol yield: 0.43 g/g	[63]
Dilute H_2SO_4 pretreatment	Wheat straw		SSF, recombinant *Escherichia coli* (FBR5), 35°C, 83 h.	Ethanol: 36.0 g/L, Ethanol yield: 0.29 g/g	[64]
N-methylmorpholine N-oxide- (NMMO) and phosphoric acid	Rice straw	Batch, 20 FPU/g substrate, 30% solids, 72 h	SSF, *Mucor indicus,* 72 h.	Ethanol: 63 g/L, Ethanol yield: 0.38 g/g	[65]
Two-step: H_2SO_4 (100°C, 2 h) and sulfomethylation (160°C, 5 h) treatment	Rice straw		SSF, 72 h.	Ethanol: 40.6 g/L, Ethanol yield: 0.45 g/g	[66]
CELF pretreatment 150°C for 25 min 7.5% solids loading, 0.5% H_2SO_4)	Corn stover	Batch, 5 mg protein/g glucan, 15.5% solids, 72 h	SSF, *S. cerevisiae* (D_5A strain), 37°C, 72 h	Ethanol: 56.4 g/L, Ethanol yield: 0.46 g/g	[67]
Hydrothermal treatment (160°C, 5.7% solids loading, 50 min)	Tissue paper	Batch, 50 FPU/g substrate, 8% solids, 72 h	SHF, co-culture *(S. cerevisiae* and *C. shehatae)*, 72 h	Ethanol: 28.1 g/L Ethanol yield: 0.35 g/g	[68]
Two-step: 1.5% H_2SO_4 (108°C, 6h), 2% NaOH (80°C, 75 min)	Corn stover	Batch, 20 FPU/g substrate, 72 h	SHF, recombinant *S. cerevisiae* (ZU-10), 30°C	Ethanol: 40.4 g/L Ethanol yield: 0.41 g/g	[69]

SHF: separate hydrolysis and fermentation, SSF: simultaneous saccharification and fermentation, SSCF: simultaneous saccharification and co-fermentation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data will be made available on request.

References

[1] M. Mujtaba, L.F. Fraceto, M. Fazeli, S. Mukherjee, S.M. Savassa, G.A. de Medeiros, F. Vilaplana, Lignocellulosic biomass from agricultural waste to the circular economy: a review with focus on biofuels, biocomposites and bioplastics, J. Clean. Prod. 402 (2023) 136815, https://doi.org/10.1016/j.jclepro.2023.136815.

[2] T.B. Bonenkamp, L.M. Middelburg, M.O. Hosli, R.F. Wolffenbuttel, From bioethanol containing fuels towards a fuel economy that includes methanol derived from renewable sources and the impact on European Union decision-making on transition pathways, Renew. Sustain. Energy Rev. 120 (2020) 109667, https://doi.org/10.1016/j.rser.2019.109667.

[3] A. Kazmi, T. Sultana, A. Ali, A. Nijabat, G. Li, H. Hou, Innovations in bioethanol production: a comprehensive review of feedstock generations and technology advances, Energy Strategy Rev. 57 (2025) 101634, https://doi.org/10.1016/j.esr.2024.101634.

[4] P. Di Donato, I. Finore, A. Poli, B. Nicolaus, L. Lama, The production of second generation bioethanol: the biotechnology potential of thermophilic bacteria, J. Clean. Prod. 233 (2019) 1410–1417, https://doi.org/10.1016/j.jclepro.2019.06.152.

[5] I. Bukhari, F. Haq, M. Kiran, T. Aziz, S. Mehmood, M. Haroon, Lignocellulosic biomass as a renewable resource: driving second-generation biofuel innovation from agricultural waste, Biomass Bioenergy 201 (2025) 108133, https://doi.org/10.1016/j.biombioe.2025.108133.

[6] P. Bhattacharyya, D. Bhaduri, T. Adak, S. Munda, B.S. Satapathy, P.K. Dash, H. Pathak, Characterization of rice straw from major cultivars for best alternative industrial uses to cutoff the menace of straw burning, Ind. Crops Prod. 143 (2020) 111919, https://doi.org/10.1016/j.indcrop.2019.111919.

[7] S. Muthayya, J.D. Sugimoto, S. Montgomery, G.F. Maberly, An overview of global rice production, supply, trade, and consumption, Ann. N. Y. Acad. Sci. 1324 (2014) 7–14, https://doi.org/10.1111/nyas.12540.

[8] Y.T. Zewide, T.A. Yemata, A.A. Ayalew, H.J. Kedir, A.A. Tadesse, A.Y. Fekad, M. T. Mihiret, Application of response surface methodology (RSM) for experimental optimization in biogenic silica extraction from rice husk and straw ash, Sci. Rep. 15 (2025) 132, https://doi.org/10.1038/s41598-024-83724-6.

[9] P. Pal, H. Li, S. Saravanamurugan, Removal of lignin and silica from rice straw for enhanced accessibility of holocellulose for the production of high-value chemicals, Bioresour. Technol. 361 (2022) 127661, https://doi.org/10.1016/j.biortech.2022.127661.

[10] N. Vivek, L.M. Nair, B. Mohan, S.C. Nair, R. Sindhu, A. Pandey, P. Binod, Bio-butanol production from rice straw–recent trends, possibilities, and challenges, Bioresour. Technol. Rep. 7 (2019) 100224, https://doi.org/10.1016/j.biteb.2019.100224.

[11] Z. Yuan, Y. Wen, N.S. Kapu, R. Beatson, Evaluation of an organosolv-based biorefinery process to fractionate wheat straw into ethanol and co-products, Ind. Crop. Prod. 121 (2018) 294–302, https://doi.org/10.1016/j.indcrop.2018.05.028.

[12] B. Nath, M.M. Ahmmed, S. Paul, M.D. Huda, M.A. Hossain, S. Islam, Unlocking the potential of rice straw: sustainable utilization strategies for Bangladesh, Circular Econ (2025) 100126, https://doi.org/10.1016/j.cec.2025.100126.

[13] P. Binod, R. Sindhu, R.R. Singhania, S. Vikram, L. Devi, S. Nagalakshmi, A. Pandey, Bioethanol production from rice straw: an overview, Bioresour. Technol. 101 (2010) 4767–4774, https://doi.org/10.1016/j.biortech.2009.10.079.

[14] M. Nazar, L. Xu, M.W. Ullah, J.M. Moradian, Y. Wang, S. Sethupathy, D. Zhu, Biological delignification of rice straw using laccase from *Bacillus ligniniphilus* L1 for bioethanol production: a clean approach for agro-biomass utilization, J. Clean. Prod. 360 (2022) 132171, https://doi.org/10.1016/j.jclepro.2022.132171.

[15] L.I. Igbojionu, C. Laluce, Optimization of high-solid enzymatic hydrolysis of two-step alkaline and dilute acid-pretreated sugarcane bagasse at low enzyme loadings by response surface methodology, Biomass Convers. Biorefinery 13 (2023) 5821–5830, https://doi.org/10.1007/s13399-021-01544-4.

[16] [a] R.S. Abolore, S. Jaiswal, A.K. Jaiswal, Green and sustainable pretreatment methods for cellulose extraction from lignocellulosic biomass and its applications: a review, Carbohydr. Polym. Technol. Appl. 7 (2024) 100396, https://doi.org/10.1016/j.carpta.2023.100396;
[b] M.M. Contreras-Gámez, Á. Galán-Martín, N. Seixas, A.M. da Costa Lopes, A. Silvestre, E. Castro, Deep eutectic solvents for improved biomass pretreatment: current status and future prospective towards sustainable processes, Bioresour. Technol. 369 (2023) 128396, https://doi.org/10.1016/j.biortech.2022.128396.

[17] N. Wang, K. Liu, Z. Hou, Z. Zhao, H. Li, X. Gao, Microwave-assisted deep eutectic solvents pretreatment for lignocellulose: Kinetics, intensified mechanism and

process optimization, Chem. Eng. Sci. 302 (2025) 120881, https://doi.org/10.1016/j.ces.2024.120881.
[18] D. Mikulski, G. Kłosowski, Microwave-assisted pretreatment with aqueous solutions of deep eutectic solvents for enhanced enzymatic saccharification of oat hull and brewer's spent grain, Ind. Crops Prod. 241 (2026) 122826, https://doi.org/10.1016/j.indcrop.2026.122826.
[19] Y. Mao, E. Purvis, L.I. Igbojionu, H.R. Danesh-Azari, E. Binner, Effective wheat straw pre-treatment and saccharification using p-toluenesulfonic acid (pTSA)-based deep eutectic solvents and microwave heating, Biomass Bioenergy 209 (2026) 108995, https://doi.org/10.1016/j.biombioe.2026.108995.
[20] L.I. Igbojionu, Y. Mao, E. Binner, A. Fernandez-Castane, A comparative study of effects of microwave-assisted deep eutectic solvents pretreatment of rice straw on cellulose enrichment and enzymatic digestibility, Cellulose 32 (2025) 10513–10528, https://doi.org/10.1007/s10570-025-06834-y.
[21] Y. Mao, A. Gerrow, E. Ray, N.D. Perez, K. Edler, B. Wolf, E. Binner, Lignin recovery from cocoa bean shell using microwave-assisted extraction and deep eutectic solvents, Bioresour. Technol. 372 (2023) 128680, https://doi.org/10.1016/j.biortech.2023.128680.
[22] S. Ethaib, Microwave-assisted pretreatment for lignocellulosic biomass energy conversion path, Bioresour. Technol. Rep. 28 (2024) 102006, https://doi.org/10.1016/j.biteb.2024.102006.
[23] Y. Hu, A. Priya, C. Chen, C. Liang, W. Wang, Q. Wang, W. Qi, Recent advances in substrate-enzyme interactions facilitating efficient biodegradation of lignocellulosic biomass: a review, Int. Biodeterior. Biodegrad. 180 (2023) 105594, https://doi.org/10.1016/j.ibiod.2023.105594.
[24] R. Ningthoujam, P. Jangid, V.K. Yadav, R. Zairov, S.M. Hasnain, A. Patel, H. K. Dhingra, Recent advances in bioethanol production from rice straw and husks by physical, chemical, and biological pretreatment methods, Results Eng. (2026) 109162, https://doi.org/10.1016/j.rineng.2026.109162.
[25] C. Sun, X. Meng, F. Sun, J. Zhang, M. Tu, J.S. Chang, A.J. Ragauskas, Advances and perspectives on mass transfer and enzymatic hydrolysis in the enzyme-mediated lignocellulosic biorefinery: a review, Biotechnol. Adv. 62 (2023) 108059, https://doi.org/10.1016/j.biotechadv.2022.108059.
[26] N. Arshad, E.J. Panakkal, P.B. Kalivarathan, A. Tawai, S. Chuetor, W. Wanmolee, M. Sriariyanun, Emerging technologies in pretreatment and hydrolysis for high-solid-loading bioethanol production from lignocellulosic biomass, Fermentation 11 (2025) 613, https://doi.org/10.3390/fermentation11110613.
[27] C. Hierro-Iglesias, C.O. Fatokun, A. Chimphango, R. Bayitse, P.H. Blanco-Sanchez, P. Thornley, A. Fernandez-Castane, Process integration for efficient conversion of cassava peel waste into polyhydroxyalkanoates, J. Environ. Chem. Eng. 12 (2024) 111815, https://doi.org/10.1016/j.jece.2023.111815.
[28] G. Nikaeen, S. Yousefinejad, S. Rahmdel, F. Samari, S. Mahdavinia, Central composite design for optimizing the biosynthesis of silver nanoparticles using Plantago major extract and investigating antibacterial, antifungal and antioxidant activity, Sci. Rep. 10 (2020) 9642, https://doi.org/10.1038/s41598-020-66357-3.
[29] F. da Silva Fernandes, E.S. de Souza, L.M. Carneiro, J.P. Alves Silva, J.V.B. de Souza, J. da Silva Batista, Current ethanol production requirements for the yeast *Saccharomyces cerevisiae*, Internet J. Microbiol. 2022 (2022) 7878830, https://doi.org/10.1155/2022/7878830.
[30] I.A. Ndubuisi, C.O. Amadi, T.N. Nwagu, Y. Murata, J.C. Ogbonna, Non-conventional yeast strains: unexploited resources for effective commercialization of second generation bioethanol, Biotechnol. Adv. 63 (2023) 108100, https://doi.org/10.1016/j.biotechadv.2023.108100.
[31] S.S. Queiroz, M.G.A.F. Maria, S.I. Mussatto, An overview of xylose valorization through its conversion into high-value chemicals by yeast, Biomass Bioenergy 190 (2024) 107428, https://doi.org/10.1016/j.biombioe.2024.107428.
[32] L. Wang, S.W. York, L.O. Ingram, K.T. Shanmugam, Simultaneous fermentation of biomass-derived sugars to ethanol by a co-culture of an engineered Escherichia coli and *Saccharomyces cerevisiae*, Bioresour. Technol. 273 (2019) 269–276, https://doi.org/10.1016/j.biortech.2018.11.016.
[33] S.R. Kim, S.J. Ha, N. Wei, E.J. Oh, Y.S. Jin, Simultaneous co-fermentation of mixed sugars: a promising strategy for producing cellulosic ethanol, Trends Biotechnol. 30 (2012) 274–282, https://doi.org/10.1016/j.tibtech.2012.01.005.
[34] Y. Chen, Development and application of co-culture for ethanol production by co-fermentation of glucose and xylose: a systematic review, J. Ind. Microbiol. Biotechnol. 38 (2011) 581–597, https://doi.org/10.1007/s10295-010-0894-3.
[35] A. Sluiter, B. Hames, R. Ruiz, C. Scarlata, J. Sluiter, D. Templeton, D. Crocker, Determination of Structural Carbohydrates and Lignin in Biomass, Laboratory Analytical Procedure (LAP), National Renewable Energy Laboratory, 2012. NREL/TP-510–42618, http://www.nrel.gov/biomass/pdfs/42618.pdf. (Accessed 24 July 2024).
[36] T.K. Ghose, Measurement of cellulase activities, Pure Appl. Chem. 59 (1987) 257–268, https://doi.org/10.1351/pac198759020257.
[37] N. Boudechiche, H. Yazid, M. Trari, Z. Sadaoui, Valorization of *Crataegus azarolus* stones for the removal of textile anionic dye by central composite rotatable design using cubic model: optimization, isotherm, and kinetic studies, Environ. Sci. Pollut. Res. 24 (2017) 19609–19623, https://doi.org/10.1007/s11356-017-9606-0.
[38] S. Agarwal, I. Tyagi, V.K. Gupta, A.R. Bagheri, M. Ghaedi, A. Asfaram, A. A. Bazrafshan, Rapid adsorption of ternary dye pollutants onto copper (I) oxide nanoparticle loaded on activated carbon: experimental optimization via response surface methodology, J. Environ. Chem. Eng. 4 (2016) 1769–1779, https://doi.org/10.1016/j.jece.2016.03.002.
[39] M. van Dijk, F. Mierke, Y. Nygård, L. Olsson, Nutrient-supplemented propagation of Saccharomyces cerevisiae improves its lignocellulose fermentation ability, AMB Express 10 (2020) 157, https://doi.org/10.1186/s13568-020-01070-y.
[40] A.S. da Silva, R.P. Espinheira, R.S.S. Teixeira, Constraints and advances in high-solids enzymatic hydrolysis of lignocellulosic biomass: a critical review, Biotechnol. Biofuels 13 (2020) 58, https://doi.org/10.1186/s13068-020-01697-w.
[41] C. Huang, Y. Zhan, J. Cheng, J. Wang, X. Meng, X. Zhou, G. Fang, A.J. Ragauskas, Facilitating enzymatic hydrolysis with a novel guaiacol-based deep eutectic solvent pretreatment, Bioresour. Technol. 326 (2021) 124696, https://doi.org/10.1016/j.biortech.2021.124696.
[42] C. Zhu, X. Liu, Optimization of extraction process of crude polysaccharides from pomegranate peel by response surface methodology, Carbohydr. Polym. 92 (2013) 1197–1202, https://doi.org/10.1016/j.carbpol.2012.10.073.
[43] J. Hou, C. Qiu, Y. Shen, H. Li, X. Bao, Engineering of *Saccharomyces cerevisiae* for the efficient co-utilization of glucose and xylose, FEMS Yeast Res. 17 (2017) fox034, https://doi.org/10.1093/femsyr/fox034.
[44] K. Simpson-Lavy, M. Kupiec, Carbon catabolite repression in yeast is not limited to glucose, Sci. Rep. 9 (2019) 6491, https://doi.org/10.1038/s41598-019-43032-w.
[45] S. Li, J. Du, J. Sun, J.M. Galazka, N.L. Glass, J.H. Cate, H. Zhao, Overcoming glucose repression in mixed sugar fermentation by co-expressing a cellobiose transporter and a β-glucosidase in *Saccharomyces cerevisiae*, Mol. Biosyst. 6 (2010) 2129–2132, https://doi.org/10.1039/C0MB00063A.
[46] A.J.A. van Maris, D.A. Abbott, E. Bellissimi, J. van den Brink, M. Kuyper, M.A. H. Luttik, H.W. Wisselink, W.A. Scheffers, J.P. van Dijken, J.T. Pronk, Alcoholic fermentation of carbon sources in biomass hydrolysates by *Saccharomyces cerevisiae*: current status, Antonie Leeuwenhoek 90 (2006) 391–418, https://doi.org/10.1007/s10482-006-9085-7.
[47] M. Láinez, H.A. Ruiz, M. Arellano-Plaza, S. Martínez-Hernández, Bioethanol production from enzymatic hydrolysates of *Agave salmiana* leaves comparing S. cerevisiae and K. marxianus, Renew. Energy 138 (2019) 1127–1133, https://doi.org/10.1016/j.renene.2019.02.058.
[48] T.W. Jeffries, Y.S. Jin, Metabolic engineering for improved fermentation of pentoses by yeasts, Appl. Microbiol. Biotechnol. 63 (2004) 495–509, https://doi.org/10.1007/s00253-003-1450-0.
[49] A.J. Mattam, A. Kuila, N. Suralikerimath, N. Choudary, P.V. Rao, H.R. Velankar, Cellulolytic enzyme expression and simultaneous conversion of lignocellulosic sugars into ethanol and xylitol by a new *Candida tropicalis* strain, Biotechnol. Biofuels 9 (2016) 157, https://doi.org/10.1186/s13068-016-0575-1.
[50] M. Shin, J.W. Kim, S. Ye, S. Kim, D. Jeong, D.Y. Lee, S.R. Kim, Comparative global metabolite profiling of xylose-fermenting *Saccharomyces cerevisiae* SR8 and *Scheffersomyces stipitis*, Appl. Microbiol. Biotechnol. 103 (2019) 5435–5446, https://doi.org/10.1007/s00253-019-09829-5.
[51] S. Larsson, E. Palmqvist, B. Hahn-Hägerdal, C. Tengborg, K. Stenberg, G. Zacchi, N. O. Nilvebrant, The generation of fermentation inhibitors during dilute acid hydrolysis of softwood, Enzym. Microb. Technol. 24 (1999) 151–159, https://doi.org/10.1016/S0141-0229(98)00101-X.
[52] R.E.H. Sims, W. Mabee, J.N. Saddler, M. Taylor, An overview of second generation biofuel technologies, Bioresour. Technol. 101 (2010) 1570–1580, https://doi.org/10.1016/j.biortech.2009.11.046.
[53] B. Hahn-Hägerdal, K. Karhumaa, C. Fonseca, I. Spencer-Martins, M.F. Gorwa-Grauslund, Towards industrial pentose-fermenting yeast strains, Appl. Microbiol. Biotechnol. 74 (2007) 937–953, https://doi.org/10.1007/s00253-006-0827-2.
[54] A. Matsushika, H. Inoue, K. Murakami, O. Takimura, S. Sawayama, Bioethanol production performance of five recombinant strains of *Saccharomyces cerevisiae* with different xylose utilization pathways, Bioresour. Technol. 100 (2009) 2392–2398, https://doi.org/10.1016/j.biortech.2008.11.047.
[55] B. Sharma, C. Larroche, C.G. Dussap, Comprehensive assessment of 2G bioethanol production, Bioresour. Technol. 313 (2020) 123630, https://doi.org/10.1016/j.biortech.2020.123630.
[56] T.Y. Nguyen, C.M. Cai, O. Osman, R. Kumar, C.E. Wyman, CELF pretreatment of corn stover boosts ethanol titers and yields from high solids SSF with low enzyme loadings, Green Chem. 18 (2016) 1581–1589, https://doi.org/10.1039/C5GC01977J.
[57] E. Palmqvist, B. Hahn-Hägerdal, Fermentation of lignocellulosic hydrolysates. I: inhibition and detoxification, Bioresour. Technol. 74 (2000) 17–24, https://doi.org/10.1016/S0960-8524(99)00160-1.
[58] P.C. Lopez, I.A. Abeykoon Udugama, S.T. Thomsen, C. Bayer, H. Junicke, K. V. Gernaey, Promoting the co-utilisation of glucose and xylose in lignocellulosic ethanol fermentations using a data-driven feed-back controller, Biotechnol. Biofuels 13 (2020) 190, https://doi.org/10.1186/s13068-020-01829-2.
[59] Y. Zhang, C. Wang, L. Wang, R. Yang, P. Hou, J. Liu, Direct bioethanol production from wheat straw using xylose/glucose co-fermentation by co-culture of two recombinant yeasts, J. Ind. Microbiol. Biotechnol. 44 (2017) 453–464, https://doi.org/10.1007/s10295-016-1893-9.
[60] M.J. Brauer, A.J. Saldanha, K. Dolinski, D. Botstein, Homeostatic adjustment and metabolic remodeling in glucose-limited yeast cultures, Mol. Biol. Cell 16 (2005) 2503–2517.
[61] N. Rodrussamee, P. Sattayawat, M. Yamada, Highly efficient conversion of xylose to ethanol without glucose repression by newly isolated thermotolerant *Spathaspora passalidarum* CMUWF1–2, BMC Microbiol. 18 (2018) 73, https://doi.org/10.1186/s12866-018-1218-4.
[62] J.K. Saini, Enhanced cellulosic ethanol production via fed-batch simultaneous saccharification and fermentation of sequential dilute acid-alkali pretreated sugarcane bagasse, Bioresour. Technol. 372 (2023) 128671, https://doi.org/10.1016/j.biortech.2023.128671.
[63] Z. Ziaei-Rad, M. Pazouki, J. Fooladi, M. Azin, S.N. Gummadi, A. Allahverdi, Investigation of a robust pretreatment technique based on ultrasound-assisted, cost-effective ionic liquid for enhancing saccharification and bioethanol

production from wheat straw, Sci. Rep. 13 (2023) 446, https://doi.org/10.1038/s41598-022-27258-9.
[64] B.C. Saha, N.N. Nichols, N. Qureshi, G.J. Kennedy, L.B. Iten, M.A. Cotta, Pilot scale conversion of wheat straw to ethanol via simultaneous saccharification and fermentation, Bioresour. Technol. 175 (2015) 17–22, https://doi.org/10.1016/j.biortech.2014.10.060.
[65] M. Molaverdi, S. Mirmohamadsadeghi, K. Karimi, M. Aghbashlo, M. Tabatabaei, Efficient ethanol production from rice straw through cellulose restructuring and high solids loading fermentation by *Mucor indicus*, J. Clean. Prod. 339 (2022) 130702, https://doi.org/10.1016/j.jclepro.2022.130702.
[66] S. Zhu, W. Huang, W. Huang, K. Wang, Q. Chen, Y. Wu, Pretreatment of rice straw for ethanol production by a two-step process using dilute sulfuric acid and sulfomethylation reagent, Appl. Energy 154 (2015) 190–196, https://doi.org/10.1016/j.apenergy.2015.05.008.
[67] T.Y. Nguyen, C.M. Cai, R. Kumar, C.E. Wyman, Overcoming factors limiting high-solids fermentation of lignocellulosic biomass to ethanol, Proc. Natl. Acad. Sci. U. S. A. 114 (2017) 11673–11678, https://doi.org/10.1073/pnas.1704652114.
[68] J. Zhou, P. Lv, B. He, J. Wu, G. Wang, H. Ma, G. Chen, Optimisation of the ethanol fermentation process using hydrothermal pretreatment of cellulose waste—effect of fermentation pattern and strain, Molecules 29 (2024) 5266, https://doi.org/10.3390/molecules29225266.
[69] J. Zhao, L. Xia, Bioconversion of corn stover hydrolysate to ethanol by a recombinant yeast strain, Fuel Process. Technol. 91 (2010) 1807–1811, https://doi.org/10.1016/j.fuproc.2010.08.002.