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RESEARCH ARTICLE

Strategies to achieve high productivity, high conversion, and high yield in yeast fermentation of algal biomass hydrolysate

Xing-Feng Huang | **Kenneth F. Reardon**

Department of Chemical and Biological Engineering, Colorado State University, Fort Collins, CO, USA

Correspondence
Kenneth F. Reardon, Department of Chemical and Biological Engineering, Colorado State University, Fort Collins, CO 80523–1370, USA.
Email: Kenneth.Reardon@colostate.edu

Abstract

The conversion of carbohydrates in biomass via fermentation is an important component of an overall strategy to decarbonize the production of fuels and chemicals. Owing to the cost and resources required to produce biomass hydrolysates, the economic and environmental sustainability of these fermentation processes requires that they operate with high yields, sugar conversion, and productivity. Immobilized-cell technology in a continuous bioprocess can achieve significantly higher volumetric productivities than is possible from standard batch fermentation using free cells. Here, we demonstrate approaches for improvement of ethanol yield from algal hydrolysates and a mock hydrolysate medium. *Saccharomyces cerevisiae* was immobilized in alginate and incorporated into a two-column immobilized cell reactor system. Furthermore, the yeast quorum-sensing molecule, 2-phenylethanol, was added to improve ethanol yield by restricting growth and diverting sugar to ethanol. The bioreactor system could achieve high ethanol volumetric productivity (>20 g/L$_{reactor}$·h) and high glucose conversion ($>99\%$) in mock hydrolysate, while the addition of 0.2% 2-phenylethanol resulted in 4.9% higher ethanol yield. With an algal hydrolysate of <10 g/L sugar, the ethanol volumetric productivity reached 9.8 g/L$_{reactor}$·h, and the addition of 0.2% 2-phenylethanol increased the ethanol yield by up to 7.4%. These results demonstrate the feasibility of novel strategies to achieve sustainability goals in biomass conversions.

KEYWORDS
fermentation, immobilized cells, productivity, yeast, yield

1 | INTRODUCTION

The conversion of biomass carbohydrates to fuels and other chemicals via fermentation is an important component of an overall strategy to decarbonize the chemical production. Owing to the cost and resources required to produce biomass hydrolysates, the economic and environmental sustainability of these fermentation processes requires that they operate with high yields, sugar conversion, and productivity.

Microalgae are a promising biomass source since this crop grows quickly and all of the biomass can be converted to products [1,2]. Algal biomass avoids the major drawbacks of first- and second-generation biofuels such as competition with agricultural food and feed production

Abbreviations: CCE, carbon conversion efficiency; DMSO, dimethyl sulfoxide; PheOH, 2-phenylethanol; RT, retention time; TrpOH, tryptophol; YPD, yeast extract-peptone-dextrose

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PRACTICAL APPLICATION

Key factors in the economic and environmental sustainability of biomass conversion to products are the yield, conversion extent, and productivity. The research presented here demonstrates a simple method of increasing yield by reducing biomass production, along with the use of immobilized cell technology to increase conversion and productivity.

[3]. Fermentation of carbohydrates in microalgal biomass is an alternative pathway for biofuel production, especially since some microalgal species are more than 50% starch, glucose, and/or cellulose on a dry weight basis, with no lignin content [4,5]. Various methods have been developed to hydrolyze algal biomass carbohydrates into fermentable compounds [2,6,7]. Although carbohydrates account for 40% or higher of microalgal biomass by dry weight, algal hydrolysates typically contain low sugar concentrations. For instance, hydrolysis of *Chlorella* biomass using H_2SO_4 resulted in 15 g/L fermentable sugars [8]. Therefore, fermentation processes must be effective with hydrolysates that have relatively low sugar concentrations to achieve high yield, sugar conversion, and productivity.

Traditional fermentations with free cells are limited in the volumetric productivity and extent of sugar conversion that can be achieved. Batch fermentations have high sugar conversions but low volumetric productivities, especially when the time for draining, cleaning, and filling the bioreactor is considered. Fed-batch fermentations can increase productivity but are only suitable for feedstocks that have high sugar concentrations, which is not always possible with biomass hydrolysates. Finally, the volumetric productivity of continuous cultivations with free cells is limited by the specific growth rate of the biocatalyst, especially for hydrolysates with lower sugar concentrations. Sugar conversion in continuous cultivations is also low when free cells are used.

Since cells are retained within the reactor, decoupling operation from growth rate, Immobilized-cell technology in a continuous cultivation system has the potential to achieve significantly higher volumetric productivities than those using free cells [9,10]. Cell immobilization can also facilitate other strategies for increasing the yield (carbon conversion efficiency) of sugar-to-product conversion as well as lower costs of downstream processing [11]. Immobilized yeast cells have been observed with activation of yeast metabolism, increased storage polysaccharides, altered growth rates, increased substrate uptake and product yield, higher intracellular pH values, and increased tolerance against toxic and inhibitory compounds [12,13]. Immobilized yeast have been used for ethanol production in different reactor configurations including continuous stirred tank bioreactor, flow-through column, packed-bed column, and rotating bed bioreactor [14]. Due to the ease of preparation and the mild conditions, cell immobilization in calcium alginate beads has been used in several studies to produce ethanol from biomass hydrolysates [9,15–18].

A general strategy to improve ethanol yield is to redirect the flux of substrate carbon from other products, including biomass, and toward ethanol. Recently, we demonstrated that three *S. cerevisiae* quorum-sensing molecules, 2-phenylethanol, tryptophol, and tyrosol, increased the ethanol yield of *S. cerevisiae* JAY 270 [19] and seven other yeast strains by as much as 15%. These findings demonstrate the ethanol yield can be improved by adding yeast quorum sensing molecules to reduce the cell growth of *S. cerevisiae*, suggesting a strategy to improve the yield of ethanol and other yeast fermentation products by manipulating native biological control systems.

The goal of the research reported here was to determine the increases in yield, volumetric productivity, and sugar conversion levels that could be achieved from an algal hydrolysate by implementing immobilized cell bioreactor technology and the use of a quorum-sensing molecule.

2 | MATERIALS AND METHODS

2.1 | Yeast strains and media

S. cerevisiae JAY 270 was maintained as frozen stocks at -80°C. Fresh culture on yeast extract-peptone-dextrose (YPD) agar (10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose, 15 g/L agar) plates were prepared and kept at 4°C before use. Liquid YPD medium was used as a mock hydrolysate.

2.2 | Algae biomass hydrolysate

The Arizona Center for Algae Technology and Innovation (AzCATI) at Arizona State University (ASU) provided *Desmodesmus armatus*. The algae were grown in flat panel PBR's using ammonium chloride as the nitrogen source and the cultivation was performed in artificial seawater [2]. The algal strain was harvested 5-days post N-depletion using a continuous centrifuge.

Pretreatment of *D. armatus* was performed in a batch-type reactor, a 4-L (2-L working volume) ZipperClave® reactor (Autoclave Engineers) by researchers at the National Renewable Energy Laboratory. Algal biomass was

treated in batches, each with 300 g of wet algal paste per pretreatment run and additions of H_2SO_4 and water to achieve a final solids loading of 20% w/w, and an acid concentration of 2% w/w. The biomass was pretreated for 15 min at 155°C, then cooled in an ice bath. The algal hydrolysate was adjusted to pH 5.0 using NH_4OH. Algal biomass residue was removed by centrifuging at 12,500 rpm for 30 min at 4°C. Then the hydrolysate was filtered through a 0.2 μm membrane. The primary sugars in the hydrolysate were glucose and mannose. Each batch had a different composition, as reported in Section 3.

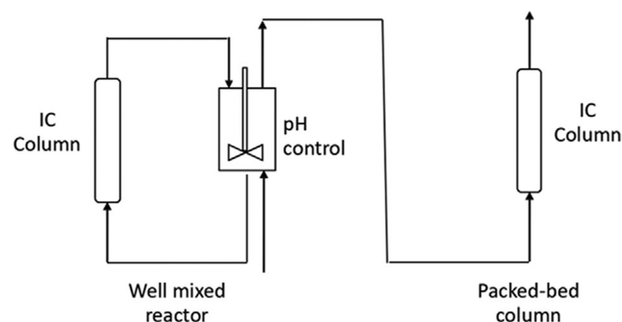

FIGURE 1 Schematic of the two-column, immobilized-cell bioreactor system

2.3 | Yeast immobilization

S. cerevisiae JAY 270 was immobilized in alginate beads to produce ethanol from both YPD medium and algal hydrolysates. Sodium alginate from brown algae (Sigma 71238) was dissolved into deionized water at 4% w/w. A 120-mL volume of 4% alginate solution was autoclaved at 121°C for 15 min and transferred into an anaerobic chamber overnight before use. *S. cerevisiae* JAY270 cells were precultured in 200 mL YPD medium at 30°C overnight and collected by centrifuging a culture at 3000 × *g* for 10 min at 4°C. The cell pellet was washed twice by resuspending in sterilized 40 mL 0.8% NaCl and centrifuged to remove any medium residue. Then, the cells were resuspended in 40 mL 0.8% NaCl and mixed with the 4% alginate solution to achieve a final alginate concentration of 3%. The cell mixture was delivered dropwise into sterilized 2% $CaCl_2$ on ice to form spheres ("beads"). These immobilized-cell beads were kept in the $CaCl_2$ solution at 4°C until use. To ensure that the calcium alginate gel remained intact, 0.6% $CaCl_2$ was added to all media for experiments involving immobilized cells.

2.4 | Two-column immobilized cell bioreactor system

The immobilized cell reactor system (Figure 1) consisted of two glass cylindrical columns, a vessel with agitation containing fermentation medium (controlled temperature, pH, and dissolved oxygen), and a pump for delivery of the feed to the column. The glass column had dimensions of OD = 32, ID = 29, and L = 160 mm; screens were placed above and below the alginate bead layer to retain the cells in the column. The flow through the first column was a rapid recirculation from a well-mixed vessel (a 50 mL bottle containing 20 mL medium) in which pH was controlled. The high recycle rate (approximately 60 mL/min) allowed this column to mimic a well-mixed bioreactor [20]. The second column operated as a single-pass, plug-flow bioreactor that was fed from the well-mixed vessel. The total liquid volume in the system was about 120 mL, including the medium in both columns (47.5 mL of each at the start of each experiment), the vessel (20 mL), and the tubing (5 mL). At the same time point, the total liquid volume in the Column 1 subsystem (well-mixed reactor system) was approximately 70 mL. Values of the volumetric productivity ($g/L_{reactor}\cdot h$) were calculated based on the liquid volumes of the Column 1 subsystem and the total system.

2.5 | Fermentation experiments

2.5.1 | Batch fermentation of algal hydrolysate in bioreactor with free cells

Approximately 250 mL of hydrolysate was loaded into a 500 mL vessel of a New Brunswick BioFlo 115 Benchtop Bioreactor. *S. cerevisiae* JAY270 was precultured in YPD medium for 24 h. Then, 0.5 mL was inoculated into the bioreactor. During the anaerobic fermentation, temperature was controlled at 30°C and pH was controlled at 5.0.

2.5.2 | Immobilized yeast fermentations of algal hydrolysate in serum bottles

Twenty-five milliliter algal hydrolysate was mixed with 15 mL alginate beads and transferred into 50 mL serum bottles and incubated in a shaker at 150 rpm and 30°C. Samples were collected at 0, 12, and 24 h for sugar and ethanol analysis.

To test the effects of inhibitor compounds on the immobilized cell fermentation, 2-phenylethanol (PheOH) and tryptophol (TrpOH) was added in the *D. armatus* hydrolysate in serum bottles at final concentration 0.2% w/v, respectively, (batch growth). TrpOH was dissolved in dimethyl sulfoxide (DMSO) at 20%. The same amount of DMSO was added to the reference medium. *S. cerevisiae*

JAY270 cells were immobilized in calcium alginate. The immobilized cell beads were incubated in YPD medium overnight at 30°C to acclimate and grow the immobilized cells. Twenty-five milliliter of *D. armatus* hydrolysate was mixed with 15 mL immobilized cell beads (three biological replicates in each treatment) and incubated with shaking at 30°C.

2.5.3 | Determination of retention time effects

For the retention time (RT) test, the two-column bioreactor system was fed with YPD medium at 2 mL/min for 20 h to acclimate and grow the immobilized cells. Then, the feed flow rate was adjusted to obtain a range of RT values. At each flow rate, the system was fed with YPD for 6 RTs and samples were collected after 4, 5, and 6 RTs to determine glucose and ethanol concentrations. The fermentation was conducted at pH 5.0 and 30°C. Ultrapure N_2 was sparged at 0.1 standard L/min in the bioreactor vessels to maintain anaerobic conditions.

2.5.4 | Determination of PheOH effects in YPD in the 2-column bioreactor system

To determine the effects of PheOH concentration on immobilized cells, the two-column bioreactor system was first fed with YPD medium at 2 mL/min for 20 h to acclimate and grow the immobilized cells. The system was then fed with YPD or YPD plus PheOH at 0.1, 0.2, and 0.3% in alternating 2-hour periods (YPD, YPD+0.1%PheOH, YPD, YPD+0.2%PheOH, YPD, YPD+0.3%PheOH, YPD). The feed flow rate was 3.4 mL/min. Samples were collected from the effluents of both columns.

To test the PheOH effects on the yeast fermentation over a longer period, a newly prepared two-column bioreactor system was first fed with YPD medium at 2 mL/min for 20 h to acclimate and grow the immobilized cells. YPD medium was then fed for 24 h, after which YPD medium containing 0.2% PheOH was fed for another 26 h, and finally the feed was changed to YPD medium for 6 h. The flow rate of YPD medium during the test was 2.5 mL/min. Samples were collected from the effluents of both columns.

2.5.5 | Continuous fermentation of algal hydrolysate using alginate-immobilized yeast in the two-column bioreactor system

Alginate-immobilized yeast cells in the two-column bioreactor system were used to convert sugars in the algal hydrolysate to ethanol. Initially, the system was fed with YPD medium for 20 h to acclimate and grow the immobilized cells. *D. armatus* hydrolysate containing 0.6 g/L $CaCl_2$ was then fed to the system for 3 h, followed by algal hydrolysate with 0.2% PheOH v/v for 3 h, followed by algal hydrolysate without PheOH for another 3 h. The flow rate of algal hydrolysate was 2.5 mL/min. Samples were collected from the effluents of both columns.

2.6 | GC-MS analysis of algal hydrolysate

A 200-μL sample of algal hydrolysate was mixed with 800 μL methanol in a 1.5-mL centrifuge tube and kept in ice overnight. Then, the tube was centrifuged at 14,000 rpm 4°C for 10 min and an 800-μL aliquot was transferred into a new centrifuge tube and dried under N_2. The dried sample was combined with 50 μL of 25 mg/mL of methoxyamine hydrochloride (in pyridine), incubated at 60°C for 45 min, sonicated for 10 min, and incubated for an additional 45 min at 60°C. Next, 50 μL of *N*-methyl-*N*-trimethylsilyltrifluoroacetamide with 1% trimethylchlorosilane (Thermo Scientific) were added to the sample. The sample was incubated at 60°C for 45 min, briefly centrifuged, then cooled to room temperature. Finally, 100 μL of the supernatant were transferred to a 150-μL glass insert in a GC-MS autosampler vial.

Metabolites were detected using a Trace 1310 GC coupled to a Thermo ISQ mass spectrometer (Thermo Scientific). One μL was injected in a 1:10 split ratio. Separation occurred using a 30-m TG-5MS column (Thermo Scientific, 0.25 mm i.d., 0.25 μm film thickness) with a 1.2 mL/min helium gas flow rate, and the program consisted of 80°C for 30 s, a ramp of 15°C per min to 330°C, and an 8 min hold. The transfer line was maintained at 300°C and the ion source at 260°C. Masses between 50 and 650 *m/z* were scanned at 5 scans/s after electron impact ionization. The GC-MS spectra were identified using the NIST17 (https://chemdata.nist.gov/) Golm Library M2.

2.7 | Analytical methods

S. cerevisiae JAY270 cell concentration was evaluated by monitoring absorbance at 600 nm (OD600). The concentrations of glucose, ethanol, and glycerol were measured via HPLC (Shimadzu LC20A series) outfitted with a refractive index detector and using an Aminex HPX-87H (300 × 7.8 mm) organic acid column and Cation H^+ guard cartridge (Bio Rad Laboratories, Hercules, CA). The column was maintained at 65°C and the mobile phase was 5 mM sulfuric acid with a flow rate of 0.6 mL/min. Standard compound solutions were used to calibrate the HPLC. Each sample was analyzed three times by HPLC.

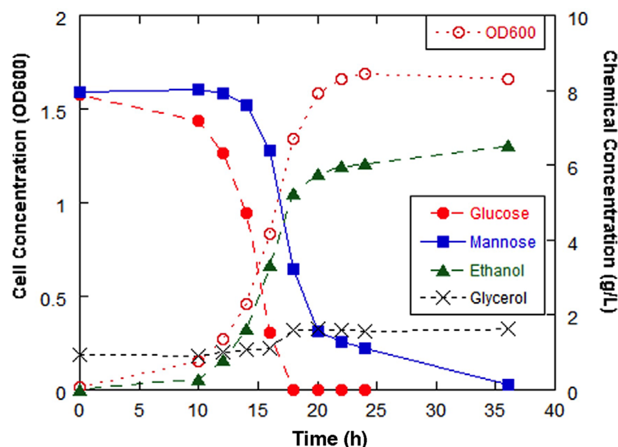

FIGURE 2 Time course of batch fermentation of *D. armatus* hydrolysate by *S. cerevisiae* JAY270

2.8 | Statistical analysis

Student's t-tests were carried out to compare the statistical difference between different treatments.

3 | RESULTS

3.1 | Free-cell batch fermentation of algal hydrolysate fermentation in bioreactor

The algal hydrolysate used for this experiment contained about 8 g/L glucose and 8 g/L mannose as the primary sugars (Figure 2). During the fermentation, yeast cells used glucose earlier than mannose. After 36 h, the yield of ethanol from glucose and mannose combined was 0.41 g ethanol/g sugar (80.7% of theoretical yield). This corresponds to a carbon conversion efficiency of 53.5%. The ethanol volumetric productivity was 0.18 $g/L_{reactor}\cdot h$.

3.2 | Effects of yeast growth inhibitors on immobilized cells fermenting algal hydrolysate in serum bottles

Batch cultivations in serum bottles were conducted to evaluate the effects of the growth inhibitors PheOH and TrpOH on immobilized cells in *D. armatus* hydrolysates. The concentrations of the primary sugars in the hydrolysate used in this experiment were 12.1 g/L glucose, 3.7 g/L mannose, and 0.5 g/L galactose. Immobilized *S. cerevisiae* JAY270 completely consumed both glucose and mannose but did not consume galactose to a detectable extent. As shown in Table 1, the ethanol yield from immobilized cells in the control cultivations was found to be 0.45 ± 0.01 g ethanol/g sugar at 24 h in the control samples. This value is about 10% higher than the value of 0.41 g ethanol/g sugar for a free-cell fermentation grown on a different preparation of *D. armatus* hydrolysate. Furthermore, both PheOH and TrpOH additions led to an increase in the ethanol yield in comparison to their control cultivations. The addition of TrpOH at 0.2% resulted in an ethanol yield of 0.49 g ethanol/g sugar, 11.4% higher than that of the DMSO reference treatment and about 20% higher than the value for free-cell fermentation of *D. armatus* hydrolysate.

TABLE 1 Effects of PheOH and TrpOH on ethanol yield of immobilized yeast cells in triplicate batch cultivations in serum bottles on *D. armatus* hydrolysate

Medium	EtOH yield (g/g)	Standard deviation	EtOH yield %Δ from control
Hydrolysate	0.45	0.01	
PheOH-0.2%	0.46	0.01	1.54
DMSO-Hydrolysate	0.44	0.03	
TrpOH-0.2%	0.49	0.00	11.42*

The mean values and standard deviations from three replicates are presented. Asterisk indicates significant difference from control at $p < 0.05$ (Student's *t*-test).

3.3 | Continuous conversions of YPD medium in the two-column bioreactor system

To characterize the system and its kinetics, immobilized yeast cells were loaded in the two columns of the bioreactor system and fed with YPD medium at a range of retention times. As the retention time increased, the ethanol volumetric productivity decreased and the sugar conversion increased (Figure 3). Data from the experiments are also presented in Table S1. For a system RT of 0.5 h, the ethanol volumetric productivity was 28 $g/L_{reactor}\cdot h$, the sugar conversion was 98.8% and the ethanol concentration in the effluent was 8.9 g/L. The column volumes were modified in accordance with the cell growth and bead swelling behavior measured in the experiment described in Section 3.4.

3.4 | Effects of PheOH on immobilized cells in continuous fermentation of YPD medium

To evaluate the use of the growth inhibition strategy in a continuous cultivation, the two-column immobilized cell bioreactor system shown in Figure 1 was used. Owing to the limited supply of algal hydrolysate, the test was conducted using YPD medium. The addition of PheOH at 0.1% did not result in significant effects on the fermentation

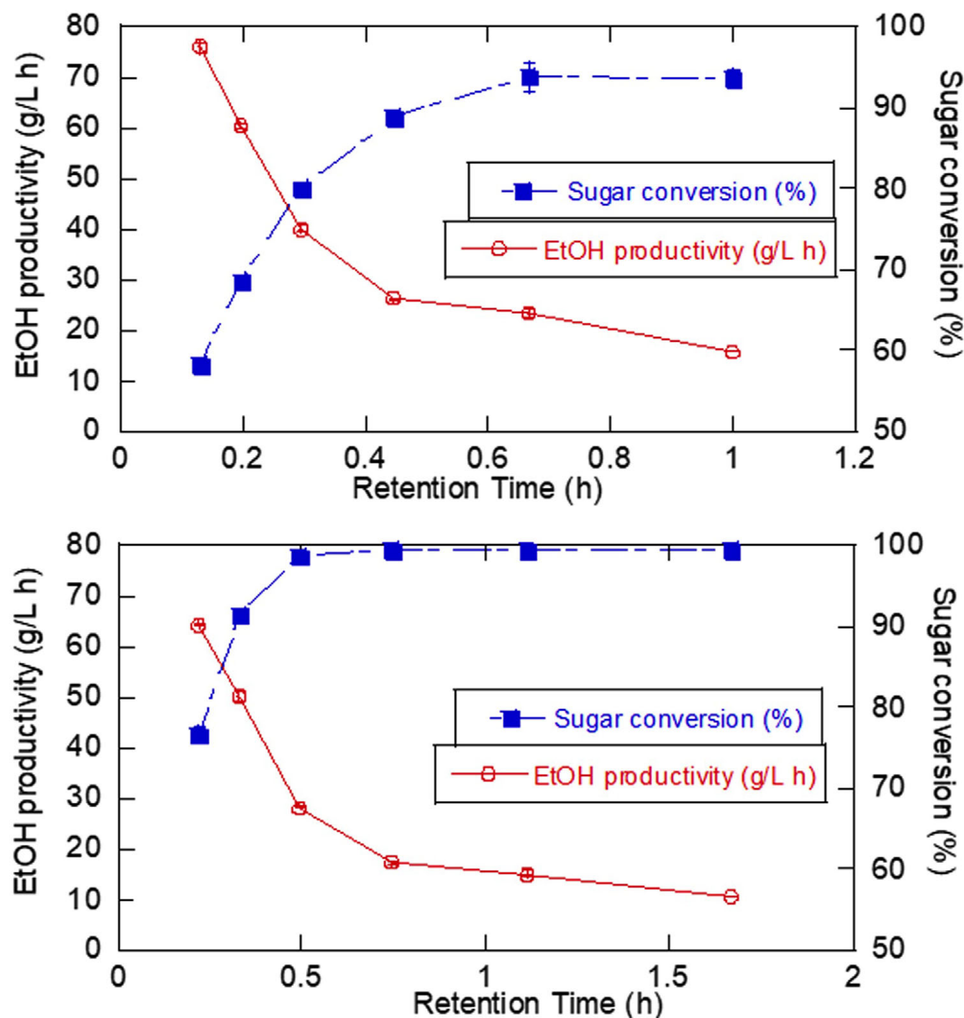

FIGURE 3 The ethanol volumetric productivity and sugar conversion in the two-column immobilized *S. cerevisiae* JAY270 reactor system at different retention times (RT) in YPD medium. Top: Ethanol volumetric productivity and sugar conversion in Column 1. Bottom: Ethanol volumetric productivity and sugar conversion in the entire system

(Figure 4, Table S1). However, the addition of PheOH at 0.2% and 0.3% resulted in higher ethanol yield but lower ethanol volumetric productivity and sugar conversion rates compared to YPD medium alone. Measurements of the liquid volume in each column and the concentration of immobilized cells during the experiment demonstrated that the yeast grew and the alginate bead volume increased during the experiment, causing the retention time to be lower than expected (Figures S1 and S2). A linear fit of the bioreactor volume vs. time was used to correct for this effect in all experiments reported here (Figure S1).

To determine whether the effects of PheOH on improving the ethanol yield last for longer periods, the same system was used and 0.2% PheOH-containing medium was fed for 26 h. Over the course of Phase 1 (no inhibitor), the ethanol yield was 0.40-0.41 after the well-mixed reactor column (C1) and in effluent of the complete system (C1+C2) (Figure 5, Table S1). The glucose conversion was about 80% after C1 and was 98% or higher for the complete system throughout this phase. Moreover, the ethanol volumetric productivity was 30.0 $g/L_{reactor}\cdot h$ in C1 and 24.9 $g/L_{reactor}\cdot h$ for the system at the end of Phase 1, with an effluent ethanol concentration of 9.4 g/L. In Phase 2, 0.2% PheOH was supplied in the medium and ethanol yield was about 0.45 and 0.43 for C1 and C1+C2, respectively, during the 26-h test (Figure 5, Table S1). This corresponds to a 4.9% increase in the ethanol yield in the system. The glucose conversion was 60% after C1 and 91% for C1+C2. The ethanol volumetric productivity was 25.3 $g/L_{reactor}\cdot h$ in C1 and 23.7 $g/L_{reactor}\cdot h$ in the whole system, and the ethanol concentration in the effluent was 9.1 g/L.

When the feed was switched back to YPD medium in Phase 3, the ethanol concentration in the effluent was 8.9 g/L, and the ethanol yield returned to 0.39-0.40, similar to the values in Phase 1. The glucose conversion was 70% and 98% and the ethanol volumetric productivity was 26.7

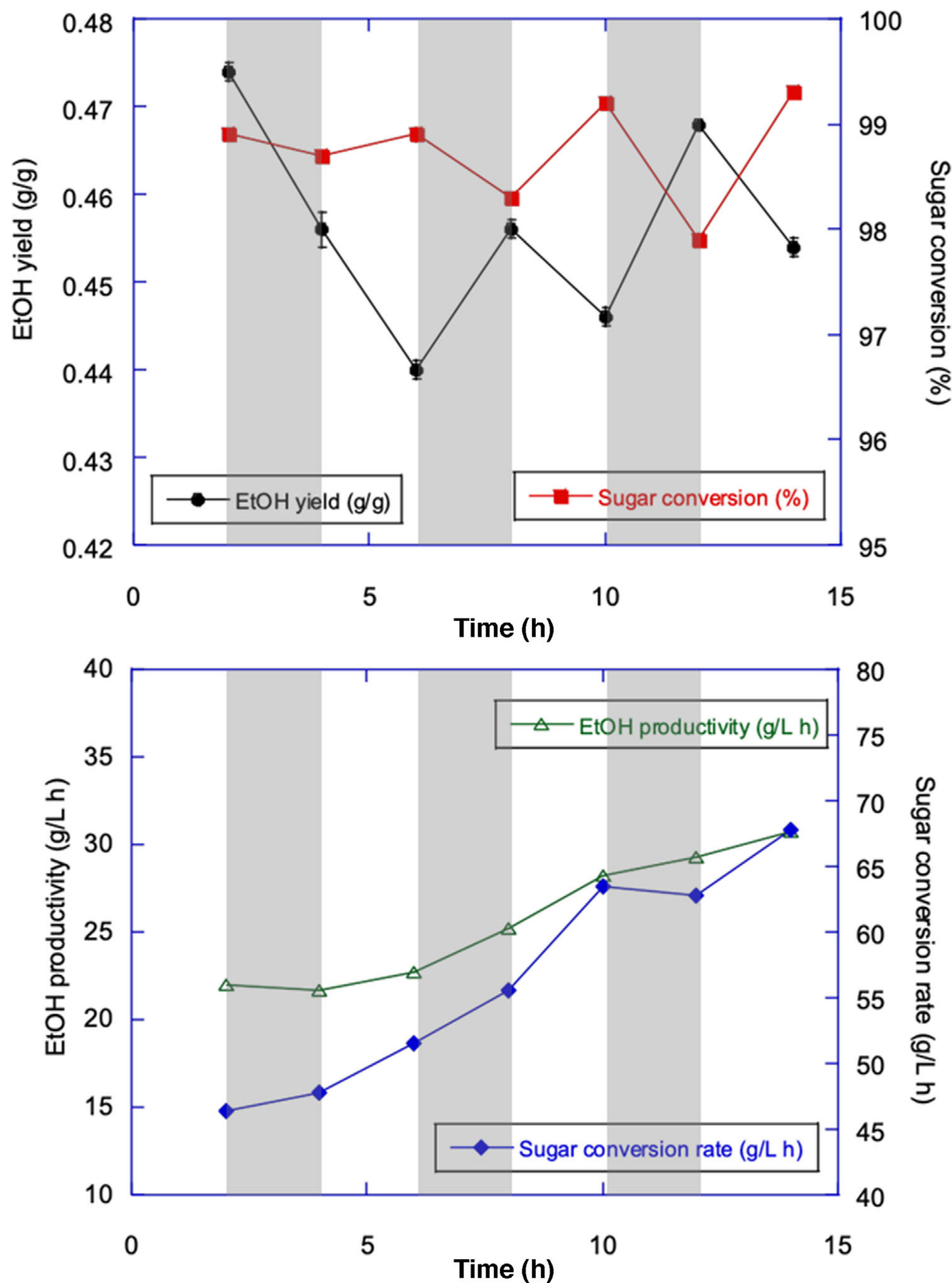

FIGURE 4 The effects of PheOH during *S. cerevisiae* JAY270 fermentation of YPD medium in the two-column immobilized cell bioreactor system. Shaded regions indicate periods in which the feed contained PheOH (0.1, 0.2, or 0.3%, in that sequence). Top: Ethanol yield and sugar conversion. Bottom: Ethanol volumetric productivity and sugar conversion rate

and 23.0 g/$L_{reactor}$·h for the C1 subsystem and the whole system, respectively (Figure 5, Table S1).

3.5 | Effects of PheOH on immobilized cells in continuous fermentation of algal hydrolysate

The concentrations of the primary sugars in the hydrolysate used in this experiment were 4.4 g/L glucose and 4.8 g/L mannose. As with YPD medium, the addition of PheOH in algal hydrolysate increased the ethanol yield but the ethanol volumetric productivity and sugar conversion were lower for the same RT (Figure 6, Table S1). In the first phase (algal hydrolysate without PheOH), the ethanol concentration in the effluent was 4.0 g/L and the ethanol yield was 0.54 ± 0.03 in the whole system. In the second phase (algal hydrolysate with 0.2% PheOH), the ethanol concentration in the effluent was 3.4 g/L and the ethanol yield was 0.58 ± 0.02 in the

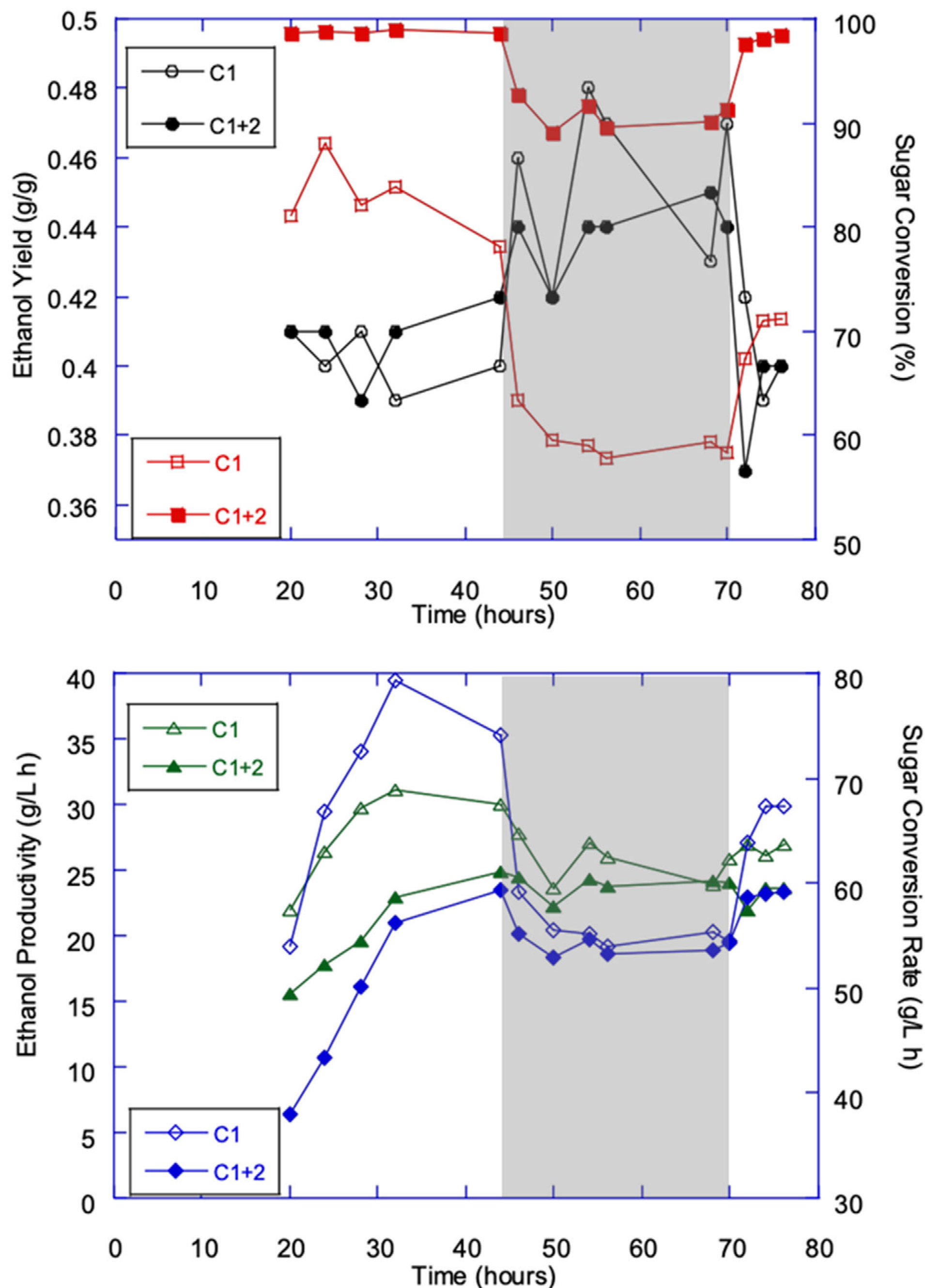

FIGURE 5 Ethanol volumetric productivity, sugar conversion, ethanol yield, and sugar conversion rate during the three-phase continuous immobilized *S. cerevisiae* JAY270 fermentation in YPD medium with 0.2% PheOH added in Phase 2 (shaded region). Top: the ethanol yield and sugar conversion. Bottom: the ethanol volumetric productivity and sugar conversion rate. C1 indicates value following conversion in the first immobilized-cell column and C1+2 indicates values following conversion in the complete bioreactor system

whole system. Then, in the third phase (return to algal hydrolysate without PheOH), the ethanol concentration in the effluent was 3.9 g/L and the yield was 0.55 ± 0.01 in the whole system. These results indicate that the addition of 0.2% PheOH in algal hydrolysate reversibly increased the ethanol yield by 5.5-7.4%. Since the theoretical yield of ethanol from glucose (and mannose) is 0.51 g/g, the higher values of the yield obtained here indicate that additional substances in the algal hydrolysate were fermented to ethanol by *S. cerevisiae* JAY 270. A metabolomics analysis of the composition of the hydrolysate revealed the presence of 28 saccharides (Table S2) and many other compounds (Table S3).

The ethanol volumetric productivity for the system ranged from 8.1 to 9.8 $g/L_{reactor}{\cdot}h$. During the test, the extent of sugar conversion ranged from 70% (the addition

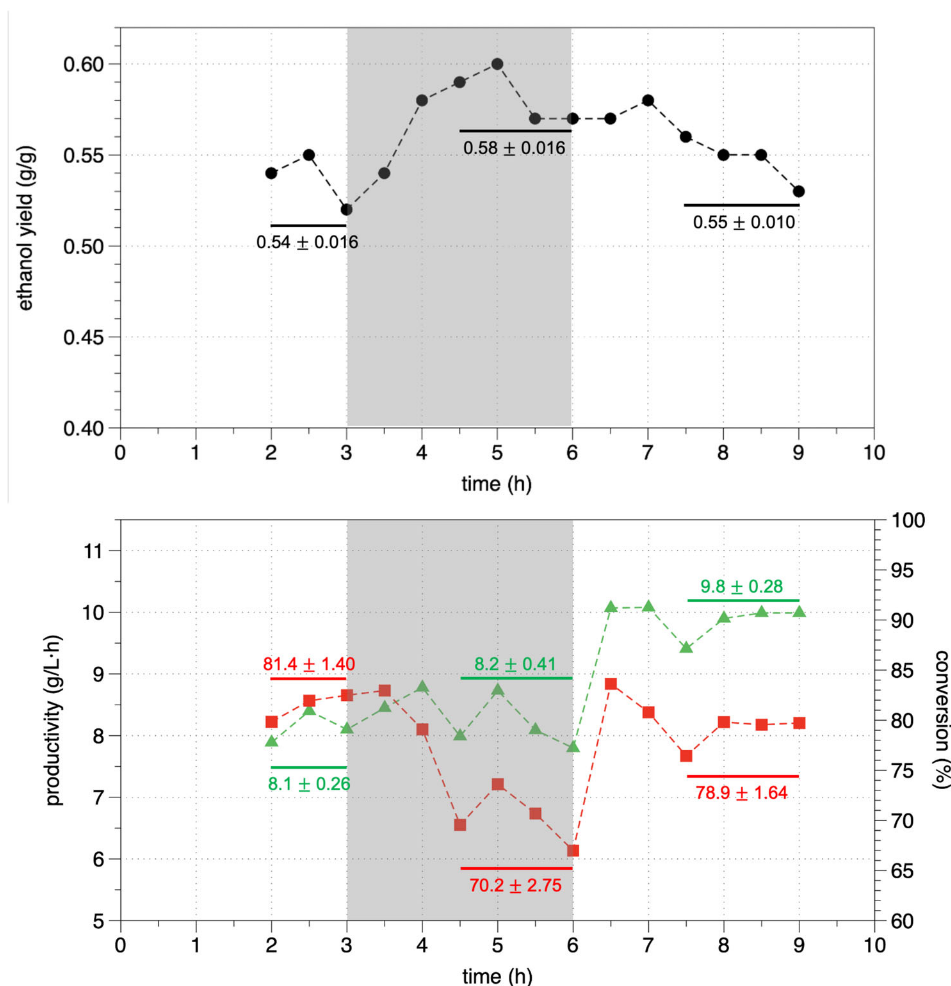

FIGURE 6 Effects of PheOH on immobilized *S. cerevisiae* JAY270 cells during continuous fermentation of algal hydrolysate. Between 3 and 6 h of the experiment, indicated with gray shading, the system was fed algal hydrolysate + 0.2% PheOH. Top: Ethanol yield in the two-column system. Bottom: Ethanol volumetric productivity (green triangles) and sugar conversion (red squares) in the two-column system. Values indicated are the mean and standard deviation for the period

of 0.2% PheOH in algal hydrolysate) to 81% (algal hydrolysate without PheOH).

4 | DISCUSSION AND CONCLUSION

4.1 | Strategies to improve productivity and sugar conversion

Productivity is an important consideration for the economic sustainability of a process because it directly indicates the capital costs required to accomplish the process at the desired scale. Continuous processes have advantages over batch and fed-batch operations because there is much less down time to drain, clean, and fill the bioreactor. Fed-batch processes can be effective when the substrate can be supplied at high concentrations but that is often not possible with biomass hydrolysates, as in the case with the algal hydrolysates used in this study.

The extent of sugar conversion is a key factor in determining both economic and environmental sustainability because producing the biomass hydrolysate is resource intensive. Batch and fed-batch cultivations typically achieve complete sugar conversion, as do appropriately-sized plug-flow bioreactors, but well-mixed bioreactors cannot be operated at high sugar conversions unless the volume is large. On the other hand, plug flow bioreactors do not allow for pH control, which can be very important for many microbial cultivations.

For these reasons, the two-column bioreactor system was evaluated for fermentation of the algal biomass hydrolysate. The system was designed to accomplish most of the sugar conversion in the well-mixed subsystem in order to control pH, while the plug-flow column was used

to increase sugar conversion. The use of immobilized cells allows operation at RTs shorter than the inverse of the maximum specific growth rate of *S. cerevisiae* and the retention of higher cell concentrations within the bioreactor volume than is possible with free cells. Using a mock hydrolysate with 20 g/L glucose, this two-column bioreactor system operated at both high volumetric productivity (28 $g/L_{reactor}{\cdot}h$) and high sugar conversion (98.8%) (Figure 3). By comparison, a free-cell batch cultivation would have an ethanol volumetric productivity of approximately 0.3 $g/L_{reactor}{\cdot}h$ if the initial 20 g/L of glucose were consumed in about 24 h to produce 8 g/L ethanol, and the overall volumetric productivity would be much lower if draining, cleaning, and filling phases are considered. As shown in Table 2, previous studies on ethanol fermentation of different feedstocks showed that the ethanol volumetric productivity was less than 1 $g/L_{reactor}{\cdot}h$ using free cells and in the range of 0.33 to 7.5 $g/L_{reactor}{\cdot}h$ with immobilized cells.

Fermentation of the algal hydrolysate (containing a low sugar content of 4.4 g/L glucose and 4.8 g/L mannose) in the two-column system enabled an ethanol volumetric productivity of up to 10.1 $g/L_{reactor}{\cdot}h$ (Figure 6), higher than obtained from other studies (Table 2). This is notable because productivity is a function of the sugar concentration in the feed. As a comparison, the volumetric productivity of the batch cultivation of a similar algal hydrolysate but 70% higher sugar concentration was only 0.18 $g/L_{reactor}{\cdot}h$ (Figure 2).

The extent of sugar conversion ranged from 69% (the addition of 0.2% PheOH in algal hydrolysate) to 81% (algal hydrolysate without PheOH) during the fermentation of the algal hydrolysate (Figure 6). Higher sugar conversion could readily be achieved by increasing the volume (and RT) of the plug-flow column.

4.2 | Strategies to improve carbon conversion efficiency (CCE)

As ethanol is the bio-based product produced the largest quantity and the global bioethanol production is expected to increase to 134.5 billion L by 2024 [29], increases of only a few percentage points represent substantial value to the ethanol industry. One strategy for increasing ethanol yield (carbon conversion efficiency) is to reduce biomass growth during fermentation in a manner that allows more substrate to be converted into product. Previously, we evaluated yeast growth inhibition compounds for their ability to redirect carbon flux from biomass to ethanol and found that the *S. cerevisiae* quorum-sensing molecules, 2-phenylethanol (PheOH), tryptophol (TrpOH), and tyrosol (TyrOH), decreased yeast biomass and glycerol yields and increased ethanol yield [19]. Notably, these effects were observed using *D. armatus* hydrolysate in the current study. In batch cultivations, the addition of 0.2% TrpOH resulted in an ethanol yield of 0.49 g ethanol/g sugar, 11.4% higher than that of the DMSO reference treatment and about 20% higher than the value for free cell fermentation of *D. armatus* hydrolysate. Increased ethanol yields (5.5–7.4%) were also demonstrated when 0.2% PheOH was added to the algal biomass hydrolysate feed to an immobilized cell bioreactor.

Interestingly, ethanol has been regarded as a growth-associated product; the finding that the relationship between growth and ethanol production can be altered indicates that this process is more complex. The addition of PheOH in the continuous-flow bioreactor experiments was found to lower the rates of ethanol production (Figures 4–6) and glucose consumption, indicating an impact on catabolic rates.

An alternative strategy for increasing CCE taken via limiting carbon flux to pathways other than ethanol production is to eliminate the production of glycerol [30–32]. For instance, an engineered *S. cerevisiae* strain, deletion of *GPD2*, which encodes an isoenzyme of NAD^+-dependent glycerol-3-phosphate dehydrogenase, combined with overexpression of enzymes of the non-oxidative pentose-phosphate pathway, exhibited a 86% lower glycerol yield and a 15% higher ethanol yield [32]. It is possible to further improve CCE by using *S. cerevisiae* strains modified to reduce glycerol production in combination with the strategies we studied including immobilized cell bioreactors and addition of yeast growth inhibitors.

4.3 | Perspectives for bioconversions of biomass hydrolysates

The results presented here show the ability of bioreactor design strategies and a novel growth inhibition approach to simultaneously achieve high productivity, sugar conversion, and yields, all of which are important to meet the environmental and economic sustainability goals for the bioeconomy. The use of immobilized cells in a combination of a well-mixed bioreactor and a plug-flow bioreactor can provide higher productivities at high conversion than either bioreactor type alone, and with far higher volumetric productivities than can be achieved with free cells. The application of immobilized cell technology at industrial scale requires additional work to develop robust immobilization methods and operational strategies.

The use of quorum-sensing molecules to inhibit growth while still allowing ethanol production resulted in higher ethanol yields. Since the quorum-sensing molecules slow cell growth, a continuous process would have very low productivity with free cells. In contrast, cell immobilization

TABLE 2 Comparison of yeast fermentations of different biomass feedstocks

Yeast strain	Feedstock	Free/ immobilized cells	Fermentation mode	Initial sugar concentration (g/L)	Ethanol yield (g/g)	Volumetric productivity ($g/L_{reactor}·h$)	Sugar conversion (%)	References
S. cerevisiae MTCC 173	Sorghum Stover	Free cells	Batch	200	0.34	0.94	83	[21]
S. stipitis CBS 6054	Giant reed	Free cells	Batch	33.4	0.33	0.17	100	[22]
S. cerevisiae KL17	Galactose and glucose	Free cells	Fed-batch	500	0.19	3.03	100	[23]
S. cerevisiae ZU-10	Corn stover	Free cells	Batch	99	0.42	0.95	100	[24]
S. cerevisiae ZU-10	Corn stover	Immobilized	Batch	99	0.41	1.7	100	[24]
S. cerevisiae RPRT90	*Ipomea carnea*	Free cells	Batch	72.1	0.4	0.87	78.5	[25]
S. cerevisiae CHY1011	Cassava starch	Free cells	Batch	585	0.46	1.35	95.6	[26]
S. cerevisiae GIM-2	Paper sludge	Free cells	Batch	27.8	0.34	0.59	100	[27]
S. cerevisiae	Cassava flour	Immobilized	Batch	66.6	0.473	0.328	100	[28]
S. cerevisiae	Sugarcane bagasse	Sugarcane bagasse immobilized	Batch	50	0.44	0.42	70	[16]
S. cerevisiae	Sugarcane bagasse	Ca-alginate immobilized	Batch	50	0.38	0.32	62	[16]
S. cerevisiae	Sugarcane bagasse	Agar-agar immobilized	Batch	50	0.33	0.26	56	[16]
S. cerevisiae	Carrot must	Ca-alginate immobilized	Batch	73.1	0.409	7.45	100	[15]
S. cerevisiae	*Aloe vera* leaf	Free cells	Batch	42.5	0.37	2.41	91.4	[17]
S. cerevisiae	*Aloe vera* leaf	Immobilized yeast in suspension	Batch	42.5	0.38	2.55	94.8	[17]
S. cerevisiae	*Aloe vera* leaf	Immobilized yeast in PBR	Continuous	42.5	0.49	2.75	77.8	[17]
S. cerevisiae *D5A*	Algal hydrolysate	Free cells	Batch	54	0.41	0.92	98.5	[2]
S. cerevisiae *D5A*	Algal hydrolysate	Free cells	Batch	62.04	0.41	0.54	100	[6]
S. cerevisiae *D5A*	Algal hydrolysate	Free cells	Batch	21.07	0.694[a]	0.30	100	[6]
S. cerevisiae JAY270	Algal hydrolysate	Free cells	Batch	15.9	0.41	0.18	100	This study
S. cerevisiae JAY270	Algal hydrolysate	Immobilized	Batch	15.8	0.45	0.30	100	This study
S. cerevisiae JAY270	Algal hydrolysate	Immobilized	Continuous	9.2	0.54[a]	10	81	This study
S. cerevisiae JAY270	Algal hydrolysate	Immobilized	Continuous+PheOH	9.2	0.58[a]	9.3	69	This study

[a]Measured ethanol yields of >0.508 may reflect fermentation of additional carbohydrates beside glucose and mannose, which was not accounted for in the theoretical calculations.

allows the biocatalysts to be retained in the bioreactor system. Further investigation of the metabolic and physiological shifts associated with exposure to these growth inhibitors and the optimal timing and level of growth inhibitor addition to achieve long-term bioreactor operation with high CCE is needed.

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CONFLICT OF INTEREST

The authors have filed a US patent application on the use of yeast quorum-sensing molecules and related molecules to increase ethanol yield.

DATA AVAILABILITY STATEMENT

Some data are available in article supplementary material; additional data are available on request from the authors.

DEDICATION

In honor of Prof. Dr. Thomas Scheper upon his retirement after an extraordinarily productive and visionary career in many areas of biotechnology.

ORCID

Kenneth F. Reardon https://orcid.org/0000-0002-7753-4049

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SUPPORTING INFORMATION

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