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ORIGINAL RESEARCH

Biochemical conversion of *Brassica carinata* biomass to organic acids

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Abstract

The economics of the inedible oilseed crop *Brassica carinata* as a source of renewable fuels can be enhanced by converting its cellulosic biomass to value-added chemicals, such as organic acids. We investigated the biochemical conversion of carinata biomass to propionic acid by first pretreating the carinata meal (CM), which is obtained after extraction of the oil from carinata seeds, with concentrated phosphoric acid to remove hemicellulose and gain access to the cellulose constituent of the meal. We then subjected the pretreated meal to enzymatic hydrolysis with cellulase enzyme to depolymerize cellulose to glucose. The overall conversion of carinata cellulose to glucose was 85%, which is promising. Finally, the recovered glucose was successfully fermented primarily to propionic acid using the bacterium *Propionibacterium freudenreichii* with a yield of 0.57 g of produced propionic acid per gram of consumed cellulosic glucose. The biobased propionic acid and other co-produced organic acids can serve as renewable building blocks for manufacturing industrial chemicals and food preservatives replacing fossil-derived organic acids. Hence, CM constitutes a renewable source of fermentable carbohydrates potentially improving the economics and sustainability of the carinata value chain.

KEYWORDS

Brassica carinata, carinata meal, cellulosic biomass, fermentation, hydrolysate, propionibacteria, propionic acid

1 | INTRODUCTION

Demand for renewable resources has intensified as a means of combating climate change through the development of biofuels and bioproducts. The US Department of Energy has identified the need for utilizing biobased jet fuel from terrestrial and aquatic biomass to help reach a sustainable carbon-neutral growth for the aviation sector (US Department of Energy, 2020) and research is actively pursued into assessing various biomass feedstocks as potential sources for sustainable biofuels. Among those feedstocks, *Brassica carinata* has already been certified as a sustainable resource (Roundtable on Sustainable Biomaterials, 2020).

Carinata is an oilseed plant of interest because it is an inedible high-yield cover crop that can be cultivated on fallow land in winter time in the southeastern United States. Its cultivation advances sustainable agriculture as it is intended to complement, not replace, summer food crops like soybeans, corn, peanuts, and corn and hence provide farmers with additional income, while reducing top soil erosion

Ehab M. Ammar and Jessica Martin contributed equally to this study.

and enhancing the soil microbiome. It has a high oil content of almost 45% that can be readily used in the production of jet and other biofuels and a plethora of bioproducts (Kumar et al., 2020; Seepaul et al., 2019). As seen in Figure 1, carinata seed crushing leads to two product streams: (1) crude carinata oil and (2) carinata meal (CM). The carinata oil is cleaned up and eventually converted to renewable jet fuel and other distillates using a series of chemical catalytic processes, whereas one of the oil's main ingredients, erucic acid, can serve as a feedstock for production of surfactants, lubricants, plasticizers, herbicides, lacquers, and nylon fibers. CM, on the other hand, which is mostly composed of protein and lignocellulosic biomass, can serve as an animal feed ingredient and as a source of cellulosic biomass and sinapic acid.

Reportedly large-scale carinata cultivation in the southeastern United States has the potential to produce between 980 and 2045 million liters of jet biofuel annually (Alam & Dwivedi, 2019). However, to improve the economics of carinata biofuels and make large-scale carinata deployment feasible, more components of the carinata seed, in addition to the oil, will need to be valorized, including the biomass content of CM. CM is the solid residue obtained after oil is extracted from the seeds and contains primarily proteins and biomass (Kumar et al., 2020). To date, CM has been successfully tested and approved for use as an animal feed ingredient (Schulmeister et al., 2019) but use of its cellulosic and hemicellulosic content has not been tested. Given that hydrolysates from a wide range of agricultural biomass residues, such as corncob, artichoke hearts, sugarcane bagasse, and sweet sorghum bagasse, can be converted to value-added products (Ammar et al., 2020; Krzyżaniak et al., 2020; Lo et al., 2020), it behooves the carinata industry to assess the potential of carinata biomass. Through thermochemical pretreatment and subsequent enzymatic hydrolysis, the biomass carbohydrates in CM could potentially be converted to valuable products, like propionic acid and other organic acids, thus strengthening the overall economics of the carinata value chain (Figure 1).

Propionic acid demand is around 300,000 metric tons annually with over half of that being used as preservatives in grain and animal feed, while the rest is used to synthesize plastics, herbicides, fungicides, flavoring, fragrances, and pharmaceuticals (Independent Commodity Intelligent Services, 2007). The most common way to produce propionic acid is through petrochemical routes, but these methods are neither sustainable nor environmentally friendly (Ekman & Börjesson, 2011; Sharma et al., 2017). A new way to synthesize propionic acid is through microbial synthesis, as various bacteria have been shown to synthesize propionic acid, including *Propionibacterium*, *Fusobacterium*, and *Clostridium* species (Ahmadi et al., 2017).

Unfortunately, these microbial routes are not ready for scale-up and commercialization as they still require significant improvements in yield and productivity to become cost competitive with the petrochemical propionic acid. A key way of reducing the cost of propionic acid biosynthesis is by utilizing low-cost feedstocks, so CM could potentially be a suitable candidate. The goal of this study is to investigate the technical feasibility of biochemically converting CM to propionic acid using *Propionibacterium freudenreichii*.

2 | MATERIALS AND METHODS

2.1 | Phosphoric acid pretreatment and enzymatic hydrolysis

Carinata meal was obtained from Nuseed (a subsidiary of Nufarm Australia Ltd) in the form of small pellets. The pellets were blended mechanically using a food processer, then sieved using size 10 mesh to obtain small particles ready for use in pretreatment. CM particles were analyzed

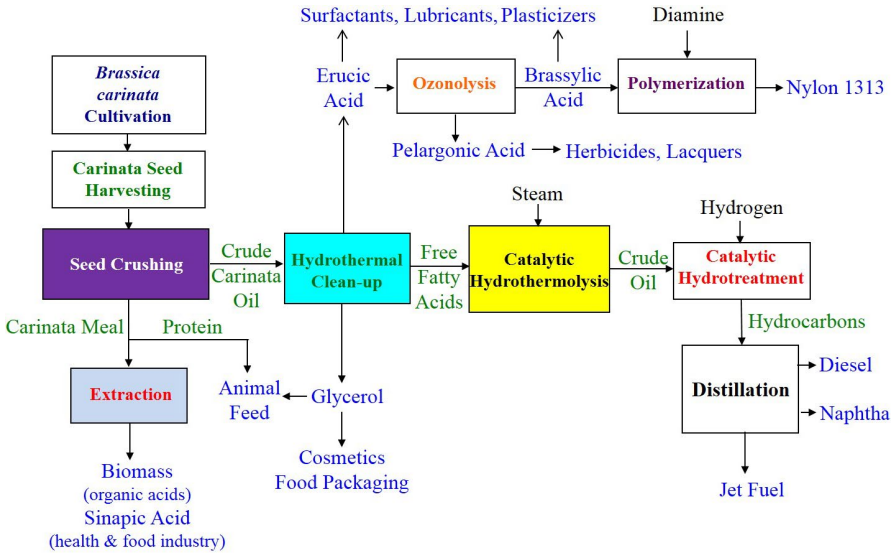

FIGURE 1 Block diagram of the envisioned carinata seed conversion to jet biofuel, other biofuels, and a range of bioproducts, including organic acids from carinata meal, using physical and thermochemical processes

for their moisture content to ensure proper meal concentration (solids loading) calculations. Pretreatment experiments were designed by employing Minitab software (Minitab Inc.) with a two-level factorial design using full factorial and center points. CM was weighed in 50 ml centrifuge tubes and mixed with concentrated phosphoric acid (85% v/v) to achieve solids loadings of 10%, 12.5%, and 15% (g/dl) on a dry basis. The mixtures were incubated in a temperature-controlled heating block at mild temperatures of 80–120°C for 30–60 min (Figure 2). After the specified time elapsed, samples were removed from the heating block and placed into an ice bath for cooling before being neutralized to pH 6.5 using NaOH and then filtered using vacuum filtration. Prehydrolysate (the filtrate from the vacuum filtration step) wash samples were collected for analysis and the remaining pretreated solids were placed into an oven at 50°C to remove all moisture. For enzymatic hydrolysis, 0.1 g of pretreated CM solids was incubated with 1 ml of the cellulase enzyme CTec2 (40 filter paper units per ml) in acetic acid buffer of pH 5.0 (Novozymes) at a 1:10 solid-to-liquid ratio, according to the manufacturer's recommendations, and the mixture was then placed in a heating block at 50°C for 3 days (Figure 2). To prepare enough hydrolysate for the fermentation experiments, 50 g of pretreated CM in total was hydrolyzed per each hydrolysis batch.

2.2 | Bacterial strain, media, and cultivation

Propionibacterium freudenreichii DSM 4902 was kindly provided by Professor S.-T. Yang and was cultivated as described before (Ammar et al., 2020). Briefly, all experiments were carried out in 120-ml sealed serum bottles under anaerobic conditions at 32°C without shaking. Each bottle contained 25 ml of medium purged with nitrogen gas to create and maintain anaerobic conditions. Glycerol stock cultures (−80°C) were first activated by growing the bacteria (1% v/v inoculum) on sodium lactate broth (NLB) containing 10 g/L sodium lactate, 10 g/L yeast extract, and 10 g/L trypticase soy broth for 72 h, and the resulting cultures were stored at 4°C (Ammar, 2013). For propionic acid fermentation experiments, NLB bottles were inoculated with 5% v/v inoculum using the 4°C NLB stored cultures and were allowed to grow for 48 h until the optical density at 600 nm (OD_{600}) reached a value of 2.0. Then, the resulting culture served as inoculum (5% v/v) for the fermentation medium, which comprised 10 g/L yeast extract, 5 g/L trypticase soy broth, 0.25 g/L K_2HPO_4, 0.05 g/L $MnSO_4$, and approximately 20 g/L of carbon source (glucose). The pH of the fermentation medium was set at 6.5 before autoclaving and $CaCO_3$ (2% w/v) was added to the medium as pH buffer (Ammar, 2013). In cases where cell growth was monitored, $CaCO_3$ was omitted to avoid interference with OD_{600} measurements.

2.3 | Analytical methods

The optical density was measured in 1.5-ml cuvettes (1-cm light path length) using a DU 730 UV/Vis spectrophotometer (Beckman Coulter) at 600 nm. The pH was measured using a benchtop pH meter Orion 3-Star (Thermo Scientific). The concentrations of glucose and organic acids (propionate, acetate, and succinate) in the fermentation broth were determined by UltiMate 3000 Ultra HPLC (Thermo Scientific) equipped with a refractive index detector using an organic column (Bio-Rad Aminex HPX-87H). The mobile phase was 5 mM H_2SO_4 at a flow rate of 0.6 ml/min and the column and detector temperature were set at 50°C. Chromatograms were analyzed using HPLC software Chromeleon 7.2.6 Chromatography Data System (Lo et al., 2020). All fermentation samples were centrifuged before analysis for 10 min at 10,621 *g* using an Eppendorf 5430 R centrifuge (Eppendorf). The supernatant was then filtered through a 0.2-μm syringe filter. The concentrations of glucose and xylose in the prehydrolysate were determined using D-glucose

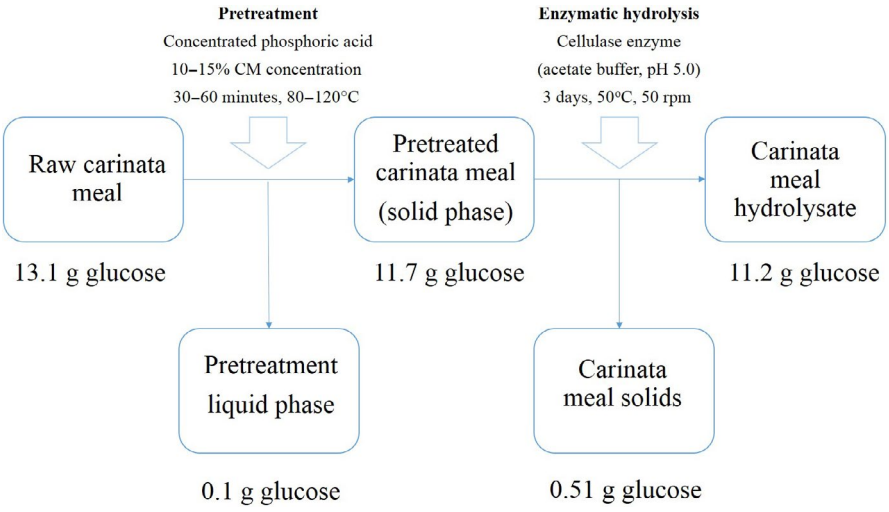

FIGURE 2 Block diagram of the integrated pretreatment and enzymatic hydrolysis process and mass balance of glucose on the basis of 100 g of raw carinata meal (dry basis) processed

and D-xylose assay kits (Megazyme Ltd) according to the manufacturer's recommended protocols.

2.4 | Statistical analysis and calculation of fermentation kinetic parameters

All experiments were carried out in duplicate and the mean and standard deviation values (error bars) were calculated and plotted. Propionic acid yield (g/g), expressed in grams of propionic acid produced per gram of glucose consumed, was determined by dividing the concentration of propionic acid (g/L) produced by the concentration of glucose (g/L) consumed during the same period of time. The propionic-to-acetic acid ratio P/A (no units) was determined by dividing the amount of propionic acid (g/L) produced by the amount of acetic acid produced (g/L) over the same period of time. Propionic acid volumetric productivity (g/L.h) was determined by dividing the concentration of propionic acid (g/L) produced by the fermentation time (h) it took to reach this concentration. Similarly, glucose consumption rate (g/L.h) was calculated by dividing the decrease in glucose concentration (g/L) over a certain period of time by the elapsed amount of time (h).

3 | RESULTS

3.1 | Pretreatment and enzymatic hydrolysis

Raw CM was found to contain 13.1 g of glucose (based on 16.2 g of cellulose content) and 7.7 g of xylose (based on 8.7 g of hemicellulose content) per 100 g of raw biomass on a dry basis. Figure 2 illustrates the integrated pretreatment and enzymatic hydrolysis of CM biomass and the mass balance of glucose derived from this biomass. Phosphoric acid pretreatment was evaluated at a range of temperatures, residence times, and biomass concentrations (solids loadings), as shown in Figure 3. On average, released glucose during pretreatment was less than 1 g/L confirming that cellulose in CM remained intact but amenable to subsequent enzymatic hydrolysis, whereas CM hemicellulose was readily hydrolyzed to xylose (data not shown). As seen in Figure 3, glucose released from the cellulosic portion of CM upon enzymatic hydrolysis by cellulase ranged from 22.8 to 37.9 g/L in the hydrolysate. The pretreatment conditions that yielded the lowest amount of glucose (22.8 g/L) upon enzymatic hydrolysis were 80°C, 30 min, and 15% solids loading. On the contrary, the highest glucose yield upon enzymatic hydrolysis (37.9 g/L) was obtained at 100°C, 45 min, and 12.5% solids loading. Only low levels of xylose (<5 g/L) were found in the hydrolysate under the tested conditions with the sole exception

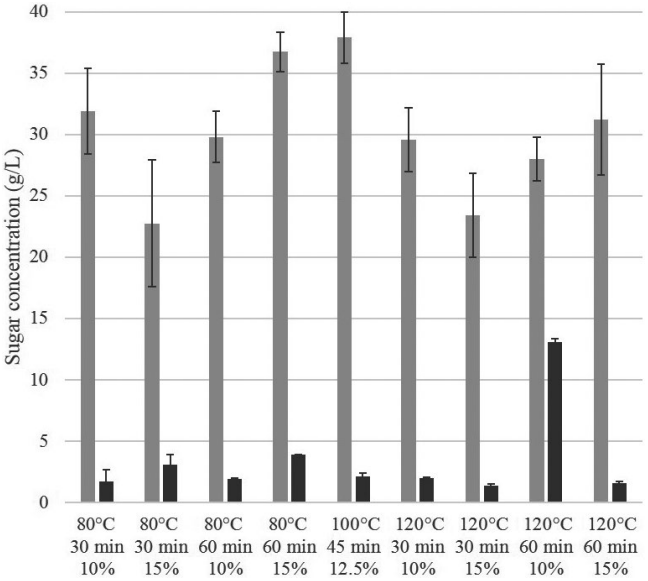

FIGURE 3 Concentrations of glucose (gray bars) and xylose (black bars) released upon enzymatic hydrolysis in the carinata meal hydrolysate at each of the tested pretreatment conditions

at the harshest pretreatment conditions at 120°C, 60 min, and 10% solids loading.

3.2 | Fermentation of CM hydrolysate with pH buffering

Carinata meal hydrolysate was tested as a renewable source of cellulosic glucose for the fermentative production of propionic acid and was compared to pure glucose as control in the presence of $CaCO_3$ for pH buffering (Figure 4). During CM hydrolysate fermentation, propionic acid reached a concentration of 8.76 g/L after 96 h, corresponding to a yield of 0.53 g/g, propionate-to-acetate ratio (P/A) was 2.65, and volumetric productivity was 0.091 g/L.h. Interestingly, the fermentation performance of CM hydrolysate exceeded that of the glucose control, which after 96 h produced 8.47 g/L propionic acid, corresponding to a yield of 0.47 g/g, P/A ratio of 2.36, and volumetric productivity of 0.088 g/L.h. In addition to propionic acid, the bacterium also produced smaller amounts of acetic acid and succinic acid in both CM hydrolysate and glucose fermentation (Figure 4) but selectivity clearly favored propionic acid.

3.3 | Fermentation of CM hydrolysate without pH buffering

The same experiment was then repeated without the pH buffering agent $CaCO_3$. As seen in Figure 5a,b, after 96 h CM hydrolysate produced 8.55 g/L propionic acid, corresponding

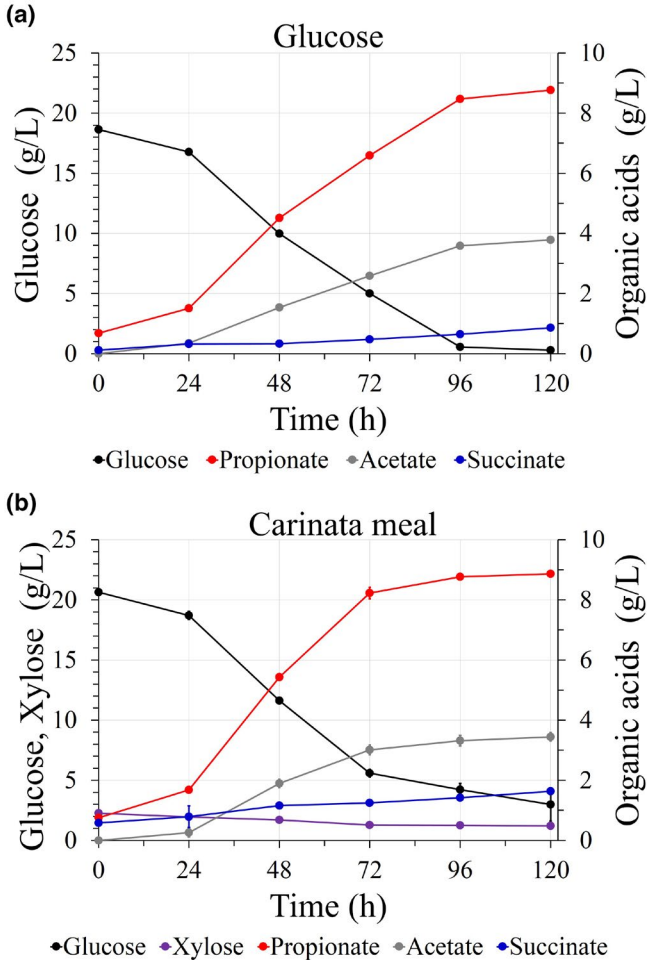

FIGURE 4 Time course of glucose and xylose consumption and organic acid production during fermentation of (a) glucose and (b) carinata meal hydrolysate in the presence of $CaCO_3$ as pH buffering agent. Each experiment was conducted in duplicate and the mean values and standard deviations (error bars) are depicted in the graphs

to a yield of 0.57 g/g, P/A of 2.48, and volumetric productivity of 0.089 g/L.h. The fermentation performance of CM hydrolysate was markedly better than that of the glucose control, even though some residual glucose (5 g/L) remained in the CM hydrolysate even after 120 h. On the other hand, the glucose fermentation was much slower and took 120 h to produce just 5.34 g/L of propionic acid, corresponding to a yield of 0.50 g/g, with a P/A of 2.50, and a volumetric productivity of 0.044 g/L.h, which was half of the propionic acid productivity in CM hydrolysate. Moreover, the growth rate of *P. freudenreichii* and the maximum OD_{600} reached were both higher in CM hydrolysate than in glucose, as seen in Figure 5d. Again, smaller amounts of acetic and succinic acid were co-produced during both fermentations.

3.4 | Effect of cellulase buffer

To investigate whether the enhanced performance of *P. freudenreichii* during CM hydrolysate fermentation in the absence of pH buffering by $CaCO_3$ was a direct result of the presence of cellulase buffer in the CM hydrolysate (carried over from the enzymatic hydrolysis that preceded fermentation), the experiment in glucose was repeated but this time the fermentation medium was supplemented with the exact same amount of cellulase buffer as that present in the CM hydrolysate. Although the presence of cellulase buffer mitigated the drop in pH during fermentation, as seen in Figure 5d, the buffered fermentation kinetics in glucose (Figure 5c) were very similar to those in glucose without buffer (Figure 4a). Likewise, growth rate and maximum OD_{600} were very similar in glucose with and without cellulase buffer (Figure 5d).

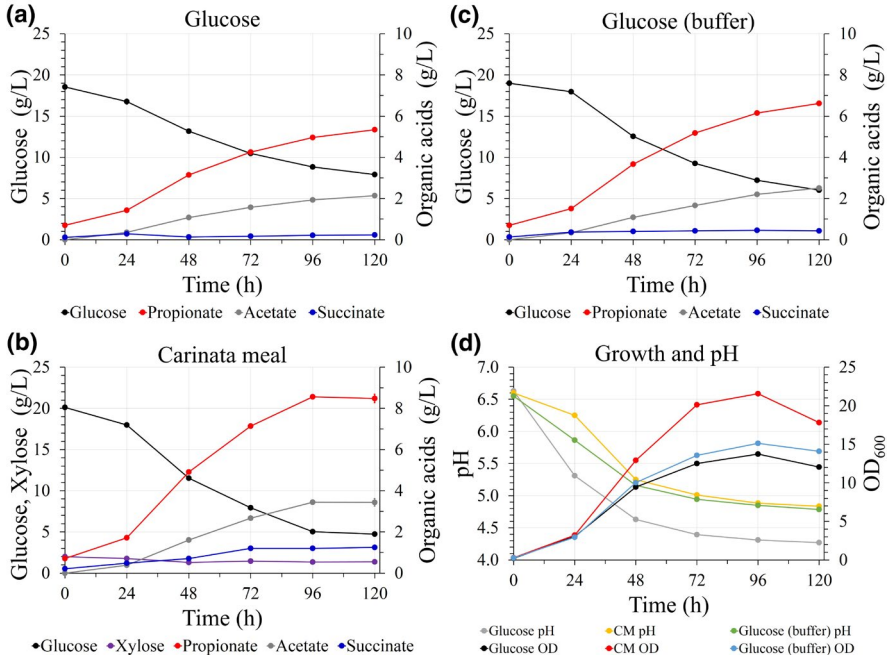

FIGURE 5 Time course of glucose and xylose consumption and organic acid production during fermentation of glucose and carinata meal (CM) hydrolysate in the absence of $CaCO_3$ as pH buffering agent (a, b) and in the presence of cellulase buffer (c). Panel (d) compiles the pH and growth (OD_{600}) profiles of runs a, b, and c. Each experiment was conducted in duplicate and the mean values and standard deviations (error bars) are depicted in the graphs

4 | DISCUSSION

If the CM biomass is monetized through conversion to value-added biomaterials, then the economics of jet biofuel production from carinata seeds can potentially improve helping make a carinata-based bioeconomy, as depicted in Figure 1, not only more sustainable, but also more cost-competitive for commercial deployment. The present study evaluated the use of CM cellulose as a renewable carbon source for the fermentative production of propionic acid and other organic acids. The effectiveness of thermochemical pretreatment on CM using concentrated phosphoric acid was determined by analyzing the release of cellulosic glucose (upon subsequent enzymatic hydrolysis) under a range of incubation temperatures, residence times, and solid loadings (Figure 3). The optimal pretreatment conditions are the ones that generate the highest concentration of glucose in the CM hydrolysate to allow for the maximum yield during fermentation. Phosphoric acid was chosen over sulfuric acid for sustainability purposes given that it also serves as a source of phosphorus and buffering capacity during the subsequent fermentation and in concentrated form (85% v/v) it is effective at mild temperatures (80–120°C), hence the pretreatment is less energy intensive than with sulfuric acid (Ammar et al., 2020).

The pretreatment wash contained less than 1 g/L of glucose indicating that cellulose remained rather intact in the solid phase, whereas hemicellulose was readily hydrolyzed primarily to xylose in the liquid phase. Based on Figure 3, the most influential factor during pretreatment of CM was residence time. The highest glucose yield was achieved at the middle point of 100°C, 12.5% solids loading, and an incubation time of 45 min. A mass balance analysis performed for glucose within the integrated pretreatment and enzymatic hydrolysis process (Figure 2) indicated that the overall glucose yield from CM was 85%, indicating a high efficiency for the entire process. A small glucose loss of 11% appears to happen during pretreatment (Figure 2) and is likely the result of glucose decomposition caused by heat and acidity of the pretreatment process.

When CM hydrolysate was utilized as carbon source for production of propionic acid by *P. freudenreichii*, the key fermentation parameters, such cell growth and propionic acid production, were better in CM hydrolysate than in glucose, as summarized in Table 1. Actually, this effect was more pronounced in the absence of $CaCO_3$ as pH buffering agent during fermentation, hence it was potentially due to the buffering capacity of the CM hydrolysate itself. The CM hydrolysate's buffering capacity, in turn, can at least partially be attributed to the carried-over acetate buffer in which the cellulase enzyme was supplied by the manufacturer (Figure 5). Nonetheless, the presence of cellulase buffer did not seem to directly affect cell growth or propionic acid fermentation kinetics suggesting that, in addition to its buffering capacity, the CM hydrolysate may also contain nutrients or components that enhance the metabolic performance *of P. freudenreichii*. Similar observations have been reported for *P. freudenreichii* and *P. acidipropionici* grown on sweet sorghum bagasse hydrolysate (Ammar et al., 2020) and corn meal hydrolysate (Wang et al., 2017), respectively. Based on Table 1, CM hydrolysate appears to be a better carbon source than pure glucose, enhancing propionic acid biosynthesis even at full strength, an indication that no cell growth inhibitors are present in CM hydrolysate prepared at the optimal pretreatment conditions.

It is anticipated that even better fermentation kinetics can be achieved at larger scale in a bioreactor compared to serum bottles because of the tight pH control and enhanced mass transfer conditions that are prevalent in bioreactors, as previously reported for sweet sorghum hydrolysate (Ammar et al., 2020). Maintaining the pH at 6.5 during the entire fermentation course, as is possible in a bioreactor, will force most propionic acid to stay in the dissociated form, which is less toxic to propionibacteria, whereas in serum bottles, when the pH drops below 5, most of the propionic acid exists in the

TABLE 1 Values of fermentation parameters during organic acid production from carinata meal (CM) hydrolysate by *Propionibacterium freudenreichii* in the presence and absence of calcium carbonate as a pH buffering agent

	With $CaCO_3$		Without $CaCO_3$		
	Glucose	CM	Glucose	Glucose (buffer)	CM
Propionate (g/L)	8.47 ± 0.07	8.76 ± 0.09	5.34 ± 0.10	6.62 ± 0.05	8.55 ± 0.05
Acetate (g/L)	3.59 ± 0.02	3.32 ± 0.18	2.14 ± 0.03	2.5 ± 0.04	3.44 ± 0.03
Succinate (g/L)	0.64 ± 0.01	1.42 ± 0.03	0.23 ± 0.00	0.43 ± 0.01	1.21 ± 0.03
Propionate yield (g/g)	0.47 ± 0.00	0.53 ± 0.01	0.50 ± 0.01	0.51 ± 0.00	0.57 ± 0.00
P/A ratio	2.36 ± 0.01	2.65 ± 0.11	2.5 ± 0.01	2.65 ± 0.02	2.48 ± 0.00
Propionate productivity (g/L.h)	0.088 ± 0.001	0.091 ± 0.001	0.044 ± 0.001	0.055 ± 0.000	0.089 ± 0.001
Glucose consumption (g/L.h)	0.188 ± 0.002	0.171 ± 0.006	0.089 ± 0.001	0.108 ± 0.001	0.157 ± 0.000

Abbreviation: P/A, propionic-to-acetic acid.

undissociated form that is more toxic to propionibacteria (Jin & Yang, 1998).

A number of studies have investigated the use of various other biomass hydrolysates as feedstocks for propionic acid production from propionibacteria, with lower, similar, or higher yields than those reported in the present study. Lower propionic acid yields were reported for Jerusalem artichoke hydrolysate (0.38–0.48 g/g; Liang et al., 2012), sugarcane bagasse hydrolysate (0.29 and 0.37 g/g; Zhu et al., 2012), corn stover hydrolysate (0.44–0.50 g/g; Wang et al., 2017), and sorghum bagasse hemicellulosic hydrolysate (0.41 g/g; Castro et al., 2020), when used as carbon sources for *P. acidipropionici*. Similarly, a lower yield was reported for sweet sorghum bagasse hydrolysate (0.51 g/g; Ammar et al., 2020) and apple pomace extract (up to 0.38 g/g; Piwowarek et al., 2019) using *P. freudenreichii*.

Propionate yields similar to those reported in the present study were reported for wheat flour hydrolysate (0.54 g/g) using *P. acidipropionici* (Kagliwal et al., 2013) and for cassava bagasse hydrolysate and co-fermented with glycerol (0.57 g/g; Wang & Yang, 2013) and corn stalk hydrolysate (~0.54 g/g; Wang et al., 2020) using *P. freudenreichii*. Finally, higher yields were reported for poplar hydrolysate (0.75 g/g; Ramsay et al., 1998), corn meal starch hydrolysate (0.58 g/g; Huang et al., 2002), corn mash (0.6 g/g; Stowers et al., 2014), and sorghum bagasse hemicellulosic hydrolysate after optimization (0.62 g/g; Castro et al., 2020) using *P. acidipropionici*. Higher yields were also reported for sweet sorghum bagasse and glycerol co-fermentation (0.59 g/g) by *P. freudenreichii* (Ammar et al., 2020) and corn stalk hydrolysate after optimization (0.75 g/g; Wang et al., 2020) by the same microorganism. Overall, CM hydrolysate is on par with or better than many biomass hydrolysates in terms of propionic acid yield. More importantly, its conversion to value-added organic acids has the potential, along with other carinata co-products (Figure 1), to improve the economics of carinata as a sustainable winter cover crop for biofuels and bioproducts.

5 | CONCLUSION

Propionibacterium freudenreichii grew faster reaching higher cell densities and produced more propionic acid at faster rates in CM hydrolysate fermentation compared to glucose fermentation (Table 1). This investigation successfully demonstrated that CM hydrolysate can serve as a renewable source of cellulosic glucose that can be fermented efficiently by *P. freudenreichii* for the production of propionic acid. In addition, acetic acid and succinic acid are also produced by the microorganism and can be separated and purified to generate additional sources of revenue. Moreover, having proven the fermentability of carinata-derived glucose, metabolites other than those organic acids could also be produced by selecting an appropriate fermentation microorganism. Although CM hydrolysate is a promising feedstock according to the present study, further work is needed to reduce the cost of fermentation through process optimization, including co-fermenting both glucose and xylose derived (Wei et al., 2016), co-fermenting biomass hydrolysate and glycerol under high cell density fermentation conditions (Ammar et al., 2020), and incorporating bioprocessing tools (Blanc & Goma, 1987; Jin & Yang, 1998; Selder et al., 2020; Suwannakham & Yang, 2005) and metabolic engineering techniques (Ammar et al., 2014; Guan et al., 2018; Liu et al., 2020; Wang, Ammar, et al., 2015; Wang et al., 2015; Wei et al., 2016).

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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