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Mixed Carboxylic Acid Production by *Megasphaera elsdenii* from Glucose and Lignocellulosic Hydrolysate

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Abstract: Volatile fatty acids (VFAs) can be readily produced from many anaerobic microbes and subsequently utilized as precursors to renewable biofuels and biochemicals. *Megasphaera elsdenii* represents a promising host for production of VFAs, butyric acid (BA) and hexanoic acid (HA). However, due to the toxicity of these acids, product removal via an extractive fermentation system is required to achieve high titers and productivities. Here, we examine multiple aspects of extractive separations to produce BA and HA from glucose and lignocellulosic hydrolysate with *M. elsdenii*. A mixture of oleyl alcohol and 10% (*v*/*v*) trioctylamine was selected as an extraction solvent due to its insignificant inhibitory effect on the bacteria. Batch extractive fermentations were conducted in the pH range of 5.0 to 6.5 to select the best cell growth rate and extraction efficiency combination. Subsequently, fed-batch pertractive fermentations were run over 230 h, demonstrating high BA and HA concentrations in the extracted fraction (57.2 g/L from ~190 g/L glucose) and productivity (0.26 g/L/h). To our knowledge, these are the highest combined acid titers and productivity values reported for *M. elsdenii* and bacterial mono-cultures from sugars. Lastly, the production of BA and HA (up to 17 g/L) from lignocellulosic sugars was demonstrated.

Keywords: butyric acid; hexanoic acid; caproic acid; volatile fatty acids; short-chain carboxylates; extractive fermentation; oleyl alcohol; corn stover; biochemical; biofuel

1. Introduction

The conversion of lignocellulosic sugars to bio-based products and biofuels presents an opportunity to replace non-sustainable, petroleum-based products while avoiding direct competition with food sources. Microbial conversion approaches, often enabled by the use of strains that naturally accumulate products of interest, have garnered significant interest in the emerging bioeconomy. One such strategy, the "Carboxylate Platform"—namely the production of volatile fatty acids (VFAs, specifically C2–C8 VFAs) from biomass sources via anaerobic fermentation either through mixed cultures or mono-culture—has emerged as an attractive approach to generate high yields of promising biofuel and biochemical precursors [1,2]. Most well studied VFA-producing microbes employ anaerobic chain elongation [3,4]. Both butyric acid (BA) and hexanoic acid (HA) are particularly promising VFAs that can readily be upgraded to biofuels and solvents through known chemistries [5–8]. Currently, much of the industrial production of BA and HA is from fossil-derived sources; however, the biological production of BA from non-fossil-based sources is gaining attention [9,10]. In general, homo-BA or homo-HA fermentations are pursued. However, for downstream catalytic transformations aimed at producing biofuels, mixtures are often desirable, creating an opportunity for heterofermentative production of mixed VFAs.

Biological production of BA (e.g., by *Clostridium tyrobutyricum* or *Clostridium butyricum*) has been extensively studied relative to HA production. In the case of BA production, the maximum titers reported reached up to 300 g/L in an extractive fermentation mode from glucose by *C. tyrobutyricum* [11]. Additionally, BA fermentations typically produce considerable acetic acid concentrations [12]. To date, reports of simultaneous HA and BA production are scarce. In addition, most of the substrates utilized in these studies are not lignocellulose-derived sugars. For instance, the production of HA and BA by *Clostridium kluyveri* is from ethanol and acetate (or succinate) fermentation [13], or in the case of *Clostridium* sp. BS-1 from galactitol [14]. The latter study reported one of the highest HA titers to date, 32 g/L in 16 days of fermentation. In other studies, VFAs were produced in bacterial co-cultures [15–21], and important productivity improvements have been recently shown [3,22].

Megasphaera elsdenii is a strictly anaerobic bacterium which was isolated from sheep rumen in 1956 [23]. This bacterium is able to ferment different carbon sources (e.g., glucose, fructose, sucrose, and lactic acid) to produce VFAs (C2 to C6), CO_2 and H_2 [23]. Specifically, BA and HA are the major metabolic products from glucose [20,21,23]. Recently, it has been also demonstrated that *Megasphera* sp. MH can generate C7- and C8-carboxylic acids when adding different carbon-chain length electron acceptors to the culture media [24]. *M. elsdenii* metabolism and, in particular, the steps involved in HA production are not still well understood, but it is hypothesized that HA may be formed from chain elongation of butyrate via reverse β-oxidation [25]. Furthermore, it has been shown that *M. elsdenii* utilizes acetate and butyrate for the production of HA [20]. Despite carboxylic acids being major metabolites of *M. elsdenii*, they are also often potent fermentation inhibitors [26]. The sensitivity of *M. elsdenii* to initial HA concentrations has been investigated as well as the toxicity of different VFAs, showing that HA is more toxic than shorter carboxylic acids [27]. Some of the highest reported concentrations for HA production by *M. elsdenii* (with or without simultaneous production of BA) were from sucrose (28.4 g/L HA) [20] and glucose (up to 19 g/L of HA and 3.4 g/L BA) [21] fermentations. In both cases, fermentations were coupled to extraction systems to overcome product inhibition.

Several methods to mitigate carboxylate inhibition have been previously explored, such as initial pH adjustment [20], pH control by addition of base [21] or a buffer [14], cell immobilization [21], product removal by extraction [20], gas stripping [28], or ion exchange resin in the fermentation vessel [21]. In the case of in situ product removal via organic solvent extraction, there is potential to avoid product feedback inhibition, decrease processing costs associated with neutralization, and facilitate product purification. This strategy has been utilized and demonstrated to be effective for a variety of carboxylic acids including propionic acid, BA, and HA [11,14,20,29,30]. For these highly water soluble compounds, a reactive extraction method is preferred. In a reactive extraction, a chemical complex is formed involving the carboxylate group of the product and the extraction solvent system facilitating extraction [31]. For instance, combining an alkylamine and organic solvent has been shown to improve extraction efficiency [14,20,31]. The selection of the solvent is critical to maximize extraction efficiency while minimizing toxic effects to the microorganism.

The objective of the current study was to produce heterogeneous mixtures of primarily BA and HA through anaerobic fermentation from glucose and glucose-rich lignocellulosic hydrolysate. For this purpose, two extractive fermentation strategies (liquid–liquid and pertractive) were investigated. A concentration of 57.2 g/L of a combined BA and HA mixture was obtained in the extracted fraction of the fed-batch, pertractive fermentation at an average productivity of 0.26 g/L/h. Furthermore, the robustness and efficiency of *M. elsdenii* producing BA and HA from biomass hydrolysates was demonstrated, indicating that this organism may eventually be a potential candidate strain for producing mixed VFAs from biomass sugars.

2. Materials and Methods

2.1. Hydrolysate Preparation

In the current work, two different biomass sugar streams generated at the pilot scale at the National Renewable Energy Laboratory (NREL, Golden, CO, USA) were utilized (Table 1). One stream was from deacetylated, dilute-acid (DDA) pretreated, enzymatically hydrolyzed corn stover. DDA material was prepared as previously described [32]. The second stream was obtained from deacetylated, mechanically refined (DMR), enzymatically hydrolyzed corn stover and was prepared as previously reported [33]. Enzymatic hydrolysis of both pretreated materials were run as previously detailed [33] except that 20 mg/g Novozymes Cellic® CTec2 (Franklinton, NC, USA) was used for DDA digestion and 20 mg/g of a 80:20 mixture of Novozymes Cellic® Ctec2 and Novozymes Cellic® HTec2 (Franklinton, NC, USA) was used for DMR hydrolysis. Both enzyme hydrolysates were centrifuged to remove solids, neutralized to pH 6.5 with NaOH (Sigma-Aldrich, St. Louis, MO, USA), and sterile filtered in the anaerobic chamber (Coy Laboratory Products, Grass Lake, MI, USA).

Table 1. Composition of non-diluted deacetylated, dilute-acid (DDA) and deacetylated, mechanically refined (DMR) hydrolysates.

Biomass Stream	Glucose (g/L)	Xylose (g/L)	Galactose (g/L)	Arabinose (g/L)	Acetic Acid (g/L)	Lactic Acid (g/L)	HMF * (g/L)	Furfural (g/L)
DDA	85.9	52.3	3.1	7.8	1.9	0.0	0.1	1.0
DMR	68.0	48.9	0.8	3.6	0.8	2.8	0.0	0.0

* HMF = 5-*hydroxymethylfurfural*.

2.2. Microorganism and Growth Media

M. elsdenii NCIMB702410, a strain chosen based on the screening work performed by Choi, et al. [20], was purchased from the National Collection of Industrial, Food and Marine Bacteria (NCIMB) in the United Kingdom. The strain was revived in Peptone Yeast Glucose (PYG) media, adapted from Choi et al. [20] and contained the following components in grams per liter of water: yeast extract (Becton Dickinson, Franklin Lakes, NJ, USA), 10 g; tryptone (Becton Dickinson, Franklin Lakes, NJ, USA), 5 g; peptone (Becton Dickinson, Franklin Lakes, NJ, USA), 5 g; beef extract (Sigma-Aldrich, St. Louis, MO, USA), 5 g; glucose (Caisson Labs, North Logan, UT, USA), 40 g; K_2HPO_4 (Sigma-Aldrich, St. Louis, MO, USA), 2.04 g; KH_2PO_4 (Sigma-Aldrich, St. Louis, MO, USA), 0.04 g; cysteine-HCl (Sigma-Aldrich, St. Louis, MO, USA), 0.5 g; $CaCl_2 \cdot 2H_2O$ (Sigma-Aldrich, St. Louis, MO, USA), 0.01 g; $MgSO_4 \cdot 7H_2O$ (Sigma-Aldrich, St. Louis, MO, USA), 0.02 g; $NaHCO_3$ (Sigma-Aldrich, St. Louis, MO, USA), 0.4 g; NaCl (Fisher Chemicals, Fairlawn, NJ, USA), 0.08 g; resazurin (Sigma-Aldrich, St. Louis, MO, USA), 1 mg; hemin solution, 10 mL; and vitamin K1 solution, 0.2 mL. The hemin solution was prepared by dissolving 50 mg of hemin (Sigma-Aldrich, St. Louis, MO, USA) in 1 mL of 1 M NaOH then adjusted up to 100 mL with water. The vitamin K1 solution was made by dissolving 0.1 mL of vitamin K1 stock (Sigma-Aldrich, St. Louis, MO, USA) in 20 mL 95% ethanol (Fisher Chemicals, Fairlawn, NJ, USA). After combining all ingredients except the cysteine, the media was pH adjusted to 6.5 with NaOH and sparged with nitrogen for 1 h after which the cysteine was added. Then, the media was sterile filtered inside the anaerobic chamber and dispensed into serum bottles, sealed with septa and aluminum crimp seals, and finally autoclaved at 121 °C for 20 min. Media with sugars other than glucose were made as described except the sterile sugars were added after the media was autoclaved. The bacterial frozen stock was prepared from revived bacteria in PYG media during exponential phase which corresponded to an optical density at 600 nm (OD_{600}) of approximately 5.0. A 500-µL aliquot of the culture was injected into a sealed HPLC vial containing 500 µL of 50% anoxic and autoclaved glycerol (Sigma-Aldrich, St. Louis, MO, USA). The vials were gently vortexed and stored at −70 °C.

2.3. Seed Culture Preparation

Inocula were initiated by adding 1 vial of bacterial stock in sealed serum bottles containing 50 mL of PYG and 20 g/L glucose. Cells were grown anaerobically, in a shaker incubator (New Brunswick Scientific, Edison, NJ, USA) at 37 °C and 180 rpm for 8 h. This culture was then used to inoculate the "seed culture" bottle containing the same medium at an initial OD_{600} of 0.05. This culture grew for 18–24 h until an OD_{600} of ~8.0 was obtained. When additional volume (>100 mL) of inoculum was required, the "seed culture" was prepared in a 0.5 L BioStat-Q plus bioreactor (Sartorius AG, Goettingen, Germany) at 37 °C, with 150 rpm agitation, pH controlled at 6.3 and 300 mL PYG media. Inoculation of both serum bottles and fermenters in further experiments was accomplished by approximately 10% (v/v) direct transfer to reach an initial OD_{600} of 0.5.

2.4. Fermentation in Serum Bottles to Test the Effect of Organic Solvents on M. elsdenii Growth

A variety of solvents were tested to evaluate effects on *M. elsdenii* growth. Extractive batch cultures were run using oleyl alcohol (Alpha Aesar, Ward Hill, MA, USA) or octanol (Sigma-Aldrich, St. Louis, MO, USA) as the organic solvent and either trihexylamine (THA) or trioctylaime (TOA) (Sigma-Aldrich, St. Louis, MO, USA) as reactive compounds (at 5% and 10% solvent v/v). Fermentations in serum bottles were run as described above except a 20% volume of anoxic organic extractant was injected into the bottles. This mixture was allowed to equilibrate for at least 1 day. Oxygen was removed from the organic solutions by sparging with nitrogen for 2 h. The inoculum was injected into the aqueous layer with the bottle inverted to minimize contact of the organism with the organic phase. Experiments were performed in duplicate and error bars are presented as the absolute difference between values.

2.5. Batch Extractive Fermentations at Different pHs

Batch extractive fermentations were performed in 0.5 L BioStat-Q plus bioreactors. Fermentors were filled in a sterile hood with 280 mL anoxic PYG and 40 g/L glucose overlaid with 60 mL of 10% trioctylamine diluted in oleyl alcohol and then sparged overnight with nitrogen. The pH was initially adjusted to 5.0, 5.5, 6.0, or 6.5 with NaOH and maintained with either 4 M H_2SO_4 or 4 N NaOH. After inoculation, the sparge was turned off and cultures were maintained at 37 °C and 150 rpm agitation. During sampling, a nitrogen sparge was used to provide positive pressure and to remove any oxygen contamination. Samples (~1 mL) were taken for both aqueous and organic fraction. Acids from the organic phase were extracted against 0.5 N NaOH. Equal volumes (400 µL) of organic solvent and NaOH were mixed, vigorously vortexed, and statically maintained overnight at room temperature. Acid concentrations were measured from both aqueous and NaOH fractions. Glucose and bacterial growth (OD_{600}) were analyzed from the aqueous fraction. Fermentations were performed in duplicate and error bars are presented as the absolute difference between values.

2.6. Fed-Batch Pertractive Fermentations

The pertractive fermentation system utilized in the current study consisted of two Liqui-Cel Extra-Flow 2.5 × 8 membrane contactor units (3M, Charlotte, NC, USA), a 1-L Q-plus, bioreactor (Sartorius AG, Goettingen, Germany), and three FH100 peristaltic pumps (Thermo Scientific, Waltham, MA, USA) (Figure 1). The fermentor contained 1 L of PYG and 40 g/L of glucose and was maintained at pH 6.3, 37 °C, and 180 rpm agitation. The 1-L fermentation broth was continuously circulated through the shell side of the first membrane contactor at 400 mL/min. The organic extractant was continuously sparged with nitrogen and circulated through the lumen side of both membrane contactor units, countercurrent to the aqueous phases at 150 mL/min. The 1.0 N NaOH stripping solution was circulated through the shell side of the second membrane contactor unit at 400 mL/min. Adjustable tubing clamps were used as valves, partially constricting the tubing to apply 3–4 psi of pressure to the membrane contactor units and prevent the organic extractant from bleeding through the membrane. The feed solution was delivered with a model 120U peristaltic pump (Watson Marlow,

Wilmington, MA, USA), continuously sparged with nitrogen, contained 500 g/L glucose, 5 g/L yeast extract, and 2.5 g/L each of tryptone, peptone, and beef extract.

The fermentation portion of the system was sterilized by flushing 100% isopropanol (Fisher Chemicals, Fairlawn, NJ, USA) through the shell to lumen side of the filter unit, followed by circulating 10% H_2O_2 (Sigma-Aldrich, St. Louis, MO, USA) through the shell to lumen side for 6 h. The unit was then drained and flushed with at least 2 L of sterile water from the shell to lumen to remove residual H_2O_2. The filter unit was then dried overnight by flushing sterile filtered air from the shell to lumen. Samples (~1 mL) were taken periodically from the fermentation broth, organic phase, and NaOH. Glucose and bacterial growth were tracked in the fermentor broth. Acids were individually measured in the three solutions. Acids in the organic phase were extracted with 0.5 N NaOH as reported above. Pertractive fermentations were performed in duplicate and error bars are presented as the absolute difference between values.

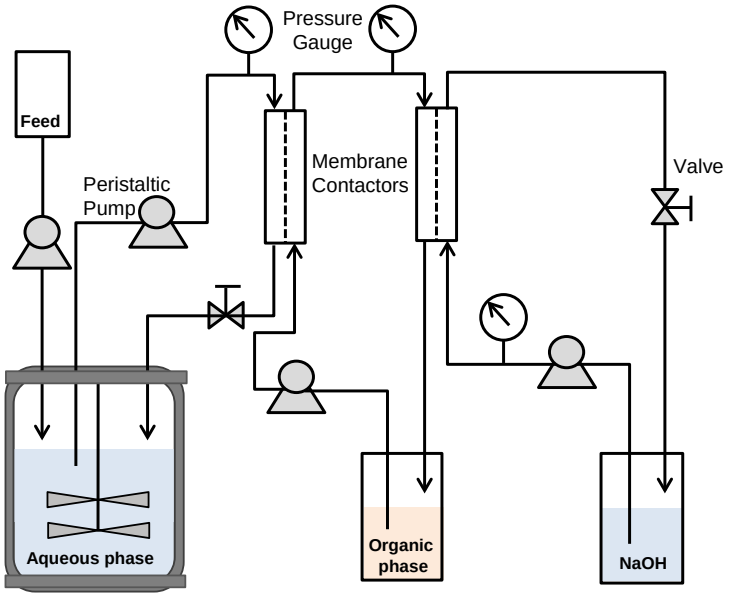

Figure 1. Scheme of the fed-batch pertractive fermentation set up utilized in the current study.

2.7. M. elsdenii Fermentations on Biomass Hydrolysate

Media was prepared in the anaerobic chamber by aseptically adding 5× yeast peptone (PY) (no glucose) media, corn stover hydrolysate (DDA and DMR), and water in sufficient quantities to make 1× PY with a glucose concentration from biomass sugars of 40 g/L. This strategy resulted in differing levels of xylose and other hydrolysate components in the two biomass streams. As a control, media was prepared as detailed above but substituting pure glucose in place of hydrolysate. Media were adjusted to pH 6.5. For extraction, 10% trioctylamine in oleyl alcohol was added at 20% the volume of media. Samples (~1 mL) were taken for both aqueous and organic fraction as reported in Section 2.5. Samples were consistently diluted 20× for OD_{600} measurements to control interference from the hydrolysate. Fermentations were performed in duplicate and error bars are presented as the absolute difference between values.

2.8. Analytical Methods

The optical density of the culture was measured at a wavelength of 600 nm and a 1-cm path length using a Genesys 10uv, UV-vis spectrophotometer (Thermo Scientific, Waltham, MA, USA). The concentrations of carboxylic acids, glucose, and other sugars concentrations were measured via HPLC (Agilent1100 series, Santa Clara, CA, USA)) outfitted with a refractive index detector and using an Aminex HPX-87H (300 × 7.8mm) organic acid column and Cation H^+ guard cartridge

(Biorad Laboratories, Hercules, CA, USA). The column was maintained at 65 °C with mobile phase consisting of 0.01 N sulfuric acid with a flow rate of 0.6 mL/min. Mobile phase was prepared from 10.0N sulfuric acid (Ricca Chemical Company, Arlington, TX). Analytes were identified by comparing retention times and spectral profiles with pure standards. The lactic, formic, acetic, butyric, valeric and hexanoic acids, along with 5-hydroxymethylfurfural, furfural, sodium propionate, arabinose and galactose used as standards were obtained from Sigma-Aldrich (St. Louis, MO, USA). Xylose was obtained from (Cascade Biochemicals, Corvallis, OR, USA).

2.9. Yields, Productivities, Carbon Yields, and Mass Balance Calculations

Acid yields were calculated as the coefficient of acetic acid (AA), BA, and HA production (g) and the sugars consumed (g). Yields were not corrected by the dilution factor caused by base addition since base volumes were less than 4% (*v*/*v*). For yield calculations, the acid sum from all aqueous, organic, and NaOH sources was considered. Lactic and acetic acids present in the biomass sugars were included in yield calculations as carbon sources. Productivity (g/L/h) was calculated as the concentration of total acids (g/L) divided by the time (h) at the end of the fermentation. Carbon yields were expressed as the percentage of moles of carbon in the products (acetic, BA, and HA) divided by the moles of carbon from glucose, lactate, and/or acetate utilized. For mass balance calculations, carbon from cell biomass and CO_2 were considered. Dry cell biomass (g/L) was estimated by multiplying the OD_{600} by the factor 0.261 g/L/OD which we previously determined correlating OD_{600} and cell dry weight (g/L). Carbon content of *M. elsdenii* biomass was 44% as measured with a LECO TruSpec CHN determinator using high temperature combustion in pure oxygen followed by infrared (IR) analysis of H_2O and CO_2. The gas was then reduced and scrubbed to measure nitrogen content via a thermal conductivity detector. CO_2 was estimated to be 33% during butyrate (Equation (1)) and HA production (Equation (2)). These equations are expressed as the maximum carbon from glucose that could lead to product without considering bacterial biomass.

$$C_6H_{12}O_6 \rightarrow C_4H_8O_2 + 2\,CO_2 + 2\,H_2 \quad (1)$$

$$3\,C_6H_{12}O_6 \rightarrow 2\,C_6H_{12}O_2 + 6\,CO_2 + 2\,H_2O + 4\,H_2 \quad (2)$$

3. Results

3.1. Evaluation of Solvent Compatibility for M. elsdenii Growth and Glucose Utilization

A reactive extraction is one where a reactive hydrophobic compound, often an alkylamine, interacts with an acid to facilitate extraction into an organic phase. The reactive compounds are typically diluted in an organic solvent. Tri-alkyl amines have been reported to be effective as reactive compounds for acid extraction [11,20,30,34]. In the current work, we use pure THA and TOA. Oleyl alcohol is one of the most common diluents for in situ extraction [11,20,30] and octanol has also been identified as highly effective for succinic acid extractions [29].

The reactive compounds mentioned above, THA and TOA, and two diluents, octanol and oleyl alcohol, were first evaluated for their compatibility in *M. elsdenii* fermentations. Bacterial growth (OD_{600}) and glucose conversion were employed as indicators of solvent toxicity (Figure 2). In the control case without extraction solvent, bacterial growth peaked at 30 h (OD_{600} = 14) and, after 48 h, the OD_{600} dropped to 4. In parallel, the pH declined from 6.5 to ~5, due to the accumulation of VFAs, which stalled the fermentation at ~30 h. In the presence of octanol, cells did not grow and glucose was not utilized, indicating high toxicity. Cultures with oleyl alcohol exhibited enhanced bacterial growth compared to the control and utilized 75% of the glucose. This result suggests that oleyl alcohol has low toxicity and that it extracts carboxylic acids, as previously described by Choi et al. [20]. The reactive compounds, THA and TOA, were also tested at two concentrations diluted in oleyl alcohol. Compared to cultures containing only oleyl alcohol, cultures grown in the presence of 5% and 10% THA exhibited significant decreases in growth and glucose utilization, indicating a moderate level of

THA toxicity. Cultures grown with 5% and 10% TOA showed the highest cell densities and complete glucose utilization. Based on the highest cell density and glucose utilization, 10% TOA in oleyl alcohol was chosen as the extraction solvent for subsequent work.

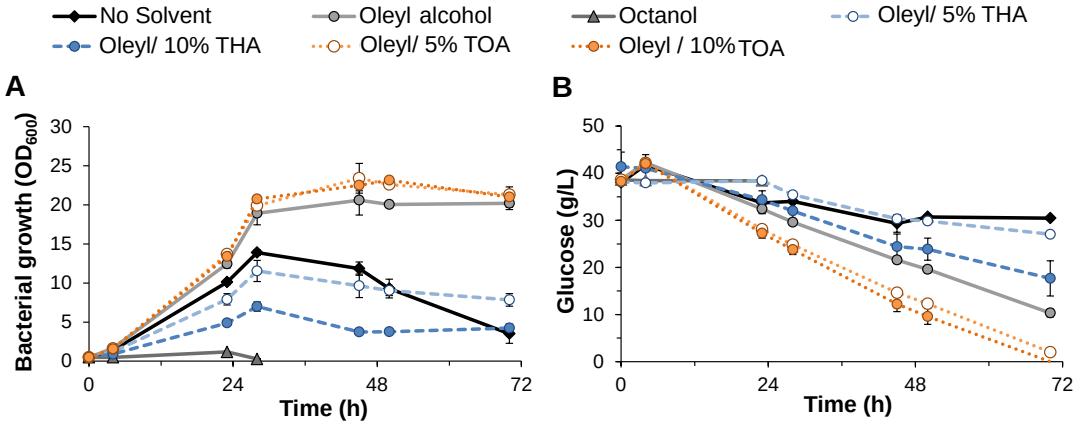

Figure 2. Serum bottle experiments without pH control to test the effect of extraction solvents on *Megasphaera elsdenii* (**A**) growth and (**B**) glucose utilization. Oleyl: oleyl alcohol; THA: trihexylamine; TOA: trioctylamine. Dry cell biomass (g/L) can be calculated by multiplying the optical density at 600 nm (OD_{600}) by the factor 0.261 g/L/OD. Results are duplicate experiments and error bars represent the data range.

3.2. Batch Liquid–Liquid Extractive Fermentation at Different pHs

One of the most important factors for effectively extracting carboxylic acids is pH. In a reactive extraction, only the protonated form of the acid is extracted and consequently, extraction is more efficient at low pH [20]. AA, BA, and HA all have similar pKa values of approximately 4.8. Above this pH, the acids become increasingly deprotonated. However, *M. elsdenii* grows better in the pH range of 5–8. To understand the balance of these requirements, we investigated the effects of different pH (5.0, 5.5, 6.0, and 6.5) in batch fermentations overlaid with 10% TOA in oleyl alcohol.

At pH 5.0, the culture grew very poorly (data not shown). At higher pH, both cell growth and glucose utilization rates were enhanced with increasing pH (Figure 3A,B). The growth rate for pH 6.5 was similar to pH 6.0 and considerably higher than at 5.5. Glucose utilization rates were 0.39, 0.27, and 0.20 g/L/h at pH 6.5, 6.0, and 5.5, respectively. These trends in *M. elsdenii* are similar to those previously reported by Miyazaki et al. [35]. At lower pH, the cells utilize energy to maintain intracellular pH [26] which is reflected as a decrease in growth. Titers of BA and HA (Figure 3C) were similar at pH 6.5 and 6.0 and lower at 5.5, reflecting incomplete glucose utilization at the lower pH. It has been also demonstrated that higher pH enhances titers and productivity due to lower concentrations of protonated acids [21]. Protonated acids in the fermentation broth can diffuse into the cells, decreasing the intracellular pH and causing microbial stress. Although the microbe is equipped to handle excess protons, the cells may not have the capacity to expel the conjugate anion, which can accumulate to toxic levels [36,37]. Carboxylic acid and molar carbon yields were similar for the three cases, ranging between 0.34–0.36 g/g and 0.51–0.55 mol/mol respectively (Table 2). In addition, these fermentations showed a carbon mass balance over 90%, accounting for most of the glucose conversion products.

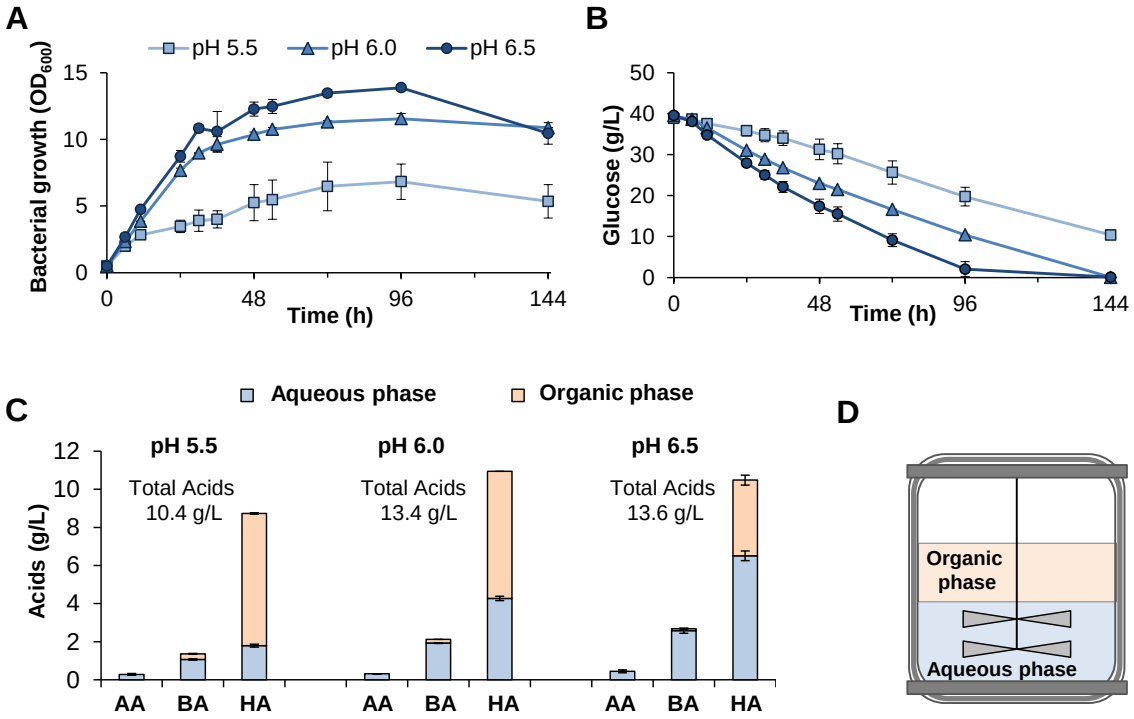

Figure 3. Production of carboxylic acids from glucose by *M. elsdenii* in a liquid–liquid extractive fermentation setup maintained at pH 5.5, 6.0, and 6.5: (**A**) bacterial growth; (**B**) glucose consumption; (**C**) acid concentration in the fermentor (aqueous phase) and extractive solvent (organic phase); and (**D**) representation of the liquid–liquid extractive set up. Results are duplicate experiments and error bars represent the data range. Dry cell biomass (g/L) can be calculated by multiplying the OD_{600} by the factor 0.261 g/L/OD. AA: acetic acid, BA: butyric acid; HA: hexanoic acid.

Table 2. Summary of end-point fermentation parameters for the production of volatile fatty acids (VFAs) by *M. elsdenii* in the different fermentation experiments presented in the current study. Results are duplicate experiments and error represents the data range.

Condition	Total Titer [1] (g/L)	Total Yield [1] (g/g)	AA Yield [2] (g/g)	BA Yield (g/g)	HA Yield (g/g)	Productivity [1,3] (g/L/h)	Carbon Yield [1] (mol/mol)	CMB [4] (%)
Liquid–liquid batch pH 5.5	10.4 ± 0.1	0.36 ± 0.00	0.01 ± 0.00	0.05 ± 0.00	0.31 ± 0.00	0.07 ± 0.00	0.55 ± 0.01	94 ± 1
Liquid–liquid batch pH 6.0	13.4 ± 0.2	0.34 ± 0.01	0.01 ± 0.00	0.05 ± 0.00	0.28 ± 0.01	0.09 ± 0.00	0.51 ± 0.01	92 ± 1
Liquid–liquid batch pH 6.5	13.6 ± 0.2	0.35 ± 0.01	0.01 ± 0.00	0.07 ± 0.01	0.27 ± 0.00	0.09 ± 0.00	0.52 ± 0.01	92 ± 1
Pertractive Fed-batch	61.3 ± 3.6	0.32 ± 0.01	0.02 ± 0.00	0.19 ± 0.01	0.11 ± 0.00	0.26 ± 0.01	0.63 ± 0.02	98 ± 1
Glucose [5]	5.2 ± 0.3	0.44 ± 0.00	0.01 ± 0.01	0.15 ± 0.01	0.28 ± 0.02	0.03 ± 0.00	0.65 ± 0.01	103 ± 1
DDA [5]	6.1 ± 0.0	0.43 ± 0.02	−0.02 ± 0.00	0.16 ± 0.01	0.30 ± 0.01	0.04 ± 0.00	0.65 ± 0.02	104 ± 2
DMR [5]	5.3 ± 0.01	0.37 ± 0.03	−0.02 ± 0.02	0.11 ± 0.00	0.27 ± 0.00	0.04 ± 0.00	0.56 ± 0.03	91 ± 3
Extracted Glucose [5]	16.9 ± 0.1	0.44 ± 0.00	0.01 ± 0.00	0.11 ± 0.01	0.32 ± 0.01	0.10 ± 0.00	0.65 ± 0.00	108 ± 0
Extracted DDA [5]	17.8 ± 0.4	0.42 ± 0.01	−0.01 ± 0.01	0.10 ± 0.00	0.33 ± 0.00	0.10 ± 0.00	0.63 ± 0.01	103 ± 1
Extracted DMR [5]	18.1 ± 0.1	0.44 ± 0.04	0.03 ± 0.00	0.10 ± 0.03	0.32 ± 0.02	0.10 ± 0.00	0.66 ± 0.06	115 ± 7

[1] These parameters account for AA, BA, and HA in aqueous and/or organic fractions; [2] Negative values indicate greater acetate utilization than production; [3] Productivity is calculated at the end of the experiment, irrespective of the time of complete sugar utilization; [4] Carbon mass balance (CMB) accounts for the carbon in acids produced, cell biomass, and a theoretical production of 33% CO_2; [5] These fermentations were performed in serum bottles.

Acid extraction was most efficient at pH 5.5 although still 30% of the carboxylic acids remained in the aqueous phase. Extraction efficiency was progressively worse at higher pH with 49% and 70% carboxylic acids remaining in the broth at pH 6.0 and 6.5, respectively. Furthermore, the high water solubility of BA may have a negative effect on its extraction relative to the less water soluble HA [20].

Although accumulation of BA in the broth would favor chain elongation, limits exist where increasing concentrations would become inhibitory to the organism. These results show that a liquid–liquid extraction process (Figure 3D) is not sufficient to provide high recoveries of extracted acids at an optimal pH for bacterial growth. Therefore, a different fermentation strategy, namely fed-batch pertractive fermentation, was further investigated.

3.3. Fed-Batch Pertractive Fermentation

During liquid–liquid extractive fermentation (Figure 3D), as acid production and extraction proceeds, the organic phase becomes increasingly loaded with acids and the extraction efficiency decreases due to a loss in driving force. Increasing the amount of organic solvent could resolve this issue; however, pertractive fermentation, where the organic solvent is back-stripped with a high pH aqueous phase to remove the acids, will alleviate acid buildup in the organic extractant. The pertractive fermentation system employed in this work (Figure 1) consists of two porous polypropylene membranes that separate the organic (oleyl alcohol + 10% TOA) phase from the aqueous fermentation broth and back stripping (NaOH) phases. Within the pores of the membrane, the two liquid phases are in contact and extraction occurs.

Pertractive fermentation (a means of recovering the product in base as a result of product stripping from the organic solvent) has been used to improve productivity and to mitigate solvent toxicity for the production of BA and propionic acids [11,30] as well as HA from mixed culture fermentations [25,38]. The current work is a demonstration of this technique for the production and extraction of both BA and HA from *M. elsdenii* fermentation. Considering the results presented in Figure 3, the pH selected for these fermentations was 6.3, prioritizing VFA productivity over extraction efficiency. The fermentation was started in a batch mode, containing 40 g/L of glucose. After the glucose concentration dropped below 10 g/L (~36 h), glucose was maintained at 5–10 g/L by continuous feeding. The feed rate was adjusted, as necessary, to maintain this concentration. The glucose utilization rate was 0.42 g/L/h at 23 h. From 23 to 59 h, a period where cells reached a maximal OD_{600}, the glucose utilization rates increased to 1.33 g/L/h. At 59 h, there was an inflection, where cells reached a stationary phase and glucose utilization rates decreased to 0.76 g/L/h (Figure 4).

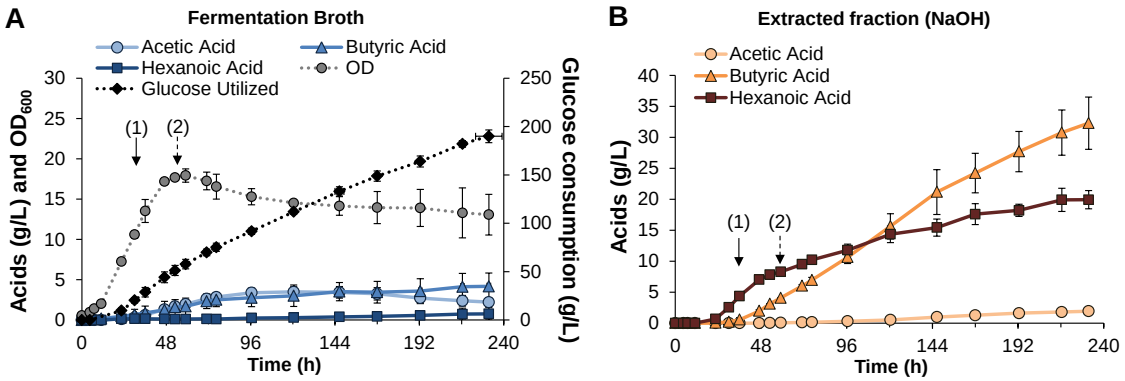

Figure 4. Fed-batch pertractive fermentation by *M. elsdenii* from glucose: (**A**) parameters evaluated in fermentation broth such as bacterial growth, glucose concentration, and acid concentration; (**B**) acid concentration in NaOH from both the organic and NaOH fraction. It is worth noting that only 0.7 and 3.0 g/L of BA and HA, respectively, remain in the organic phase at the end of the fermentation. Experiments were run in duplicate and error bars represent the data range. For samples not taken at the precise same time in the duplicate, time was averaged. Horizontal error bars on the glucose data series show the time data range. Continuous arrows show (1) the end of the batch phase and beginning of the continuous feeding and discontinuous arrow (2) the time point which corresponds to the inflection mentioned above. Dry cell biomass (g/L) can be calculated by multiplying the OD_{600} by the factor 0.261 g/L/OD.

The HA production rate also occurred in three distinct phases corresponding to the glucose utilization rates. During the first 23 h, HA was practically absent, but from 23 to 59 h, the productivity increased to 0.19 g/L/h. From that point to the end of the fermentation, HA production decreased to 0.07 g/L/h. Production of BA lagged about 12 h behind HA production but then remained relatively steady at a production rate of ~0.16 g/L/h. These results suggest that maximum HA production is associated with cell growth and that production declines when the cells enter stationary phase. In contrast, BA production is independent of the phase of the bacteria. Overall, 32 g/L of BA and 20 of g/L HA were recovered in NaOH; in addition, 0.7 and 3.0 g/L of BA and HA, respectively remained in the organic phase. Only 4.5 g/L of BA and 0.7 g/L HA remained in the fermentor broth at the end of the fermentation. AA was only a minor product reaching 2.0 and 0.5 g/L in the NaOH and fermentor broth fractions, respectively. Considering AA, BA, and HA recovered in all fractions, titers of 61.3 g/L and productivities of 0.26 g/L/h were obtained, which in total, are the highest values reported for *M. elsdenii* to our knowledge on glucose.

Although HA was mostly extracted, BA and AA accumulated in the fermentation broth at low levels, requiring neutralization to maintain the pH at 6.3. Other publications noted that the pH would autoregulate around this value due to increased extraction efficiency at lower pH [20]. This was not the case in the current work. However, it is worth noting that a previous single pertractive experiment was run without pH control and the results were similar to those presented here. In this fermentation, the initial pH was 6.5 and dropped to around 5.6 at 80 h before slowly rising to about 6.1 at 200 h. This fermentation produced 3.1, 31.2 and 20.8 g/L of AA, BA and HA respectively for a total of 55.2 g/L acids and a productivity of 0.26 g/L/h.

Carboxylic acid yields were similar to those found in batch experiments (0.32 g/g) as well as the high closure of the carbon mass balance at 98% (Table 2). Carbon yields from carboxylic acids were higher (63%) (mol/mol) for the pertractive experiment than batch experiments, partly due to more glucose being consumed by a similar amount of biomass produced.

3.4. Fermentation of Biomass Sugars with and without Acid Extraction

Subsequently, the performance of *M. elsdenii* on two relevant biomass hydrolysates in the presence and the absence of organic solvent was evaluated to understand if there was any toxicity associated with the hydrolysate. It is worth noting that xylose is a major component of these sugars streams and the *M. elsdenii* strain employed here cannot utilize xylose or other pentose sugars. Regardless, it is important to demonstrate that *M. elsdenii* can tolerate these streams since they have been reported to be toxic for other organisms due to the presence of potential bacterial inhibitors such as acetate, furfural, and phenolic compounds [39]. Considering that xylose is not utilized by this bacterium, a batch experiment in serum bottles was performed; in a fed-batch pertractive fermentation, xylose would accumulate to high concentrations in the fermentor and may present an extra inhibitory effect in the bacterium due to increased osmotic stress.

3.4.1. Non-Extracted Fermentation of Biomass Sugars

Fermentation performance on pure glucose, DDA, and DMR hydrolysates, all at initial glucose concentrations of 40 g/L, was compared. Without extraction, DDA and DMR sugars compared favorably to pure glucose and exhibited similar glucose utilization (11–12 g/L), cell growth, and BA + HA production (4.8, 5.5, and 4.9 g/L for pure glucose, DDA and DMR, respectively) (Figure 5A–C; Table 2). As a result of bacterial contamination during biomass storage and processing, DMR hydrolysates also contained a small amount of lactic acid (1.7 g/L). It is known that *M. elsdenii* can utilize lactic acid to produce propionic, BA, and valeric acids [40]. Lactic acid was included with glucose in the yield calculation, and propionic or valeric acids were not detected. Similar to lactic acid in DMR, both DDA and DMR hydrolysates contain varying levels of AA, which *M. elsdenii* can use for the production of BA and HA [20]. Acetate and lactate utilization were clearly observed during the first 12 h of fermentation in both DDA and DMR as well as a concomitant initial burst of BA

production (Figure 5B,C). For clarity, lactate was omitted from the figures. This result suggests rapid lactate utilization (from DMR) and/or chain elongation from AA to BA (from both DDA and DMR). This burst of BA does not occur in pure glucose fermentation, which lacks any initial lactate or acetate (Figure 5A). As expected, the pure glucose fermentation stalled at 72 h due to a combination of product accumulation and decrease in pH, while DDA and DMR fermentations were slower and continued over 100 h. These experiments presented the highest yields in this work, in particular, the fermentation in DMR (0.47 g/g); however, productivities in these conditions were very low (Table 2).

3.4.2. Extracted Fermentation of Biomass Sugars

In contrast to the previous fermentations, extraction resulted in essentially complete glucose utilization for all the conditions (Figure 5D–F). However, with extraction, initial cell growth was delayed compared to the previous set of experiments (Figure 5A–C). Nevertheless, maximum cell densities were 4–6 fold higher than with no extraction. Glucose utilization rates for the pure glucose condition were faster, and all of the glucose was consumed in ~80 h, while the DDA and DMR conditions required more than 100 h. Total BA + HA titers (from both aqueous and organic phases) were 16.45, 16.8, and 17.0 g/L for pure glucose, DDA, and DMR, respectively (Table 2). In this experiment, the accumulation of BA and HA was again observed in the aqueous phase although the extraction was even more efficient than that observed in the batch liquid–liquid fermentations (Figure 3), both starting at pH 6.5. Since no pH control was used in this experiment, this is likely the result of increased extraction efficiency at lower pH. No propionic or valeric acids were detected and the net AA produced was negative for DDA, probably due to the utilization of this compound to produce BA as previously noted. Carboxylic acid yields were again higher than the observed in the fermentors, around 0.44 g/g (Table 2) and carbon mass balances were all slightly over 100%. In this case, BA and HA productivities equaled those observed in the batch liquid–liquid fermentations, reaching 0.10 g/L/h (Table 2).

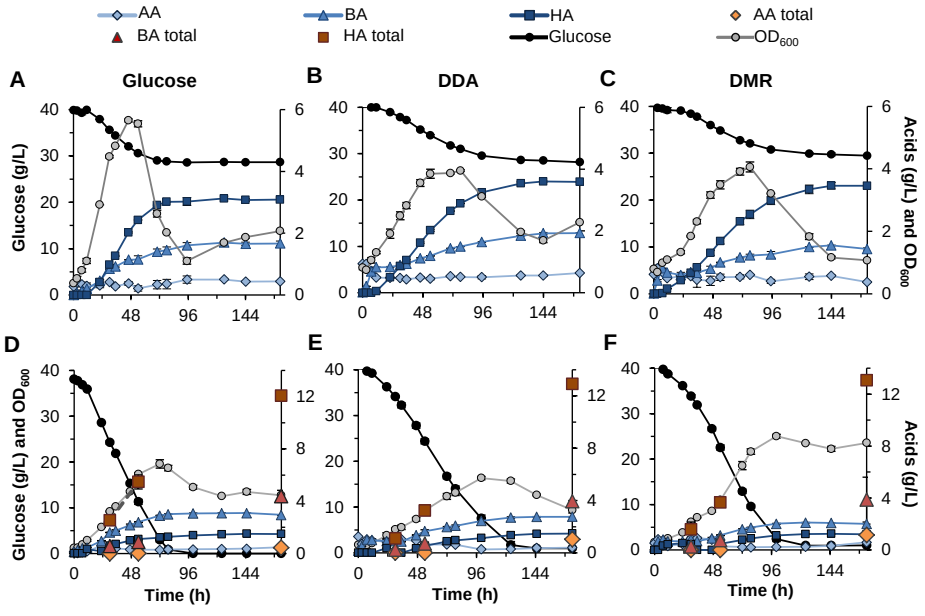

Figure 5. Glucose fermentations by *M. elsdenii* from different carbon sources in serum bottle experiments. Fermentations were performed in (**A**–**C**) the absence and (**D**–**F**) the presence of oleyl alcohol plus 10% TOA. Carbon sources consisted of (**A**,**D**) pure glucose, (**B**,**E**) DDA hydrolysate, and (**C**,**F**) DMR hydrolysate. Lines with filled markers present the variables analyzed in the aqueous fraction. Empty markers show AA, BA, and HA total concentration in both aqueous and organic phases. Experiments were run in duplicate and error bars represent the data range. Dry cell biomass (g/L) can be calculated by multiplying the OD_{600} by the factor 0.261 g/L/OD.

4. Discussion

The anaerobic production of VFAs from lignocellulosic hydrolysates is a promising route to generate biofuel precursors. However, due to the toxicity of these compounds, the development of extraction methods integrated to the fermentation system is critical to the production process. In addition, the use of extractive solvents would considerably reduce the processing cost associated with a neutralization process (including reducing cost and mitigating sustainability issues with salt generation and wastewater treatment) and would also facilitate downstream processing. In the current work, we evaluated different extractive fermentation strategies for the simultaneous production of BA and HA by *M. elsdenii* from glucose and corn stover hydrolysate.

The effects of diverse solvents on the *M. elsdenii* fitness were tested (Figure 2). Octanol was more toxic than oleyl alcohol and THA more toxic than TOA. Similar findings for octanol toxicity have been previously reported for the ethanologen *Zymomonas mobilis* [41]. Although oleyl alcohol plus TOA improved bacterial growth in liquid–liquid extractive fermentations, we cannot definitively state that this mixture does not present any negative, toxic effect on the cells. Since acid extraction clearly provides a positive effect, this could possibly mask a mild toxicity. If bacterial growth is compared between liquid–liquid extraction experiments from serum bottles (Figure 2) and fermentors (Figure 3), one can observe that the maximal OD_{600} are 25 and 15, respectively. It is possible that the increased salinity from the addition of NaOH used to control the pH might osmotically stress the cells. In the current work, the OD_{600} obtained in the pertractive fermentation was close to 20, which also does not achieve the value of 25 observed in the serum bottles. Again, this could be a result of the reduced addition of NaOH used to control pH. It is also worth noting that in the pertractive system, the cells might be subjected to additional stress, possibly due to shear or transient temperature changes as a result of recirculation of the fermentor broth through the system.

Extraction efficiency and bacterial growth are both highly dependent on the pH, but as observed in the current work, these important fermentation metrics are also a function of the fermentation configuration. For instance, BA was hardly extracted in the liquid–liquid system (Figure 3), in contrast to the efficient extraction observed in the pertractive bioreactor configuration (Figure 4). Similarly, the extraction efficiency of HA was also enhanced in the latter system. These results highlight the advantage of some means of backstripping the acids from the organic solvent, which improves extraction efficiency by preventing acid saturation of the organic phase. Apart from this advantage, the possibility of utilizing high pH in the pertractive system also favors higher titers and productivity, which are critical fermentation parameters for industrial use.

The titers reached in the current work are among the highest reported to date when considering mixed BA (36.5 g/L) and HA (20.7 g/L) production by *M. elsdenii*. Roddick et al. [21] reported BA and HA titers of 3.4 and 19 g/L respectively, from glucose by *M. elsdenii* in a fed-batch system, with cells inmobilized and using an anion exchange resin. Choi et al. [20] reached a combined BA and HA titer of 32.0 g/L from sucrose in liquid–liquid extractive fermentation also by *M. elsdenii*. Bacterial co-cultures and mono-culture fermentations of *C. kluyveri* using acetate and ethanol as carbon sources produced HA titers of < 10 and 12.8 g/L, respectively [13,15,18,19] and *Clostridium* sp. BS-1 has been reported to produce up to 32 g/L of HA in 16 days from galactitol. However, as the authors state, *Clostridium* sp. BS-1 displays poor glucose utilization, which would need to be improved if lignocellulosic sugars are to be used [14].

Interestingly, the maximum yield values were obtained in the last experiment from both glucose and biomass hydrolysates (0.42–0.47 g/g), compared to the lowest yield of 0.32 g/g obtained in the pertractive system. Overall, the production of acids was higher in the biomass experiments, including the pure sugar controls. This can be seen in the carbon mass balance values, in most cases, being near or slightly above 100%. Unfortunately, no apparent explanation was found for these acid yield increases but it is noted that one of the main differences in this experiment was the use of serum bottles instead of bioreactors. In the current work, the carbon balance was essentially closed when BA and HA were considered as the major products. It has been also reported that *M. elsdenii* T81

accumulated considerable amounts of glycogen from glucose [40]. Although not measured in this work, this type of intracellular accumulation could be a carbon sink, reducing yields. The maximum HA yield reported in literature is 0.5 g HA/g sucrose, which would be very close to the maximum yields for homo-BA fermentations from glucose (0.49 g BA/g glucose) [12]. However, reported yields in *M. elsdenii* from glucose have varied from 0.27 to 0.39 [21], which are closer to our results.

It has been also demonstrated that productivities were considerably lower in batch extractive fermentations than in the pertractive system (Table 2). This is likely due to a combination of reduced product feedback inhibition, lower salinity from product neutralization, and reduced solvent toxicity in the pertractive system. Comparing glucose utilization rates at maximal OD time points, batch extractive and pertractive fermentations at pH 6.5 yielded values of 0.39 and 0.97 g/L/h respectively. This behavior is reflected in carboxylic acids productivities where ~3-fold higher values were obtained in the pertractive system (0.26 g/L/h) compared to batch extractive (0.09 g/L/h). The pertractive productivity value in this work is the highest reported for the simultaneous production of VFAs using a mono-culture organism from sugars, greater than those obtained from sucrose 0.23 g/L/h [20] and glucose 0.13 g/L/h [21] considering both BA and HA or from acetate and ethanol 0.18 g/L/h [13] and galactitol 0.08 g/L/h [14] for exclusive HA production.

The production of some carboxylic acids from lignocellulosic substrates, such as BA and propionic acid, has been reported [12,42]. However, the production of HA from lignocellulosic sugars by a bacterial mono-culture has not been reported previously to our knowledge. Furthermore, some benefits have been found when using biomass hydrolysates compared to glucose. For instance, the presence of additional acids from biomass deconstruction, such as lactic and AA, which are inherent of some biomass hydrolysates and inhibitors for some organisms, enhance VFA production. BA and HA productivity from hydrolysates decreased compared to pure glucose fermentations since the biomass hydrolysates also contain other inhibitors (e.g., furfural, 5-hydroxymethylfurfural) that the bacterium likely needs to detoxify. The detoxification of furfural to furfuryl-alcohol has been demonstrated in other organisms while growing in anaerobic conditions [43–45]. Another interesting observation was that the HA:BA ratio increased in hydrolysates (1.73 for DDA and 2.0 for DMR, respectively) compared to glucose (1.54). However, as observed in the pertractive fermentation sytem, this ratio might change over the time. Overall, *M. elsdenii* is a robust organism for producing VFAs from biomass hydrolysates.

5. Conclusions

The efficient and simultaneous production of BA and HA by *M. elsdenii* from glucose and corn stover hydrolysates has been demonstrated in the current work. However, presently, this organism cannot utilize xylose, which is one of the main drawbacks for further lignocellulose conversion. Despite the lack of genetic tools for *M. elsdenii*, further efforts to engineer xylose and other minor sugar utilization pathways could elevate this organism as a potential platform host for the production of VFAs from lignocellulosic sugars. In addition, we acknowledge that the media utilized in the current study is rich. Thus, media development will be also critical to decrease costs and improve the economics of the process. Furthermore, cell immobilization may be another means to divert more carbon from cell biomass to product to increase metabolic yields as well as to avoid transient changes in cell recirculation systems.

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