Production of medium-chain volatile fatty acids by mixed ruminal microorganisms is enhanced by ethanol in co-culture with Clostridium kluyveri

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**Highlights**

- Clostridium kluyveri 3231B grew well with mixed ruminal bacteria in vitro.
- Co-cultures fermented mixtures of cellulosic biomass and ethanol to C4–C6 VFAs.
- Co-cultures produced up to 6.1 g caproate and 1.6 g valerate L\textsuperscript{-1} in 48 h at 39 °C.
- This VFA production system represents a useful extension of the carboxylate platform.

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**Abstract**

Mixed bacterial communities from the rumen ferment cellulosic biomass primarily to C2–C4 volatile fatty acids, and perform only limited chain extension to produce C5 (valeric) and C6 (caproic) acids. The aim of this study was to increase production of caproate and valerate in short-term in vitro incubations. Co-culture of mixed ruminal microbes with a rumen-derived strain of the bacterium Clostridium kluyveri converted cellulosic biomass (alfalfa stems or switchgrass herbage) plus ethanol to VFA mixtures that include valeric and caproic acids as the major fermentation products over a 48–72 h run time. Concentrations of caproate reached 6.1 g L\textsuperscript{-1}, similar to or greater than those reported in most conventional carboxylate fermentations that employ substantially longer run times.

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**1. Introduction**

The carboxylate platform for biofuels production is a means of converting organic wastes and biomass to organic acids, particularly volatile fatty acids (VFA) using undefined mixed cultures of anaerobic microorganisms typically derived from sewage sludge digesters or aquatic sediments (Agler et al., 2011; Chang et al., 2010). In the carboxylate platform, a conventional anaerobic digestion process is altered via pH control or addition of methanogenic inhibitors, upon which accumulating C2–C4 VFA are extended to medium chain VFA (C5–C8) via reverse β-oxidation (RBO) reactions. The medium chain VFA can be recovered by extraction or other methods (Singhania et al., 2013) and converted by catalytic chemistry or electrochemistry to various liquid fuels or industrial chemicals. Examples of potential products include ketones and secondary alcohols produced in the MixAlco process (Holtzapple and Granda, 2009); esters by a process analogous to the valeric biofuels platform of Lange et al. (2010); or hydrocarbons and alcohols produced by Kolbe or Hoefer–Moest electrolysis (Levy et al., 1983). The carboxylate platform has several attractive features, including feedstock flexibility; minimal feedstock pretreatment; utilization of most organic components of the biomass (carbohydrate, protein, nucleic acid, organic acids and some lipids); non-aseptic operation; and mixed culture stability. The main limitation of the biological (fermentation) component of the carboxylate platform based on anaerobic digester communities is the slow rate of VFA chain extension (incubation times of weeks).

As an alternative to the above configuration, biomass may be fermented in bioreactors using mixed cultures of microbes from the rumen. These “extraruminal” fermentations have some additional advantages over the originally described carboxylate platform (Weimer et al., 2009). Fermentation run times are...
considerably shorter due to a more active fermentation of plant structural polysaccharides and production of VFA as natural end products (rather than intermediates) of the fermentation. In the rumen, accumulation of VFA to concentrations of ~0.15 M occurs during a relatively short (1–3 d) retention time, which prevents the establishment of slow-growing species such as proton-reducing acetogens (which convert C3–C6 VFA to acetate) and aceticlastic methanogens (which convert acetate to methane). As a result, methane is produced exclusively by coupling H2 oxidation to CO2 reduction (Ungerfeld and Kohn, 2006; Weimer et al., 2009), and methane represents only 8–15% of the energy content of the fermentation products (Weimer et al., 2009), versus 90% or more in conventional anaerobic digestion and 0% in a “stuck” anaerobic digestion.

Ruminal fermentations of forages and other cellulosic substrates typically yield acetate (C2), propionate (C3) and butyrate (C4) in molar ratios of ~6:2:1, and produce only minor amounts of medium chain VFA. The shorter chain VFA are more difficult to extract than are the medium chain VFA (Singhania et al., 2013). Furthermore, upon chemical conversion the shorter chain VFA yield lower molecular weight fuels rather than higher molecular weight, more energy-dense products that can be formed from the medium chain VFA. Thus from a biofuels production standpoint, there is incentive to modify the extraruminal fermentation to shift VFA production toward longer chain VFA products without sacrificing its naturally rapid fermentation rate. The essential challenge, then, is to effectively couple rapid biomass fermentation by the mixed ruminal microbial culture to rapid VFA chain extension via reverse β-oxidation (RBO) reactions that define the carboxylate platform.

Ethanol, the most abundantly produced biofuel, is typically derived from fermentation of starches or sugars in grain crops, and its commercial production from cellulosic biomass is only in its nascent stages. A substantial number of cellulosic ethanol studies have been carried out at laboratory scale. Some of these fermentations have industrially appealing features (e.g., direct fermentation of biomass without added enzymes via “consolidated bioprocessing” (Lynd et al., 2002)), but often produce ethanol at concentrations below the threshold required for cost-effective recovery (e.g., by distillation). As pointed out by Kenealy et al. (1995) and more recently by others (Steinbusch et al., 2011; Agler et al., 2012; Grootscholten et al., 2013; Vasudevan et al., 2014), addition of such dilute ethanol solutions to mixed culture organic acid fermentations of biomass material (i.e., carbohydrate fermentations) favors production of medium chain VFA. Thus these VFA retain the fuel value of ethanol in a more easily recovered form. Most studies demonstrating combined conversions of biomass and ethanol to higher VFA have been conducted with microbial inocula from static or low turnover time habitats such as sewage digesters or sediments, and the resulting production rates of caproic acid (C6) have been relatively modest, with rather long incubation times. This report describes the conversion of mixtures of cellulosic biomass and ethanol to caproic acid at high yield and concentration in short term (48–72 h) fermentations by mixed cultures of ruminal bacteria supplemented with a ruminally derived strain of the ethanol-utilizing RBO bacterium Clostridium kluyveri.

2. Methods

2.1. Microorganisms

Ruminal contents were obtained from two non-lactating ruminally fistulated Holstein cows, and were processed as described previously (Weimer, 2011) to obtain the mixed ruminal microflora inoculum. The cows were maintained under protocol A1104 approved by the University of Wisconsin College of Agricultural and Life Sciences Animal Care and Use Committee. The ruminal isolate C. kluyveri 3231B was grown on EASY medium under a CO2 gas phase, without the addition of chemical reducing agents, as described previously (Weimer and Stevenson, 2012).

2.2. Substrates

Biomass samples were obtained from harvests of late-maturity switchgrass (Panicum virgatum L) whole herbage or third-cut alfalfa (Medicago sativa L) stems grown at the University of Wisconsin Arlington Agricultural Research Station. Air-dried biomass materials were ground through a 1 mm screen in a Wiley mill, but were not otherwise pretreated prior to use.

2.3. Fermentations

All experiments were carried out under a CO2 gas phase, in glass serum vials (~160 mL) that were volume-calibrated to 0.01 mL. Vessels were sealed with butyl stoppers and aluminum crimp seals. Unless indicated otherwise, culture vessels were incubated at 39 °C without shaking. Except for growth of C. kluyveri in pure culture for use in augmentation experiments, all experiments were conducted under non-aerobic conditions with unsterilized medium (Goering and Van Soest, 1970) and culture vessels, without pH control.

2.4. Analyses

Headspace H2 and CH4 were determined by gas chromatography as previously described (Weimer, 2011). Soluble fermentation end products (VFA, ethanol, lactate and succinate) were determined by HPLC following treatment of culture supernatants with Ca(OH)2 and CuSO4, followed by precipitation of excess Ca++ with H2SO4 (Weimer et al., 1991). The refractive index detector of the HPLC provided a linear response for ethanol up to at least 0.8 M, and for all other products up to at least 0.2 M, except for caproic acid, for which insolubility issues precluded accurate measurement at concentrations above 0.15 M. Data were analyzed using PROC MIXED in SAS 9.4 (SAS, Inc., Cary, NC).

Two metrics were used to compare cultures with respect to the shift in the amounts and relative proportions of VFA products:

(1) Total mM alkyl groups, the sum of all methyl and methylene groups in VFA, i.e., total mM alkyl groups = C2 + 2(C3) + 3(C4) + 4(C5) + 5(C6), where C6 corresponds to mM concentration of VFA of total carbon number 6; and

(2) Average VFA chain length = (total mM alkyl groups/total mM VFA) +1, where the 1 represents the non-energetic carbon (carboxyl group) of the VFA molecule.

3. Results and discussion

Fermentations of switchgrass (Table 1) by mixed ruminal microflora yielded the expected predominance of acetate, with successively smaller amounts (on a molar basis) of propionate, butyrate, valerate and caproate. Average VFA chain length (ACL) of these fermentations was ~2.7. Addition of ethanol to the biomass fermentations did not appreciably increase the amounts of VFA produced or the ACL over the short incubation times of the fermentations, and essentially no ethanol was consumed. However, combined addition of ethanol and C. kluyveri resulted in a vigorous conversion of ethanol and the shorter VFA (acetate and propionate) to medium chain VFA. The concentrations of butyrate, valerate and caproate after 72 h reached 32, 16 and 42 mM (2.8, 1.6 and 4.9 g L−1), respectively, and ACL of the VFAs exceeded 4.5. 
Fermentations of switchgrass and/or ethanol by mixed ruminal microflora with or without added Clostridium kluyveri 3231B.

<table>
<thead>
<tr>
<th>Culture</th>
<th>Contenta</th>
<th>Concentrations (mM) of substrates and products after 72 h b</th>
<th>mM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ethanol</td>
<td>Acetate</td>
<td>Propionate</td>
</tr>
<tr>
<td>A</td>
<td>No added</td>
<td>0a</td>
<td>37.1b</td>
</tr>
<tr>
<td>B</td>
<td>SWG</td>
<td>0a</td>
<td>57.5a</td>
</tr>
<tr>
<td>C</td>
<td>SWG + ETOH</td>
<td>220.6a</td>
<td>74.8a</td>
</tr>
<tr>
<td>D</td>
<td>SWG + ETOH+ Ck</td>
<td>24.1w</td>
<td>43.7w</td>
</tr>
<tr>
<td>E</td>
<td>ETOH</td>
<td>229.6w</td>
<td>43.3w</td>
</tr>
<tr>
<td>F</td>
<td>ETOH + Ck</td>
<td>74.5x</td>
<td>27.8x</td>
</tr>
<tr>
<td></td>
<td>S.E.D e</td>
<td>9.4a</td>
<td>1.8a</td>
</tr>
</tbody>
</table>

Carbon balance for C. kluyveri:
+SWG (culture D–culture C) Ethanol: −196.5 mM = −393.0 mM C Butyrate: 31.9 mM = 127.6 mM C
+SWG (culture D–culture C) Acetate: −31.1 mM = −62.2 mM C Valerate: 12.5 mM = 62.5 mM C
+SWG (culture D–culture C) Propionate: −14.8 mM = −44.4 mM C Caproate: 41.9 mM = 251.4 mM C
−SWG (culture F–culture E) Ethanol: −154.5 mM = −309.0 mM C Butyrate: 27.4 mM = 109.6 mM C
−SWG (culture F–culture E) Acetate: −15.5 mM = −31.0 mM C Valerate: 8.3 mM = 41.5 mM C
−SWG (culture F–culture E) Propionate: −9.0 mM = −18.0 mM C Caproate: 32.6 mM = 195.6 mM C

Calculations of net consumption of ethanol, acetate and propionate, along with net production of the C4–C6 acids (Table 1), suggest that net product formation accounted for nearly all net substrate consumption.

Fermentations of alfalfa stems (Table 2) in the presence of ethanol by mixed ruminal microbes yielded small amounts of valerate and caproate, but the amounts were increased dramatically when the culture was augmented with C. kluyveri. As in the case of the switchgrass fermentations, C4–C6 VFA production was substantially higher when both substrates (biomass plus ethanol) were added than when ethanol alone was added, reflecting the conversion of the biomass to the shorter chain VFA that serve as electron acceptors for the RBO pathway. In the alfalfa stem/ethanol fermentation, the total concentration of medium chain VFA was greater after 48 h than in the 72 h fermentations with switchgrass/ethanol, which probably reflects a greater digestibility of the alfalfa stems relative to the late-maturity switchgrass (as neither biomass was subjected to pretreatment prior to fermentation). The concentrations of butyrate, valerate and caproate after 48 h reached 26, 15 and 52 mM (2.3, 1.6 and 6.1 g L−1), respectively. VFA production resulted in a decrease in culture pH from an initial value of 6.8 to a value of 5.7–5.9 after 48 h incubation.

Ruminal fermentations have long been known to proceed rapidly in vitro (i.e., extraruminally) to produce total VFA concentrations of ~0.15 M, but a major limitation for their use in the carboxylate platform is the relatively low carbon number (expressed as an average chain length) of the VFA products. Although the molar proportion of C5–C6 VFA in typical in vivo ruminal fermentations of feeds is only a few per cent, there have been reports of higher yields. Ørskov et al. (1967) observed caproate molar proportions of up to 15 per cent and concentrations of up to 18 mM in ruminal samples from cows fed rations high in sugar and starch (21.8 per cent each on a feed dry matter basis); in these same cows, ethanol was detected within 2 h of feeding, and the authors speculated that C. kluyveri was a likely agent for caproate production, based on its known ability to couple ethanol oxidation to reductive RBO. Moomaw and Hungate (1963) and Allison et al. (1964) had also observed ethanol accumulation in the rumen, but again only under conditions of feeding diets rich in concentrates. To our knowledge there are no reports of significant ethanol accumulation in ruminants fed diets high in forages or other cellulosic materials that would likely be used in a cellulosic biofuels system. With regard to in vitro fermentations, Kenealy et al. (1995) demonstrated that defined mixed cultures of the ruminal cellulolytic bacteria Fibrobacter succinogenes or Ruminococcus flavefaciens (neither of which produce ethanol) with C. kluyveri ATCC 8527 (originally isolated from aquatic sediment) converted added cellulose and ethanol to a maximum of 4.6 g caproate L−1. Taken together, both

Table 2
Fermentation of ethanol with or without alfalfa stems by mixed ruminal microflora with or without added Clostridium kluyveri 3231B.

<table>
<thead>
<tr>
<th>Culture</th>
<th>Substrate c</th>
<th>Inoculum d</th>
<th>Concentrations (mM) of substrates and products after 48 h e</th>
<th>mM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ethanol</td>
<td>Acetate</td>
<td>Propionate</td>
<td>Butyrate</td>
</tr>
<tr>
<td>A</td>
<td>Alf + ETOH</td>
<td>MRM</td>
<td>150.6w 50.3v</td>
<td>11.3w 9.5w</td>
</tr>
<tr>
<td>B</td>
<td>Alf + ETOH</td>
<td>MRM + Ck</td>
<td>42.7w 24.1v</td>
<td>3.5w 26.4w</td>
</tr>
<tr>
<td>C</td>
<td>ETOH</td>
<td>MRM</td>
<td>171.4w 25.4v</td>
<td>4.8w 0.7w</td>
</tr>
<tr>
<td>D</td>
<td>ETOH + Ck</td>
<td>MRM</td>
<td>113.5w 14.0w</td>
<td>1.7w 10.3w</td>
</tr>
<tr>
<td></td>
<td>S.E.D f</td>
<td>17.2</td>
<td>0.7</td>
<td>0.2</td>
</tr>
</tbody>
</table>

Mean values within column differ (P < 0.05).

a Alfalfa stems (Alf) added at 9 g/L (dry matter basis). Ethanol (ETOH) added at 225 mM (10.37 g/L).
b All vials received mixed ruminal microflora (MRM) inoculum at 300 mL/L liquid volume. C. kluyveri (Ck, OD525 = 0.50) added at 80 mL/L total liquid volume.
c Values are means from triplicate vials. Protein-derived branched chain VFA not included (see text). Total liquid volume per vial was 29.5 mL.
d Average VFA chain length.
e Pooled standard error of the difference.
f Pooled standard error of the difference.
in vivo and in vitro studies suggested the feasibility of coupling mixed ruminal microflora with C. kluyveri in vitro to produce caproate, as long as a source of ethanol is provided.

In the present study, the rapid extraruminal fermentation of plant biomass to C2–C4 VFA was coupled with the highly efficient, ethanol-mediated elongation of these acids by C. kluyveri 3231B. The ease with which this strain collaborated with the diverse mixed culture from the rumen reflects the fact that the strain was originally isolated from the bovine rumen, and has a relatively high tolerance for both VFA products and low pH (Weimer and Stevenson, 2012). Simple unagitated batch cultures produced caproate at concentrations up to 52 mM (6.1 g L\(^{-1}\)), along with total alkyl carbon concentrations of >0.4 M and an ACL of the VFA products of >4, within relatively short incubation periods (48–72 h). In addition to caproate, both butyrate and valerate accumulated in ethanol-amended cultures. Butyrate is a known intermediate in the production of caproate by C. kluyveri, and valerate was likely produced by RBO reactions involving ethanol as electron donor and propionate as electron acceptor, as demonstrated by Kenealy and Wasielefsky (1985). The total millimolar concentration of butyrate plus valerate was similar to the millimolar concentration of caproate.

Average rates of caproic acid production over the 48 h incubation period reached 3.1 g L\(^{-1}\) d\(^{-1}\). These concentrations and productivities are comparable to or exceed most of those reported in the literature using other undefined mixed culture (“open culture”) systems (Table 3), but were achieved over a shorter incubation period with more recalcitrant biomass feedstocks (viz., ground forages not subjected to chemical pretreatments). The concentrations and productivities reported here are in some cases similar to those of pure culture systems in which substrates were provided at higher concentrations (Weimer and Stevenson, 2012), or fermentation conditions have been optimized to include some combination of pH control and extractive fermentation of accumulating caproate (Roddick and Britz, 1997; Jeon et al., 2013).

The augmented extraruminal fermentations described here were conducted without efforts to inhibit methanogenesis, so all fermentations produced methane as an additional product. For the experiments described above, methane was not quantified. In separate experiments with switchgrass plus ethanol and inoculated with mixed ruminal microflora augmented with C. kluyveri, and in which total VFA after a 72 h incubation averaged 132.4 mM (ACL = 3.48, total VFA C = 460.6 mM), methane averaged 6.8% of the total VFA carbon, on a molar basis. In these latter fermentations, H\(_2\) was below detectable limits. Methanogenesis probably occurred almost exclusively via CO\(_2\) reduction with H\(_2\), as the ruminal microbial community lacks acetoclastic methanogens (Russell, 2002; Zhou et al., 2009), due to the high rate of liquid passage through the rumen. By contrast, reactor microbiomes derived from long-retention time inocula (sewage digesters or sediments) contain both physiological groups of methanogens. While addition of chemical inhibitors such as iodoform (Holtzapple and Granda, 2009) or 2-bromoethanesulfonate (Steinbusch et al., 2011) have been shown to suppress methanogenesis, subsequent VFA chain elongation required long incubation times. Recent trends with inocula from long retention time systems have used low pH (~5.5) to inhibit acetoclastic methanogens, thus decreasing loss of substrate carbon and energy to methane, while still allowing some methane production via CO\(_2\) reduction (Agler et al., 2012; Grootscholten et al., 2013; Vasudevan et al., 2014). The present study confirms those reports that hydrogenotrophic methanogenesis is compatible with reasonable rates of VFA chain elongation. However, in our studies pH was not controlled, and naturally decreased over the course of the batch culture fermentation.

Low concentrations (<4 mM) of branched-chain VFA (isobutyrate, or the chromatographically co-eluting pair isovalerate and 2-methylbutyrate) were detected in all cultures in this study, including inoculated controls without added substrates. The concentrations of these acids did not differ among treatments (data not shown), reflecting their likely origin from the fermentation of tryptcase (present at 2 g L\(^{-1}\) in the Goering/Van Soest medium as a supplementary nitrogen source) by some members of the mixed ruminal inoculum. Consequently, these branched chain acids were excluded from calculations of total alkyl groups and ACL even though they add to the fuel value of the mixed VFA products.

Bioenergetic analysis of VFA formation during the ruminal fermentation has revealed that the process is primarily under

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Inoculum</th>
<th>Time (d)</th>
<th>Maximum caproate, (g L(^{-1}))</th>
<th>Productivity (g L(^{-1}) d(^{-1}))^c</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cellulosic biomass + EtOH</td>
<td>Mixed ruminal microflora + Clostridium kluyveri 3231B</td>
<td>2–3</td>
<td>4.9–6.1</td>
<td>1.7–3.1</td>
<td>This study</td>
</tr>
<tr>
<td>Purified cellulose + EtOH</td>
<td>Defined co-culture of ruminal cellulolytic bacteria + C. kluyveri ATCC 8527</td>
<td>5</td>
<td>4.4</td>
<td>NR</td>
<td>Kenealy et al. (1995)</td>
</tr>
<tr>
<td>Acetate + EtOH (contained 10 g BES/L)</td>
<td>Enriched mixed culture(^e)</td>
<td>115</td>
<td>8.2(^b)</td>
<td>3.0</td>
<td>Steinbusch et al. (2011)</td>
</tr>
<tr>
<td>Organic fraction of municipal solid waste + EtOH</td>
<td>None (^f)</td>
<td>28</td>
<td>2.7</td>
<td>NR</td>
<td>Grootscholten et al. (2013)</td>
</tr>
<tr>
<td>Clostridium ljungdahlii effluent + EtOH</td>
<td>Enriched mixed culture (2-year-old reactor microbe)</td>
<td>25</td>
<td>1.7(^c)</td>
<td>1.7</td>
<td>Vasudevan et al. (2014)</td>
</tr>
<tr>
<td>Yeast fermentation beer containing ethanol</td>
<td>Enriched mixed culture (reactor microbe)</td>
<td>5.5</td>
<td>NR(^d)</td>
<td>2.1(^c)</td>
<td>Agler et al. (2012)</td>
</tr>
<tr>
<td>EtOH + Acetate</td>
<td>C. kluyveri 3231B (pure culture)</td>
<td>3</td>
<td>12.8</td>
<td>4.2</td>
<td>Weimer and Stevenson (2012)</td>
</tr>
<tr>
<td>Glucose</td>
<td>Megasphaera elsdenii ATCC 25940 (pure culture)</td>
<td>5–8.3</td>
<td>2.6–11.4(^d)</td>
<td>0.62–3.2(^d)</td>
<td>Roddick and Britz (1997)</td>
</tr>
<tr>
<td>Galactitol + acetate + butyrate</td>
<td>Clostridium sp. BS-1 (pure culture)</td>
<td>16</td>
<td>6.96–32.0(^d)</td>
<td>0.28–0.34(^d)</td>
<td>Jeon et al. (2013)</td>
</tr>
</tbody>
</table>

\(^a\) Productivity values calculated over the entire incubation period for studies with C. kluyveri 3231B, but reported for active period of caproate production in other studies.

\(^b\) NR = not reported.

\(^c\) BES = 2-bromoethanesulfonic acid, an inhibitor of methanogenesis.

\(^d\) Contained naturally high abundance of C. kluyveri phenotypes.

\(^e\) Also produced small amounts of longer-chain VFA (C\(_7\) and/or C\(_8\)). All mixed culture fermentations produced varying amounts of butyrate and valerate.

\(^f\) Natural inoculum provided by solid waste substrate.

\(^g\) Upper levels obtained with pH control and/or product removal during fermentation.
thermodynamic rather than kinetic control [Ungerfeld and Kohn, 2006]. Observed concentrations and ratios of individual VFA produced from biomass materials both in vivo and in vitro are determined by reactions operating near equilibrium, and thus have only limited ability to produce significant amounts of C4 and C6 VFA. Calculations (Table 4) based on this analytical framework indicate that, under standard conditions (1 atm for gases, 1 M concentrations for all nongaseous substrates, except for H2 at pH 7), formation of butyrate, valerate and caproate from acetate by RBO is less thermodynamically favorable than using ethanol as electron donor than when using H2, the typical ruminal electron donor. However, H2 concentrations both in the rumen and in extraruminal fermentations are typically very low; for example, Ungerfeld and Kohn (2006) selected a value of 0.016 atm for their equilibrium calculations. Because (unlike H2) ethanol can be added to achieve substantial concentrations in solution, RBO reactions using ethanol as donor can have a more favorable ΔG values. Combined with anticipated rapid ethanol oxidation rates at high ethanol concentrations and the robust rates of production of shorter chain VFA (acetate and propionate) from fermentable carbohydrate, the coupling of ethanol oxidation to produce of C4-C6 VFA by RBO in extraruminal fermentations has the potential to proceed very rapidly. These circumstances contribute to the substantial concentrations and production rates of these acids observed in this study.

In fermentations augmented with ethanol and C. kluyveri, some ethanol, acetate and propionate remained in the cultures after 48 h and 72 h incubations with alfalfa stems and switchgrass herbage, respectively. Calculations of free energy changes for production of the C4–C6 acids (from the standard free energy values in Table 4 and the concentrations of substrates and products in Tables 1 and 2) yielded ΔG values of −14.6 to −18.9 kJ. This indicates that the reactions had not reached thermodynamic equilibrium, and suggests that further conversion to the C4–C6 acids was limited by other factors, including incubation time and/or toxicity of these acids at low pH. Product inhibition has also been noted for caproate production in several reports (Agler et al., 2012; Grootsholten et al., 2013; Roddick and Britz, 1997; Vasudevan et al., 2014).

4. Conclusion

The results of this work demonstrate a novel example of the carboxylate platform for upgrading dilute ethanol solutions to easily recoverable medium-chain VFA. Production of VFA (particularly caproic acid and valeric acids) from cellulosic biomass and ethanol was achieved using an inoculum of mixed ruminal bacteria amended with a rumen-derived strain of C. kluyveri. C. kluyveri augmentation resulted in ethanol oxidation coupled to reverse β-oxidation of C2–C4 VFA to yield a mixture of primarily C2–C4 VFA, over short (48–72 h) incubation times, at similar or greater concentrations and productivities observed using most nonruminal mixed cultures at longer incubation times.

Table 4

<table>
<thead>
<tr>
<th>Reaction</th>
<th>ΔG° (kJ/reaction)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 H2 + 2 acetate + H+ → butyrate + 2 H2O</td>
<td>-44.20</td>
</tr>
<tr>
<td>Ethanol + acetate → butyrate + H2O</td>
<td>-37.72</td>
</tr>
<tr>
<td>2 H2 + propionate + acetate + H+ → valerate + 2 H2O</td>
<td>-44.19</td>
</tr>
<tr>
<td>Ethanol + propionate → valerate + H2O</td>
<td>-37.67</td>
</tr>
<tr>
<td>2 H2 + acetate + butyrate + H+ → caproate + 2 H2O</td>
<td>-44.17</td>
</tr>
<tr>
<td>Ethanol + butyrate → caproate + H2O</td>
<td>-37.71</td>
</tr>
</tbody>
</table>

* Calculated from standard free energies of formation of reactants and products. Values corrected to 39°C using the van’t Hoff equation, according to Ungerfeld and Kohn (2006).

Acknowledgements

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References