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Short running title: SCFA to Alcohols by *Thermoanaerobacter*

Biotransformation of organic acids to their corresponding alcohols by *Thermoanaerobacter pseudoethanolicus*

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ABSTRACT

Higher order alcohols, such as 1-butanol and 1-hexanol, have a large number of applications but are currently prepared from non-renewable feedstocks. Here, the ability of *Thermoanaerobacter pseudoethanolicus* to reduce short-chain fatty acids to their corresponding alcohols using reducing potential generated by glucose catabolism with yields between 21.0 and 61.0%. ^{13}C-labelled acetate, 1-propionate and 1-butyrate demonstrates that exogenously added fatty acids are indeed reduced to their corresponding alcohols. This mode of producing primary alcohols from fatty acids using a thermophilic anaerobe opens the door for the production of such alcohols from low-value materials using an inexpensive source of reducing potential.

Organisms within the genera of *Thermoanaerobacter* and *Caldanaerobacter* have broad metabolic capabilities and are noted for their biotechnological potential such as their highly thermotolerant nature and ability to produce biofuels such as ethanol from a broad range of monosaccharides and branched-chain alcohols from branched-chain amino acids [1–5]. Also, *Thermoanaerobacter* species have been exploited for their thermostable enzymes such as xylanases, pectases, and amylases, and the utility of their enzymes in organic synthesis [6–9]. The alcohol dehydrogenases (ADHs) of *Thermoanaerobacter* species have been of particular interest; *Thermoanaerobacter pseudoethanolicus* 39E possess several ADHs, each with varying substrate specificity and cofactor preference [6]. One of the functions of these multiple ADHs seems to be to transfer an electron from NAD(P)H to NAD(P)$^+$ which can be used for the reduction of acetaldehyde to ethanol. The use of *Thermoanaerobacter* (formerly *Thermoanaerobium*) *brockii* ADHs have previously been commercially available and applied to

43 the enantioselective biological reductions of ketones to their corresponding secondary alcohols
44 [8,10,11]. However, the use of thermophilic *Clostridia* has not previously been investigated for
45 their ability to produce higher-order alcohols (C3-C7) from short-chain fatty acids (SCFAs)
46 although the addition of acetate to the fermentation medium is known to stimulate ethanol
47 production [12]. Beyond the production of 1-butanol via the acetone-butanol-ethanol (ABE)
48 process [13,14] and some limited work on the production 1-hexanol from the fermentation of
49 syngas [15–17], there has been little focus on the production of C3 and larger primary alcohols.
50 Historically, such alcohols are prepared using the oxo process or by the reduction of carboxylic
51 acids using strong reducing agents, both of which have substantial drawbacks. There have been
52 recent reports of *Clostridium saccharoperbutylacetonicum* N1-4 to convert 1-butyric acid to 1-
53 butanol in the presence of glucose in the context of ABE fermentations [18,19] as well as by
54 acetogens such as "*Clostridium ragsdaleii"* [20] and engineered *Clostridium autoethanogenum*
55 [21] and in mixed culture with propionate-producing *Clostridium propionicum* and
56 *Alkalibaculum bacchi* [22].
57
58 Recently, *Thermoanaerobacter* strains have been shown to produce branched-chain fatty acids
59 and alcohols during the fermentation of branched-chain amino acids [23–25] although no wild
60 type strains have been reported to produce 1-butanol or higher alcohols as a product of glucose
61 fermentation while the mechanism behind branched-chain alcohols formation from amino acids
62 remains unclear.
63
64 The present study investigates the ability of *Thermoanaerobacter pseudoethanolicus* (DSM
65 2355) to reduce C1-C8 short-chain fatty acids (SCFAs) to their corresponding alcohols using

66 glucose as a carbon source and as a source of reducing potential. *T. pseudoethanolicus* was
67 cultivated anaerobically with nitrogen in the gas phase in Hungate tubes (16x150 mm)
68 containing 8.3 mL of Basal Mineral (BM) medium [26] containing 20 mM of glucose and 20
69 mM of the SCFA. Cultivations were performed at 65°C and pH 7 in triplicate without agitation
70 for 5 days. All materials were purchased from Sigma Aldrich except for ^{13}C-labelled compounds
71 (Cambridge Isotope Laboratories, MA, USA).
72
73 Hydrogen was quantified by gas chromatograph equipped with a thermoconductivity detector
74 while short-chain fatty acids and alcohols were quantified by gas chromatograph with a flame
75 ionization detector as described previously [26]. The products were separated on a
76 nitroterephthalic-acid-modified polyethylene glycol (DB-FFAP, J&W Scientific) 30 m capillary
77 column, 0.32 i.d., 0.25 μm film thickness, using a temperature program of 40°C, hold time 1 min,
78 ramped to 240°C (10°C/min), hold time 10 min and a constant column head space pressure of 9.9
79 kPa. Optical densities were measured after 5 days of cultivation using a Shimadzu UV-1800 at
80 600 nm (l=1 cm). The mass spectrometer ion source was set to 260°C and the interface 250°C,
81 scanning took place once every 0.30 seconds in the range of 40 to 60 m/z. Peaks were identified
82 using the NIST mass spectral database, versions 147 and 27, with an identity threshold cut-off of
83 80. Samples were filtered prior to injection using a 0.45 μm syringe filter. ^{13}C nuclear magnetic
84 resonance (NMR) spectroscopy of fermentations containing ^{13}C1-labelled SCFAs were
85 performed on a Bruker AV400 NMR spectrometer at 298K after spiking with D_2O to obtain a
86 signal lock (0.3 mL addition to 1 mL of aqueous sample).
87

The ability of *T. pseudoethanolicus* to reduce short-chain carboxylic acids was evaluated by cultivation on single SCFAs and 20 mM of glucose and detection of the corresponding alcohol by gas chromatograph with flame ionizing detector. The ability of *T. pseudoethanolicus* to reduce fatty acids in the presence of glucose was evaluated in batch culture after 5 days (Figure 1); the addition of heptanoate, and octanoate was also attempted although alcohol end products were not detected above background (Supplemental Table 1). The data shows that the addition of external fatty acids increase the final optical density in the cultures (above controls) and carbon recoveries were between 70.8 and 104.4% (Supplemental Table 1).

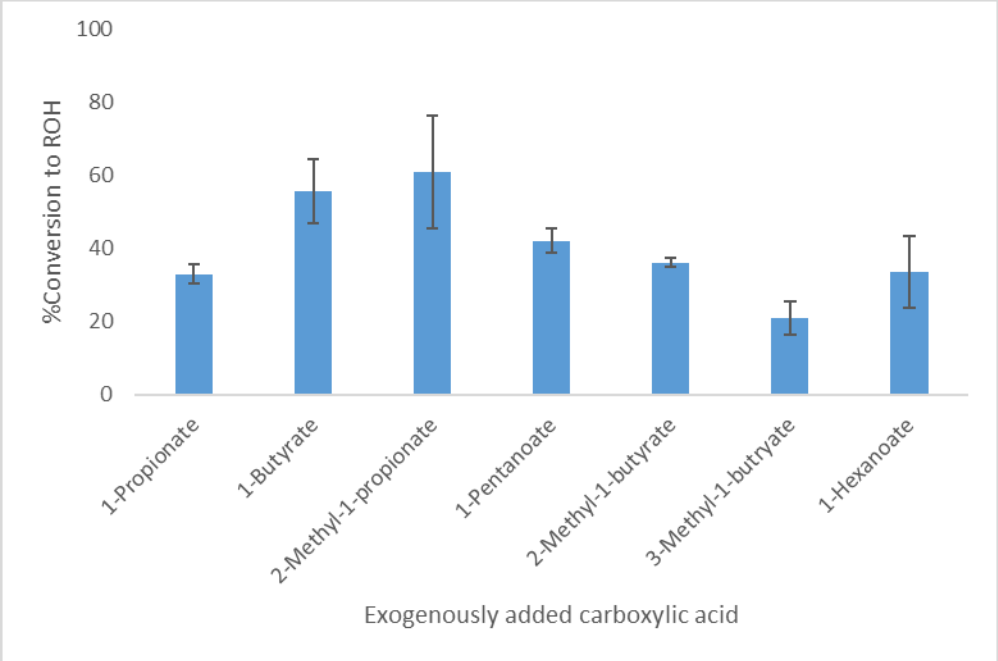
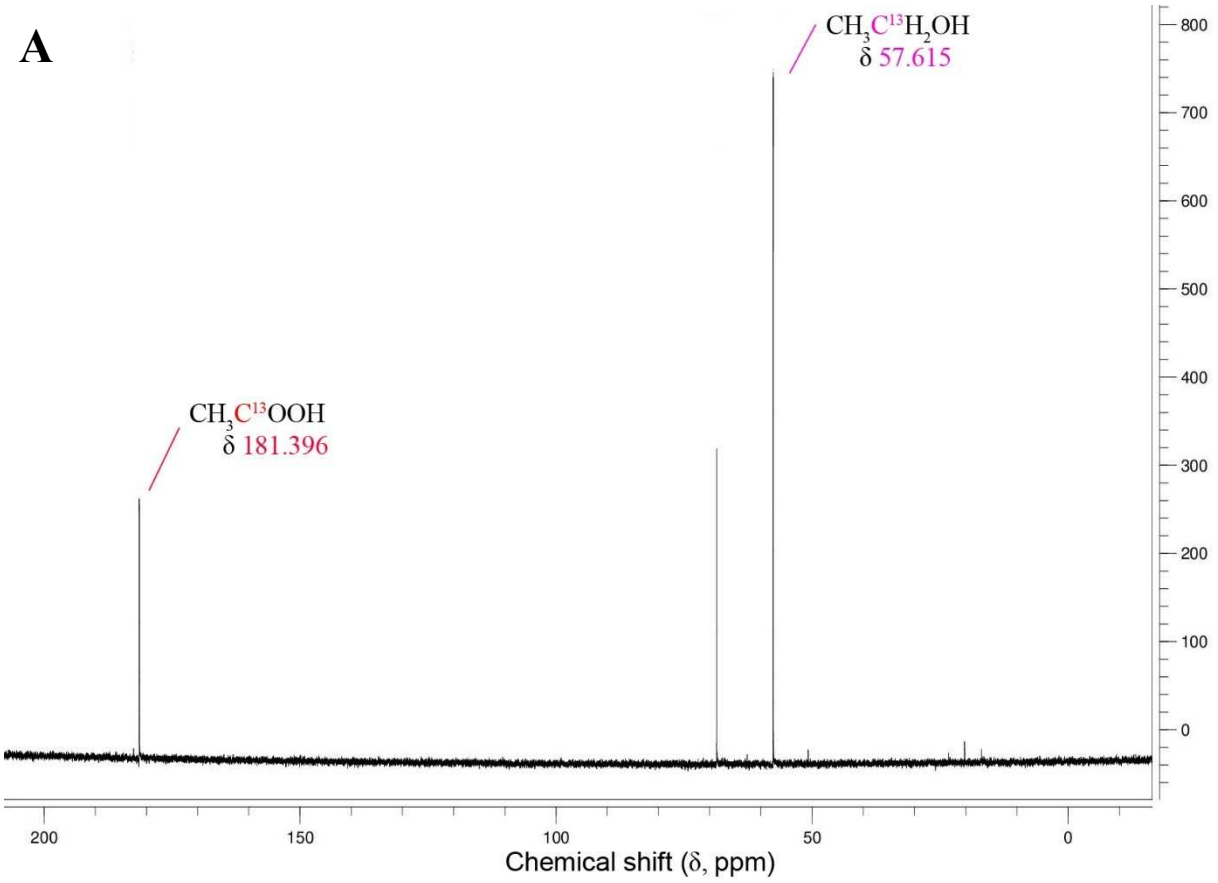


Figure 1- Conversion of exogenously added SCFAs to fermentations containing 20 mM of glucose by *T. pseudoethanolicus*. Values represent the average of triplicates with error bars presented as standard deviation.

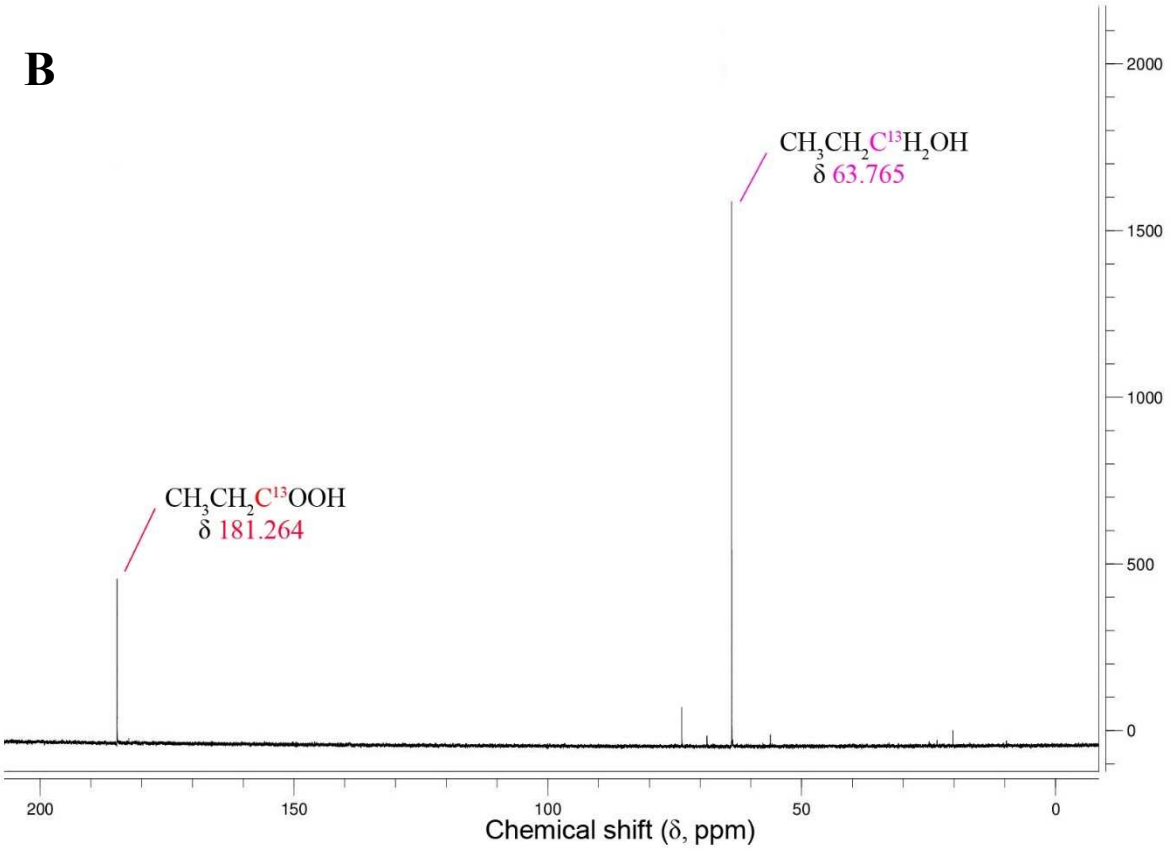
Apart from alcohol formation from the conversion of C2-C6 fatty acids, a peak attributable to methanol was detected when formate was added exogenously although it could not be quantified due to peak shouldering with ethanol. As ethanol is a normal end product of glucose fermentation from *T. pseudoethanolicus*, ethanol formation from acetate was unclear. SCFAs (C3 to C6) were converted to their corresponding alcohols with yields between 21.0 and 61.0% (Figure 1). For instance, the addition of 1-propionate gave a 1-propanol titer of 6.6 mM (33 %) and a 2-methyl-1-propionic acid addition yielded 12.2 mM of 2-methyl-1-propanol (61%). Alcohol formation from 1-hexanoic acid was observed by GC-FID with the appearance of a peak attributable to 1-hexanol which was confirmed by GC-MS (Supplemental Figure 1). As end products were only analyzed after 5 days, the rates of carboxylic acid conversion to alcohols and the impact that the addition of these exogenously added acids may have on growth is not available. However, the decreased concentration of ethanol in the presence of carboxylic acids suggest that electron flow from glucose is redirected to the corresponding alcohols.

To confirm that the organic acid was indeed reduced to the corresponding alcohol, ^{13}C1-acetate, propionate, and butyrate were added at a concentration of 20 mM in addition to 20 mM of glucose. Figure 2A-C shows the NMR spectra of fermentations containing exogenously added ^{13}C1 acetate (181.4 ppm), ^{13}C1 propionate (181.3 ppm) and ^{13}C1 butyrate (184.0 ppm), respectively. After fermentation with glucose, new peaks of alcohol formation were observed (ethanol at 57.6 ppm, propanol at 63.8 ppm and butanol at 63.7 ppm). This supports that the exogenously added SCFAs are indeed being reduced to ethanol rather than appearing direct as end products of glucose fermentation.

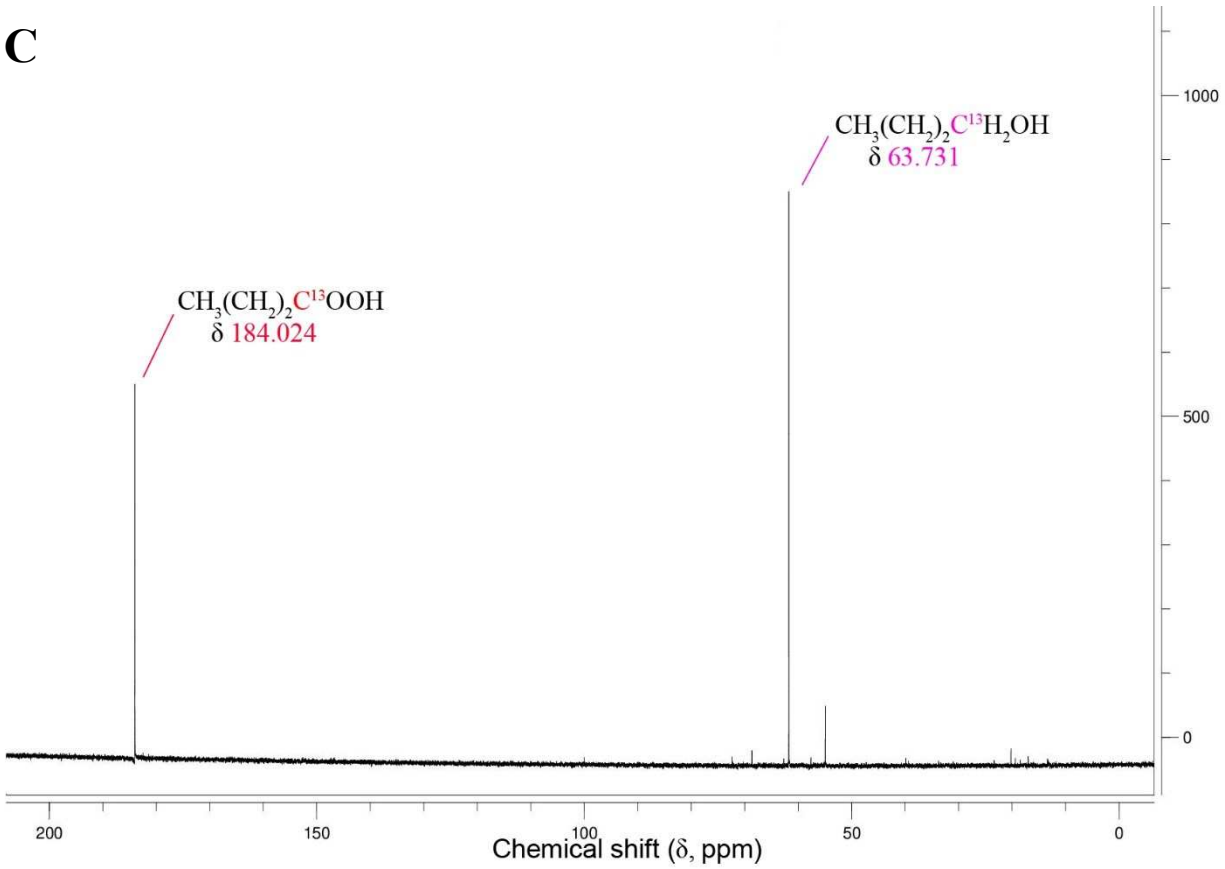
Figure 2 – ^{13}C NMR spectra of glucose fermentations (20 mM) by *T. pseudoethanolicus* with exogenously added ^{13}C1-acetate (A), ^{13}C1-propionate (B) and ^{13}C1-butyrate (C) at 20 mM final concentration.



B


CH3CH2C13H2OH
δ 63.765
CH3CH2C13OOH
δ 181.264
2000
1500
1000
500
0
200
150
100
50
0
Chemical shift (δ, ppm)

132



The ability to reduce carboxylic acids might suggest an alternative mechanism for the formation of fusel alcohols (3-methyl-1-butanol, 2-methyl-1-butanol, and 2-methyl-1-propanol) during the fermentation of branched chain amino acids [23–25]. The presence of such alcohols as end products can alter fermentation kinetics as well as cell viability due to disruption of the cellular membrane, particularly those with a more non-polar character. Thus, higher order alcohols are often more inhibitory as compared to ethanol [27]. While *Thermoanaerobacter pseudoethanolicus* is known to tolerate up to 2.5% ethanol although growth rates are diminished with 1-butanol and 2-methyl-1-butanol having a noticeable impact on growth rates at less than 1.0% v/v [28].

The capability of *Thermoanaerobacter* species to reduce carboxylic acids in the presence of glucose could present a novel route to the production of higher-order alcohols from inexpensive feedstocks using the reducing power of glucose or other inexpensive materials. *Thermoanaerobacterium* strains have been noted to rapidly reduce ^{13}C-labeled exogenously added acetate to ethanol via an acetyl-CoA intermediate [29]. Carboxylic acid reassimilation may provide insight as to why some *Thermoanaerobacterium* and *Thermoanaerobacter* strains are such efficient ethanol producers during glucose fermentation. To the author´s knowledge, this is the first demonstration of the ability of a whole cell system of *Thermoanaerobacter* to convert carboxylic acids to their corresponding alcohols using inexpensive carbohydrates as a source of reducing potential and could present a novel route to the production of higher primary alcohols, including branched-chain alcohols, from renewable substrates.

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