Exploring microbial chain elongation for production of organics and hydrogen in soils

by

Sayalee Joshi

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Graduate Supervisory Committee:

Anca G. Delgado, Chair
César I. Torres
Leon van Paassen

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ABSTRACT

This research explores microbial chain elongation as a pathway for production of complex organic compounds in soils with implication for the carbon cycle. In chain elongation, simple substrates such as ethanol and short chain carboxylates such as acetate can be converted to longer carbon chain carboxylates under anaerobic conditions through cyclic, reverse β oxidation. This pathway elongates the carboxylate by two carbons. The chain elongation process is overall thermodynamically feasible, and microorganisms gain energy through this process. There have been limited insights into the versatility of chain elongating substrates, understanding the chain elongating microbial community, and its importance in sequestering carbon in the soils.

We used ethanol, methanol, butanol, and hydrogen as electron donors and acetate and propionate as electron acceptors to test the occurrence of microbial chain elongation in four soils with different physicochemical properties and microbial communities. Common chain elongation products were the even numbered chains butyrate, caproate, and butanol, the odd numbered carboxylates valerate and heptanoate, along with molecular hydrogen. At a near neutral pH and mesophilic temperature, we observed a stable and sustained production of longer fatty acids along with hydrogen. Microbial community analysis show phylotypes from families such as *Clostridiaceae, Bacillaceae,* and *Ruminococcaceae* in all tested conditions. Through chain elongation, the products formed are less biodegradable. They may undergo transformations and end up as organic carbon, decreasing the greenhouse gas emissions, thus, making this process important to study.

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CHAPTER 1

INTRODUCTION

There are abundant, biodegradable compounds present in the soil which undergo anaerobic fermentation. Anaerobic fermentation tends to break down complex organics into simpler compounds such as short chain carboxylic acids, carbon dioxide, and hydrogen (Inglett, Reddy, and Corstanje 2005). The short chain carboxylates such as acetate can be converted further to methane by methanogenic Archaea (Spirito et al. 2014). Methane is the most reduced form of carbon with the lowest free energy content per electron, favored by long residence time in anaerobic digestion (Angenent et al. 2016). The process of breaking down complex compounds to simpler compounds such as carbon dioxide and methane in anaerobic condition has been studied extensively (Oertel et al. 2016). There is a general assumption that anaerobic metabolism leads to the breakdown of complex biodegradable compounds into simpler/smaller ones.

Other anaerobic metabolic pathways elongate rather than break down carbon compounds but have received little consideration in soils. One such process is chain elongation. Through microbial chain elongation, simpler organic compounds are combined into larger, more complex organics. As soils are rich in anaerobic microbial biota (Inglett, Reddy, and Corstanje 2005), and carbon needs to be sequestered in the soil in form of more stable organic compounds, it is important to explore more about the chain elongation pathway in soils.

Metabolic reactions, especially under anaerobic conditions, take place very close to thermodynamic equilibrium, to minimize the energy dissipation due to the inherent energy scarcity in anaerobic systems and have a potential reversibility. Small environmental changes in product and substrate concentration can reverse the driving force of the chemical reaction catalyzed (Gonzalez-Cabaleiro et al. 2013). Through one

of such reverse metabolic pathways, named reverse β oxidation, which is anaerobic oxidative reactions when operated in the opposite (reductive) direction, longer fatty acids can be produced under anaerobic conditions from short chain carbon compounds in presence of a reduced compound and mildly acidic pH (Spirito et al. 2014). Ethanol is one of the efficient reduced substrate for synthesis of caproate by the most studied microbe for chain elongation, *Clostridium kluyveri* (Xiaoyu et al. 2015). It is also a carbon and energy source, electron donor, and source for deriving acetyl-CoA so that the chain elongation pathway can proceed (Coma et al. 2016).

Microbial chain elongation is a relevant pathway of producing longer chain carbon compounds which are more stable/resistant compounds to biodegradation (Spirito et al. 2014). It is an efficient way to conserve energy under anaerobic conditions as little energy is lost in the shift of electrons from one organic molecule to another (Angenent et al. 2016). In the absence of methanogenesis, enrichment cultures of chain elongating microbes ferment substrates such as ethanol and acetate to butyrate, caproate, butanol due to reductive bio-hydrogenation pathway, along with formation of molecular hydrogen (Seedorf et al. 2008).

Reverse β oxidation, the conversion of short chain carboxylates in presence of ethanol to medium chain carboxylates (Coma et al. 2016), has been observed in a group of anaerobic bacteria that possess fatty acid synthase complex (Yin et al. 2017). The synthesis of longer chain carboxylates begins with the condensation of acetyl-CoA, which further gets reduced by adding two carbons through each subsequent round of fatty acid synthesis; thus, acetate gets elongated to butyrate (C4), and further to caproate (C6) and caprylate (C8) (Coma et al. 2016). Simple compounds such as ethanol and hydrogen, which are considered to be the common end products of fermentation, are thus used as building blocks to convert short chain fatty acids such as acetate into

medium chain fatty acid, increasing the total amount of energy recovered from the substrates (Steinbusch et al. 2011). Changes in products depend on substrates used and their concentrations. Gibbs free energy is dependent on concentration of each compound. Fermentable substrates oxidation reactions are associated with/involved in hydrogen production which has high energy requirements, but overall chain elongation reaction is kinetically feasible (Gonzalez-Cabaleiro et al. 2013), and thermodynamically favorable, thus microbes derive energy from this process (Coma et al. 2016).

Among the microorganisms that perform chain elongation, *Clostridium kluyveri*, isolated from canal mud, is the most studied anaerobe (Coma et al. 2016). Several groups of microbes which have been reported for medium chain carboxylate production were isolated from oxygen limited environments. These include *Eubacterium pyruvativorans* and *Megasphaera elsdenii* isolated from sheep rumen fluid, *Clostridium* sp. BS-1 isolated from anaerobic digester sludge, and *Rhodospirillum rubrum* (Angenent et al. 2016). Other phylotypes such have *Clostridium ljundahlii, Clostridium ragsdalei* were enriched during chain elongation reaction (Coma et al. 2016). *Clostridium* cluster IV and XIVa were dominant when lactate was added as the electron donor (Xiaoyu et al. 2015). In mixed culture fermentation, with organic wastes streams, dominant species enriched were *Azospira oryzae* and relatives of *Clostridium kluyveri* (Steinbusch et al. 2011). In the case of aerobic microorganisms, such as *Escherichia coli*, metabolic engineering and synthetic biology techniques of modifying metabolic pathways have been used to produce butyrate under aerobic growth conditions (Kataoka et al. 2017).

Our fundamental research questions include (i) does microbial chain elongation occur in soil, (ii) if yes, then is the process occurring in soil with various geochemical

characteristics , (iii) what are the effective concentrations and combinations of substrates (electron donors and electron acceptors) that form elongation products, and (iv) which microorganisms are capable of performing chain elongation? The objectivesof this research were to assess the extent and ubiquity of chain elongation in soils and to explore the enriched communities of chain elongating microbes from the soils.

We performed a metabolic microbial chain elongation exploration exercise in various/multiple soils with different physicochemical properties and diverse microbial communities. Formation of chain elongation products such as butyrate, valerate, caproate, heptanoate, butanol, and hydrogen were monitored at regular time intervals. We used deep-sequencing community analyses to compare different microbial communities and identify enriched microorganisms participating in chain elongation.

Chain elongation pathway:

Chain elongation of short chain carboxylates such as acetate, into longer chain carboxylates such as butyrate and caproate, occurs via a microbial pathway known as reverse β oxidation, where ethanol is the source of carbon, (Angenent et al. 2016) energy, and reduced equivalents (Spirito et al. 2014). It is a condensation reaction involving carbon-carbon bond formation, catalyzed by fatty acid synthesizing enzymes from the thiolase superfamily (Clementina et al. 2011). β oxidation is a reversible, cyclic, metabolic pathway unique to some anaerobes. It is likely to operate in the reverse way, when environmental conditions become favorable, such as presence of energy rich, reduced compound like ethanol, and high enough hydrogen partial pressure to prevent oxidation. Depending on how the anaerobic environmental conditions are, the

reverse β oxidation pathway can perform either oxidation or reduction (Spirito et al. 2014).

For every 5 molecules of ethanol that are used for elongation, 1 molecule of ethanol is oxidized to acetate to provide metabolic energy in the form of ATP and NADH via substrate level phosphorylation (Spirito et al. 2014, Steinbusch et al. 2011). Remaining 4 ethanol molecules get oxidized to acetaldehyde, an intermediate, and then to acetate. This acetate gets activated to a high energy intermediate (Thauer et al. 1968), acetyl-CoA (Steinbusch et al. 2011), reducing NAD^+ to NADH (Spirito et al. 2014). Oxidation of ethanol via acetaldehyde to acetyl CoA are catalyzed by NAD-dependent ethanol dehydrogenase and NAD(P)-dependent acetaldehyde dehydrogenase (Seedorf et al. 2008). Acetyl-CoA molecule derived from ethanol is added to carboxylate. This elongates the carbon chain by two carbons (C2) at each time to butyrate using acetyl-CoA, NADH, and FADH2 through coupling of two acetyl-CoA to form butyrl-CoA, if acetate is used as the electron acceptor (Steinbusch et al. 2011). Similarly, butyrate is elongated to caproate via coupling of butyl-CoA with acetyl-CoA by forming hexanoyl-CoA (Steinbusch et al. 2011, Xiaoyu et al. 2015). If propionate is used as an electron acceptor, the chain is elongated to valerate, and from valerate to heptanoate (Angenent et al. 2016). The combined pathways of ethanol oxidation and reverse β oxidation that form chain elongation are thermodynamically feasible (Spirito et al. 2014). In ethanol-acetate fermentation, hydrogen is generated via a ferredoxin-dependent hydrogenase (Seedorf et al. 2008). Ethanol oxidation coupled to chain elongation pathway is one of the most efficient ways of caproate generation (Xiaoyu et al. 2015).

$$CH_3CH_2OH + H_2O \rightarrow CH_3COO^- + H^+ + 2H_2$$
$$\Delta G^\circ = +9.7\ kJ/mol \qquad Equation\ (i)$$

$$CH_3CH_2OH + CH_3COO^- \rightarrow CH_3CH_2CH_2COO^- + H_2O$$
$$\Delta G^\circ = -38.6\ kJ/mol \qquad Equation\ (ii)$$

$$CH_3CH_2OH + CH_3CH_2CH_2COO^- \rightarrow CH_3CH_2CH_2CH_2CH_2COO^- + H_2O$$
$$\Delta G^\circ = -38.6\ kJ/mol \qquad Equation\ (iii)$$

As the reaction shown in equation (i) is highly endergonic, it must be coupled with reactions shown in equations (ii) and (iii) which are exergonic reactions from which energy is derived. Hydrogen formation from ethanol depends on butyrate formation from ethanol and acetate (Seedorf et al. 2008).

CHAPTER 2

MATERIAL AND METHODS

2.1 Soils

Soils were obtained from four different sites to investigate microbial chain elongation. The soils were designated A, B, G, and L. Relevant details and characteristics of the soils are shown in Table 1.

Table 1. Characteristics of soils used in the microbial chain elongation study.

Conductivity, salinity, pH, and total dissolved solids TDS were measured with 1 part of water to 1 part of soil by weight. Total organic carbon was measured with 250 mg of air dried soil. The data are averages with standard deviation of triplicate measurements.

Soil ID	Description and Location	Soil Type	pH	Conductivity ($\mu S\ cm^{-1}$)	Total Dissolved Solids (ppm)	Salinity (ppm)	Chemical Oxygen Demand ($mg\ g^{-1}$)	Total Organic Carbon ($mg\ kg^{-1}$)
A	Chlorinated solvent-contaminated soil; ~30 m deep; Arizona	Sand + Clay	7.6 ± 0.4	180 ± 10	130 ± 20	70 ± 10	3.5 ± 0.9	5700 ± 300
B	0.2 m deep; Montana	Peat	7.7 ± 0.2	180 ± 20	120 ± 10	70 ± 10	0.7 ± 0.4	140000 ± 7000
G	Garden soil; 0.2 m deep; Arizona	Silt + Loam	7.8 ± 0.3	170 ± 30	120 ± 10	70 ± 10	2.9 ± 1	37000 ± 3000
L	Hydrocarbon-contaminated soil; ~1.5 m deep; Texas	Clay	5.7 ± 0.5	80 ± 10	50 ± 10	40 ± 10	0.85 ± 0.6	20000 ±3000

1.2 Experimental setup of semi-batch microcosms

Soil microcosms were established in the anaerobic chamber in 250 mL glass serum bottles according to established protocols (Delgado et al. 2014). Microcosms consisted of 25 g of homogenized soil and 75 mL of medium. Reduced anaerobic mineral medium was prepared containing the following reagents per liter: 1 g NaCl, 0.06 g $MgCl_2 \times 6H_2O$, 0.2 g KH_2PO_4, 0.3 g NH_4Cl, 0.3 g KCl, 0.005 g $CaCl_2 \times 2H_2O$, and 1 mL of Trace A and 1 mL of Trace B solutions. Trace element stock solution A contained per liter: HCl (25% solution, w/w), 10 ml; $FeCl_2 \times 4H_2O$, 1.5 g; $CoCl_2 \times 6H_2O$, 0.19 g; $MnCl_2 \times 4H_2O$, 0.1 g; $ZnCl_2$, 70 mg; H_3BO_3, 6 mg; $Na_2MoO_4 \times 2H_2O$, 36 mg; $NiCl_2 \times 6H_2O$, 24 mg; $CuCl_2 \times 2H_2O$, 2 mg. Trace element stock solution B contained per liter: $Na_2SeO_3 \times 5H_2O$, 6 mg; $Na_2WO_4 \times 2H_2O$, 8 mg; NaOH, 0.5 g. The medium was buffered with 12.5 mM phosphate (initial pH = 7.5), purged with ultra-high purity (UHP) N_2 gas for 20 min, and reduced with 0.2 mM Na_2S and 0.4 mM L-cysteine (Delgado et al. 2012).

Four experimental conditions were setup in duplicates: acetate + ethanol, acetate + H_2, ethanol + H_2, and ethanol, along with two control conditions: acetate and no substrate. The initial concentrations of the substrates used for the ethanol + acetate condition were 110 ± 20 mM ethanol and 94 ± 5 mM acetate. For ethanol + hydrogen and acetate + hydrogen conditions, the initial substrate concentrations were 100 ± 10 mM ethanol, 92 ± 4 mM acetate, and ~88 mM H_2 (nominal concentration). For ethanol only condition, the initial substrate concentrations were 110 ± 8 mM ethanol. Chain elongation was also tested at low substrate concentrations, which are more realistic concentrations present in natural/environmental conditions. For this test, we setup duplicate microcosms with ethanol + acetate, with initial substrate concentrations of 12 ± 1 mM acetate and 10 ± 1 mM ethanol, and 2.7 ± 0.2 mM acetate and 2.1 ± 0.2 mM ethanol

for soils G and L. 10 mM sodium 2-bromoethanesulfonate (BES) was added to all microcosms to inhibit methanogenesis only at time 0. The microcosms were closed with butyl rubber stoppers and sealed with aluminum crimps.

The microcosms were shaken at 125 rpm and were incubated in the dark at 31℃. The microcosms were operated as semi-batch. Staring on day 14, four semi-batch cycles were performed (every 7-15 days). The semi-batch cycles consisted of first removing 25 mL of soil slurry and then replacing with 25 mL fresh anaerobic medium containing 90 ± 5 mM acetate and 92 ± 20 mM ethanol foe ethanol + acetate condition. Hydrogen was added with a gastight syringe to reach a concentration of ~88 mM.

Microcosms with control conditions: Batch soil microcosms were as described above. Two control conditions were setup for all four soils consisting of acetate only (140 ± 10 mM) and no added substrates.

Enrichment cultures were established in the anaerobic chamber in 160 mL glass serum bottles according to established protocols (Delgado et al. 2014). 10 mL of slurry from the end of the experiment with ethanol + acetate condition was transferred to triplicate bottles with 90 mL anaerobic medium containing several different conditions. The initial concentrations of the substrates for ethanol + acetate transfer1 condition were, 93 ± 2 mM ethanol and 88 ± 1 mM acetate, for soils B and G. The cultures were re-spiked with ethanol and acetate, such that the initial concentrations of the substrates were 210 ± 20 mM ethanol and 260 ± 50 mM acetate.

To test if microbial chain elongation occurs with substrates other than ethanol, acetate, and hydrogen, and to check if elongation products with odd number of carbon are formed, we used different electron donors: butanol, methanol, and electron acceptor: propionate. Butanol (C4 alcohol) + propionate (C3 carboxylate) condition microcosms

were set up with initial concentrations of the substrates, 190 ± 6 mM butanol and 120 ± 8 mM propionate, for all four soils. Ethanol + Propionate condition microcosms were set up with initial concentrations of the substrates, 81 ± 2 mM ethanol and 84 ± 0.2 mM propionate for soil B. Lastly, methanol (C1 alcohol) + acetate condition microcosms were set up with initial concentrations of the substrates, 100 ± 1 mM methanol and 91 ± 9 mM acetate for soil G.

1.3 Analytical methods for liquid and gas composition analyses

Samples for chemical liquid analyses were prepared from 1.5 mL of soil slurry, which was centrifuged at 13200 RPM for 15 min and filtered through a 0.2 μm syringe filter. Volatile fatty acids (VFAs) and alcohols were analyzed using a high-pressure liquid chromatograph (HPLC, Shimadzu LC-20AT). The HPLC was equipped with an Aminex HPX-87H column (Bio-Rad, Hercules, CA). Detection of chromatographic peaks was achieved using a photodiode array detector at 210 nm and a refractive index detector. The eluent was 2.5 mM H_2SO_4 with a total elution time of 60 min. The oven temperature was kept constant at 65℃. The eluent flow rate was 0.6 mL min^{-1} for the first 30 minutes and 0.8 mL min^{-1} for the remaining 30 min. Calibration curves were generated for ethanol (0.5-100 mM), butanol (0.5-30 mM), acetate (0.5-100 mM) and all the C1-C7 VFA standards (0.5-10 mM) during every HPLC run. The detection limit for the VFAs and alcohols was ≤0.1 mM (Esquivel-Elizondo et al. 2018) and 0.5 mM, respectively (Esquivel-Elizondo, Delgado, and Krajmalnik-Brown 2017).

H_2 and methane were quantified from 200 μL of gas sample from the headspace withdrawn from a 500 μL gas-tight syringe (SGE, Switzerland) using a gas chromatograph (GC, Shimadzu GC 2010, Columbia, USA) equipped with a thermal conductivity detector (TCD) and a fused silica capillary packed column (Carboxen™

1010 PLOT, Supelco, Bellefonte, PA) (Esquivel-Elizondo, Delgado, and Krajmalnik-Brown 2017). UHP argon was the carrier gas with a constant pressure and flow rate of 42.3 kPa and 10 mL min^{-1}, respectively. The temperature of the injector was 150℃. The TCD temperature was 180 ℃ and the current was set at 41 mA. The column temperature was initially held to 80℃ for 3 min, ramped to 160℃ at 50℃ min^{-1} and held for 1.5 min. Gas calibration curves were generated for H_2 (0.05-5 mM) and methane (0.08-1.3 mM). The detection limit for H_2 and methane were 0.02 mmol L^{-1} and 0.03 mmol L^{-1} (gas concentration), respectively. Total gas volume in the headspace of the bottles was measured with a 50 mL frictionless syringe (Perfektum R matched numbered hypodermic syringes, Sigma Aldrich) before each headspace analysis.

1.4 Soil Properties

Conductivity, salinity, total dissolved solids (TDS) were measured with a Multi-parameter PCSTestr TM 35 probe (Eutech instruments, Oakton). The pH was measured with Sartorius pH meter (Thermo Scientific, Waltham, MA), which was calibrated regularly with 4, 7, and 10 standard solutions from the manufacturer. Chemical oxygen demand (COD) was quantified using TNT 821 and TNT 822 HACH kits according to the manufacturer's protocol (Esquivel Elizondo, Delgado, et al. 2017). The total organic carbon (TOC) concentrations for the air dried soils were measured. TOC was assayed using a SSM-5000A Shimadzu total organic carbon solid sample module (Shimadzu Corp., MD, USA) (Chen et al. 2018).

2.5 Molecular microbial ecology analyses

1.5 mL of soil slurry samples were obtained from the microcosms at the beginning and at the end of the experiment. These samples were pelleted using an Eppendorf

micro centrifuge 5415R (Hauppauge, NY) for 15 minutes at a maximum speed of 16100 rcf (g) / (13200 rpm) and frozen at −20℃. DNA was extracted from 1.5 mL frozen pellets using Power Soil DNA Isolation Kit (MO BIO Laboratories, Inc., (Carlsbad, CA, USA) by following the protocol recommended by the manufacturer (Delgado et al. 2014). The DNA concentrations and purity of each sample were determined by measuring absorbance at wavelengths of 260 and 280 nm with a NanoDrop spectrophotometer (NanoDrop Technology, Rockland, DE) (Esquivel Elizondo, Ilhan, et al. 2017).

High-throughput sequencing and bioinformatics and statistical analyses: To determine the changes in the microbial community structures under various chain-elongating conditions, microbial community amplicon sequencing was performed using the Illumina MiSeq at the Microbiome Analysis Laboratory in the Swette Center for Environmental Biotechnology, Arizona State University, Tempe (http://krajmalnik.environmentalbiotechnology.org/microbiome-lab.html) (Esquivel-Elizondo et al. 2018). The universal primers were 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) (Xiaoyu et al. 2015) for the V4 hyper-variable region of the 16S rRNA gene which captures Bacteria and Archaea (Caporaso et al. 2012).

Forward and reverse sequences (2 ×150 mode) were first paired (overlap ≥ 45 base-pairs) using PANDASeq (Masella et al. 2012). Then, paired reads (average length 250 base-pairs) were processed using the bioinformatics software, Quantitative Insights into Microbial Ecology QIIME (version 1.9.1) pipeline (Caporaso, Justin, et al. 2010). For further downstream analysis, low quality sequencing data was trimmed off by quality filtering based on the following criteria: shorter than 200 base-pairs (bps), quality score of 25 or below, 1 or more primer mismatches, and more than 6 homopolymers.

Operational taxonomy units (OTUs) were picked based on 97% sequence similarity from the remaining filtered sequences using the UCLUST algorithm (Edgar 2010). The most abundant sequence of each cluster was picked as the representative sequence. Taxonomy was assigned to the representative sequences by comparing them to the Greengenes database (DeSantis et al. 2006). Representative sequences were aligned with PyNAST (Caporaso, Bittinger, et al. 2010).Chimera Slayer was used to identify chimeric sequences, gaps and chimeras were filtered from this alignment (Haas et al. 2011). UCLUST was used to assign taxonomy based on the Green genes database in order to construct a BIOM formatted OTU table. Singletons (OTUs with less than 2 sequences) were removed from the OTU table. For an equal comparison among samples, the OTU table was subsampled using the command single rarefaction.py at a depth of 12000 sequences (Matsumoto and Nishimura 1998).

2.6 Electron balance

Electron balances were performed in order to understand the distribution of electrons provided from the substrates (ethanol, acetate, and H_2) to end-products identified (fatty acids, alcohols, and H_2) (Esquivel-Elizondo et al. 2018). These electron balances were quantified as COD using 8 g COD/e−eq. For the electron balances, mmol of electron donors and end-products were converted to electron milliequivalents (e−meq.) using electron equivalents per mol values. The numbers of electron equivalents per mol were as follows: H_2, 2; acetate, 8; butyrate, 20; butanol, 24; valerate, 26; caproate, 32; and heptanoate, 38. The distribution of electron equivalents from substrates to end products was calculated by dividing the number of electron equivalents of each end product by the number of electron equivalents provided as the substrate and multiplying the result by 100 (Esquivel Elizondo, Delgado, et al. 2017).

CHAPTER 3

RESULTS AND DISCUSSION

The purpose of our study was to answer fundamental knowledge based questions such as does microbial chain elongation occur in soil, if yes, then check if the process was ubiquitous, what were effective concentrations and combinations of substrates that form elongation products and monitor those products, and lastly study the enriched phylotypes to explore which microorganisms are capable of performing chain elongation.

3.1 Substrates were metabolized to longer fatty acids, alcohol, and hydrogen

Four soils with different physicochemical properties were tested in anaerobic conditions, with different substrate concentration and combinations. The most described, preferred substrate for biological chain are ethanol (C2) (Steinbusch et al. 2011) and short chain fatty acids; acetate (C2) which leads to production of even-carbon chain carboxylates (Steinbusch et al. 2011, Agler et al. 2011) . Along with ethanol + acetate combination, we also tested ethanol + hydrogen, acetate + hydrogen, only ethanol, and only acetate substrate conditions at various initial concentrations. Semi batch experiments were performed in duplicates with consecutive four enrichments. We observed that chain elongation products were formed in all different substrate conditions and in all the soil types in different concentrations, summarized in Table 2. Common elongation products formed were the fatty acids butyrate (C4) and caproate (C6), the alcohol butanol (C4), and hydrogen when ethanol (C2) and/or acetate (C2) and hydrogen were used. Stable and sustained product formation was observed in the sequential batch tests, with the naturally present mixed microbial community in the soil.

We observed formation of small concentrations of odd numbered carbon chain carboxylates which are not easily found in nature, when substrate with C3 compound (Propionate) was used (Table 2). Medium chain carboxylates, such as valerate (C5) and heptanoate (C7) were formed via chain elongation of ethanol (C2) + propionate (C3). Valerate was detected in butanol (C4) + propionate (C3) condition. The initial, main elongation product formed was valerate which further elongated to heptanoate. All the substrate conditions tested on different soils, and chain elongation products formed are summarized in Table 2.

Table 2. Substrate combinations tested on different soils, along with monitored chain elongation products formed during the semi-batch experiments in the soil microcosms.

Soils used	Substrate combinations/condition	Products formed
A, B, G, L	190 ± 6 mM Butanol + 120 ± 7 mM Propionate	4.3 ± 0.9 mM Butyrate, 0.48 ± 0.3 mM Valerate,
B, G	(Transfer1) 92 ± 2 mM Ethanol + 89 ± 0.7 mM Acetate	81 ± 17 mM Butyrate, 12 ± 2 mM Caproate, 22 ± 28 mM Butanol
B, G	(Respike) 210 ± 20 mM Ethanol + 260 ± 50 mM Acetate	210 ± 70 mM Butyrate, 23 ± 5 mM Caproate, 36 ± 30 mM Butanol
B	(Transfer1) 80 ± 2 mM Ethanol + 84 ± 0.2 mM Propionate	14 ± 3 mM Butyrate, 45 ± 10 mM Valerate, 5.1 ± 2 mM Caproate, 2.5 ± 2 mM Heptanoate, 12 ± 5 mM Butanol
G	(Transfer1) 100 ± 1 mM Methanol + 91 ± 9 mM Acetate	7.1 ± 0.7 mM Butyrate, 1.8 ± 0.3 mM Caproate

3.2 Stoichiometry

The stoichiometry we calculated from our semi-batch experiments with ethanol and acetate for soil B is shown in equation (iv):

$$5\,CH_3CH_2OH + 2\,CH_3COO^- \rightarrow 3.5\,CH_3CH_2CH_2COO^- + 1.5H^+ + 2H_2 + 2H_2O$$

$$Equation\ (iv)$$

Which is similar to the stoichiometry reported in (Angenent et al. 2016) shown in equation (v).

$$5\,CH_3CH_2OH + 3\,CH_3COO^- \rightarrow 4\,CH_3CH_2CH_2COO^- + 1H^+ + 2H_2 + 3H_2O$$

$$\Delta G^\circ = -77\ kJ/reaction \qquad Equation\ (v)$$

3.3 Effect of different substrates used on medium chain fatty acid production

Ethanol + acetate elongation: In terms of electron donors, ethanol is the most effective substrate for microbial chain elongation into medium-chain carboxylates (Agler et al. 2012). We observed high concentrations of products such as butyrate, caproate, and butanol when the soils were fed with acetate and ethanol compared to the other substrate combinations.

It has been previously studied that, at a near neutral pH conditions, ethanol gets consumed faster (Steinbusch et al. 2011), elongating acetate initially to butyrate and later to caproate (Coma et al. 2016). Butyrate is the intermediate required to form caproate; thus, caproate concentrations increase until ethanol is completely consumed. (Barker, Kamen, and Bornstein 1945). The ratio of ethanol/acetate consumed during fermentation influences the ratio of butyrate/caproate produced (Angenent et al. 2016). The higher the relative ratio of ethanol/acetate consumed, the higher the caproate formation compared to butyrate. Therefore, the high availability of ethanol facilitates chain elongation reactions resulting in reaction going towards completion that is

formation of longer carbon chains of fatty acids such as caproate. (Angenent et al. 2016). Along with production of higher carboxylates such as butyrate and caproate, we also observed butanol (C4, alcohol) and hydrogen formed similar to studies by (Angenent et al. 2016). Butanol is formed from reduction of butyrate, mostly towards the end (Seedorf et al. 2008) in this fermentation reaction (Haas et al. 2018, Branduardi et al. 2014). The ethanol + acetate condition, where both the substrates are readily available to the chain elongating microbes, was found to be the most suitable substrate combination, for high production rates of butyrate and caproate (Angenent et al. 2016). Figure 1 shows the concentration of the elongation products formed when acetate + ethanol were used as substrates. Butyrate is observed in higher concentrations, as the initial chain elongation product, followed by caproate production. 55 mM of butyrate and 35 mM of caproate were the highest product concentrations reported in this experiment.

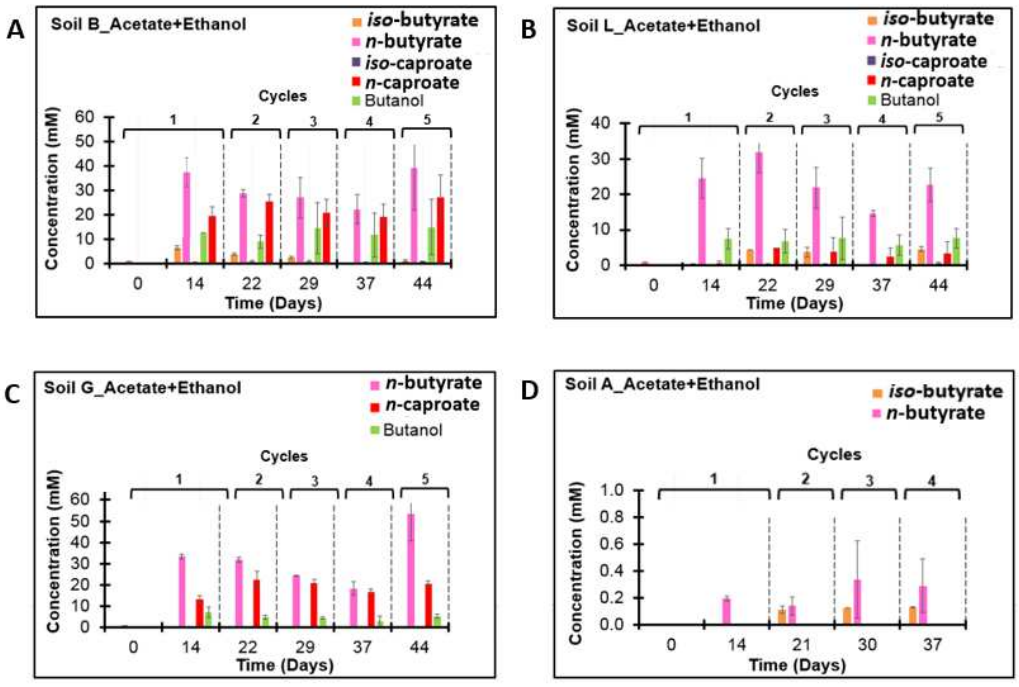


Figure 1. Formation of microbial chain elongation products in soil microcosms with ethanol + acetate condition. A, B, C, and D show the cumulative formation of elongation products during ethanol + acetate substrate combination in soils, B, L, G, and A respectively. *n*-butyrate, *n*-caproate, butanol, along with small concentrations of *iso*-butyrate and *iso*-caproate are produced during chain elongation are shown in this figure. Each dotted line represents a semi-batch cycle. The values are averages of duplicate microcosms and error bars indicate the standard deviation. Note the Y-axis differences between the panels.

Kinetics of the chain elongation metabolism with ethanol + acetate combination: Intensive HPLC data analysis was performed during cycle 4 of this experiment by daily testing concentrations of substrates consumed and products formed. Figure 2A shows the trend of substrate consumption. We observed that ethanol was consumed at a rate of 6.2 ± 3 mmol L^{-1} $day^{-1}$and acetate was consumed at a rate of 2.8 ± 1 mmol L^{-1} day^{-1}, showing that ethanol was getting consumed faster. Similar trends for faster ethanol consumption were reported in (Thauer et al. 1968). Figure 2B shows the concentration of the elongation products as a function of time. We observed that butyrate was produced at a rate of 3 ± 2 mmol L^{-1} day^{-1}, caproate was at a rate of 1 ± 0.5 mmol L^{-1} day^{-1}, along with butanol which was produced at a rate of 1 ± 0.4 mmol L^{-1} day^{-1}, showing that butyrate was produced faster and that it was the intermediate for caproate formation. There are inequalities of acetate consumption and the formation of butyrate and caproate, because it has been observed before that not only 1 ethanol + 1 acetate go to form 1 butyrate, but 2 ethanol can also form 1 butyrate shown in equation (vi) (Thauer et al. 1968).

$$2CH_3CH_2OH \rightarrow CH_3CH_2CH_2COO^- + H^+ + 2H_2 \qquad Equation\ (vi)$$

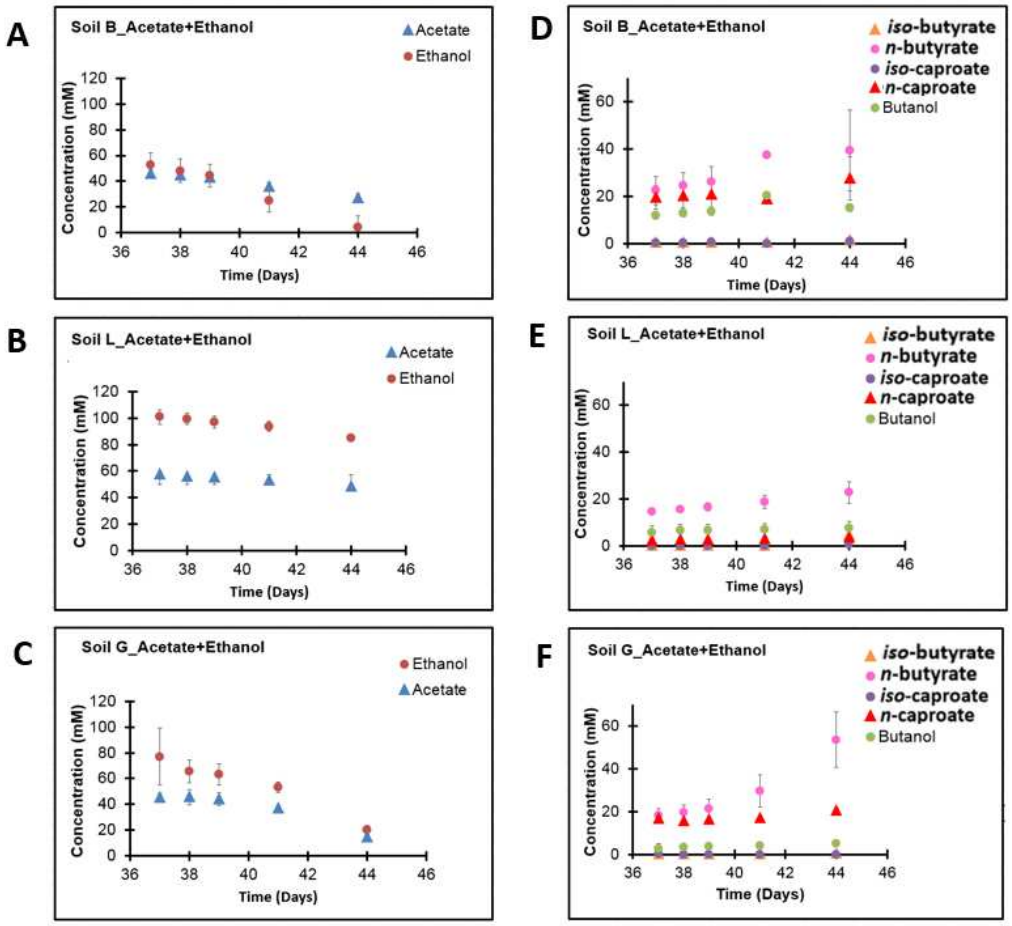


Figure 2. Consumption of substrates and formation of products in soil microcosms with ethanol + acetate condition during cycle 4.Figure A, B, C show substrate consumption and figure D, E, F show product formation in ethanol + acetate substrate combination for soils B, L, and G during cycle 4. The values are average of duplicate conditions and error bars indicate the standard deviation.

Ethanol + hydrogen and acetate + hydrogen substrate combinations: In ethanol + hydrogen condition, some ethanol got initially oxidized to acetate. Alcohol oxidation should not take place according to the unfavorable thermodynamics, such as oxidation of ethanol to acetate requires external source of energy but the energy requirement under standard biological conditions is close to equilibrium, thus any small

modification on the environment promotes oxidation of ethanol to acetate and hydrogen as shown in equation (i) (Spirito et al. 2014, Coma et al. 2016).

Acetate can also be formed by elongation of carbon dioxide through homoacetogenesis shown in equation (vii), which is a thermodynamically favorable pathway at near neutral pH conditions (Spirito et al. 2014, Steinbusch et al. 2011).

Homoacetogenesis:

$$4H_2 + 2CO_2 \rightarrow CH_3COO^- + H^+ + 2H_2O$$

$$\Delta G^\circ = -94.96\ kJ/mol \qquad Equation\ (vii)$$

This acetate formed is further used for elongation reaction, forming butyrate and eventually caproate. As acetate is directly absent in form of substrate added, slower product formation with low concentrations is observed compared to ethanol + acetate condition where required substrates are readily available.

In acetate + hydrogen condition, we detected ethanol. Some acetate got reduced to ethanol (Montel 2012); (Gonzalez-Cabaleiro et al. 2013) as shown in equation (viii). Hydrogen which is the electron donor, reduces acetate to ethanol, however conversion of acetate to ethanol is characterized by sluggish kinetics (Spirito et al. 2014); (Steinbusch et al. 2011). It has been studied, that acetate reduction to butyrate in presence of hydrogen is not as feasible as it is in presence of ethanol as an electron donor due low acetoacetyl-CoA concentration. Condensation of two acetyl-CoA is a highly endergonic reaction (Gonzalez-Cabaleiro et al. 2013).

Acetate Reduction:

$$CH_3COO^- + H^+ + 2H_2 \rightarrow CH_3CH_2OH + H_2O$$

$$\Delta G^\circ = -8.6\ kJ/mol \qquad Equation(viii)$$

When ethanol, which is a required chain elongation precursor is formed, acetate is elongated to butyrate. We observed an increase in butyrate concentration gradually,

with a decrease in concentration of the ethanol formed. A higher medium chain fatty acid production is limited due to shortage of ethanol (Steinbusch et al. 2011). Ethanol and hydrogen can be produced from anaerobic bioconversion of second generation biomass (Steinbusch et al. 2011), that is biomass which is not edible. It mostly includes cellulosic based biomass and agricultural wastes, which in turn contribute to some organic matter in the soils. However the direct addition of these substrates makes the microbial chain elongation reaction faster and easier, maximizing the formation of stable, longer compounds such as medium chain fatty acids. Figure 3A and 3B show concentration of products formed in acetate + hydrogen, and ethanol + hydrogen substrate combination. We observed that 3.8 ± 0.1 mM butyrate was formed at the end of the experiment for soil microcosms with acetate + hydrogen condition. Similarly, 2.1 mM of butyrate and 4.1 mM of caproate concentrations were observed at the end of the experiment with ethanol + hydrogen condition only in soil L. These low concentrations of elongation compounds show that acetate + hydrogen, and ethanol + hydrogen substrate combinations are less favorable for formation of longer chain fatty acids, and have slower kinetics, compared to ethanol + acetate. Possible reason for this is because ethanol is a better electron donor than hydrogen. Ethanol might be consumed easily because of less mass transfer resistance between ethanol and reaction phase, which in this case is the soil slurry in the microcosms, compared to hydrogen and reaction phase (Yin et al. 2017).

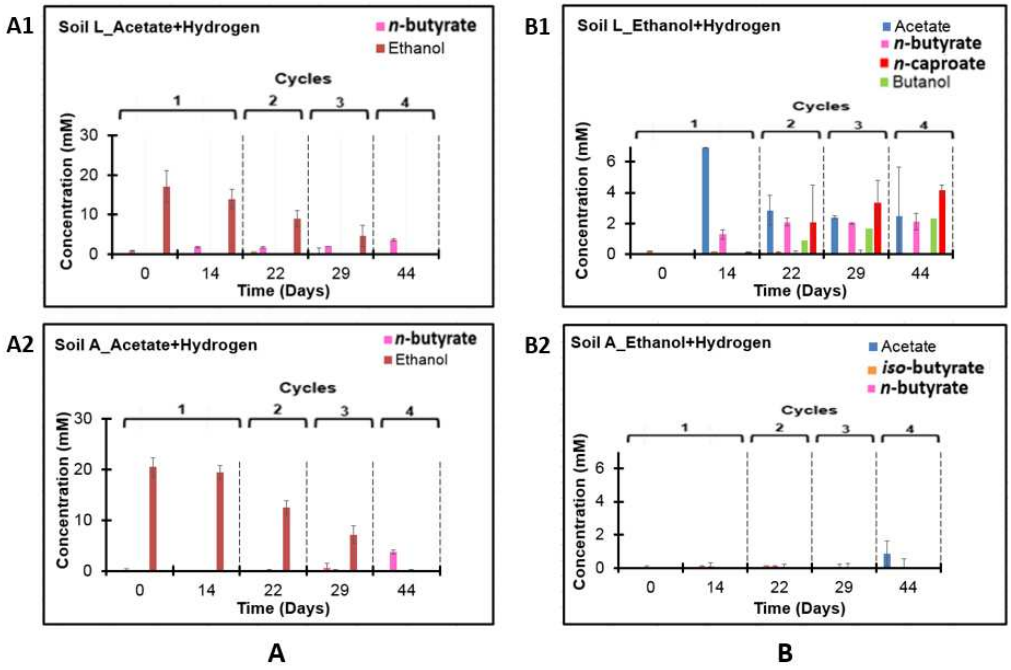


Figure 3. Formation of intermediates: ethanol, and chain elongation products in soil microcosms with acetate + hydrogen and ethanol + hydrogen condition. Figure A (A1, A2) shows the intermediate, ethanol formation along with formation of elongation product, butyrate in acetate + hydrogen substrate combination for soil L and A. Figure B (B1, B2) shows the intermediate, acetate formation along with formation of elongation products, butyrate, caproate, and butanol in ethanol + hydrogen substrate combination for soil L and A. The values are average of duplicate conditions, and error bars indicate the standard deviation.

Ethanol only condition: In this condition, acetate along with molecular hydrogen are seen to form as ethanol get oxidized (Coma et al. 2016) shown in equation (xi).

Ethanol oxidation:

$$CH_3CH_2OH + H_2O \rightarrow CH_3COO^- + H^+ + 2H_2$$

$$\Delta G^\circ = +8.6\ kJ/mol \qquad Equation\ (xi)$$

In Figure 4. we observed that acetate is formed, and it initiates chain elongation reaction with the ethanol added/present in the microcosms, to form butyrate. Butyrate was later elongated to caproate with ethanol as the electron donor (Coma et al. 2016). As the ratio of ethanol/acetate consumed is more than any other condition, higher concentrations of caproate were detected towards the end of the semi batch experiment, in microcosms which were fed only with ethanol as the substrate. High concentrations of ethanol make it energetically favorable for the microorganisms to use the volatile fatty acids (acetate) as electron acceptors (Steinbusch et al. 2011). Also, high availability of ethanol compared to acetate, facilitates favorable chain elongation, resulting in longer carbon chains (Angenent et al. 2016).

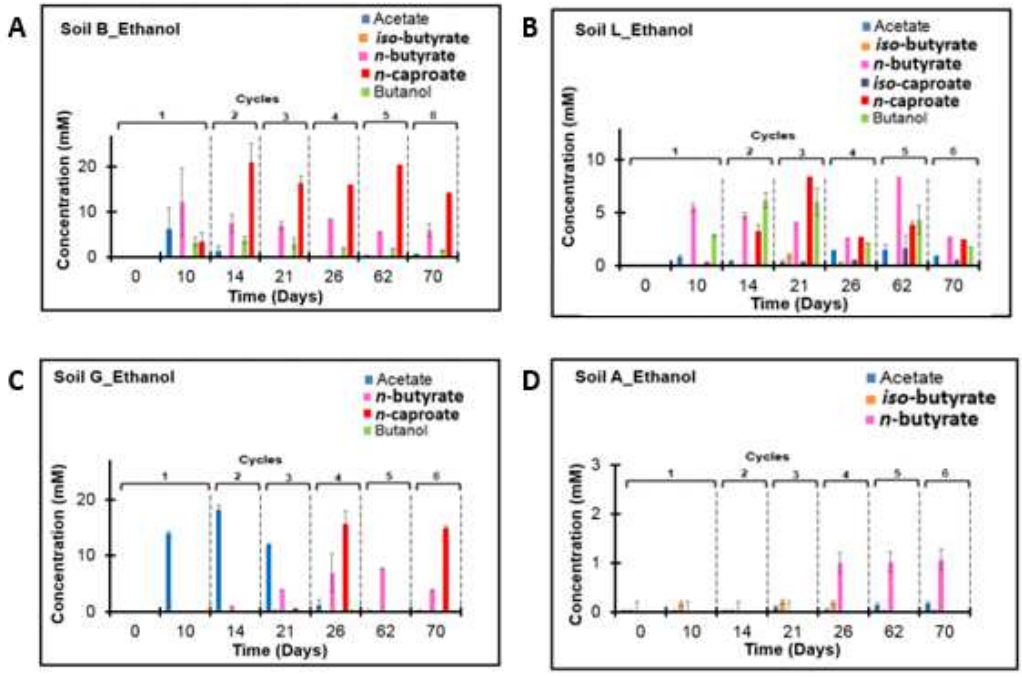


Figure 4. Formation of intermediate: acetate, and chain elongation products in soil microcosms with only ethanol condition. A, B, C, and D show the cumulative formation of elongation products during only ethanol condition in soils, B, L, G, and A respectively. Acetate, butyrate, caproate, along with butanol produced during chain

elongation are shown in this figure. Each dotted line represents a cycle, indicating a fill and draw event. It involves removing one third of slurry from the microcosm, and adding equal volumes of anaerobic media with substrates in the semi batch experiment to avoid shortage of substrates and to obtain an enrichment culture of chain elongating microbes. The values are average of duplicate conditions, and error bars indicate the standard deviation. Note that Y-axis is different for each soil.

To confirm if chain elongation happens at a very low substrate concentration, two sets of microcosms with ~10 mM and ~2 mM ethanol + acetate each were set up with soils G and L. We observed formation of the same elongation products such as butyrate, caproate, along with hydrogen. For the condition which had initial 12 ± 1 mM acetate and 10 ± 0.7 mM ethanol, after 15 days, 3.7 ± 2 mM of butyrate and 0.6 ± 0.4 mM of caproate was formed. Similarly, for the conditions with 2.7 ± 0.2 mM acetate and 2.1 ± 0.2 mM ethanol, 1.1 ± 0.1 mM of butyrate and 0.3 ± 0.1 mM of caproate was formed.

Control conditions: We observed**,** that there was no consumption of acetate in the soil microcosms that were fed only with acetate as the substrate. No elongation compounds or intermediates, such as ethanol, were detected (initial 15 days). Similarly, we did not detect any elongation compounds or intermediates, such as acetate and ethanol in soil microcosms fed with no substrate. This confirmed that both electron donor, either ethanol or hydrogen and electron acceptor, acetate are essential for all tested conditions to initiate the microbial chain elongation reaction.

Maximum concentrations of the C4 and C6 products: We observed 4.9 g L^{-1} of butyrate (C4) and 4.1gL^{-1} caproate (C6) produced at the end of our experiments with ethanol +

acetate condition using the mixed microbial community from the soil. These concentration are similar to the trends seen from literature, which have been summarized in Table 3.

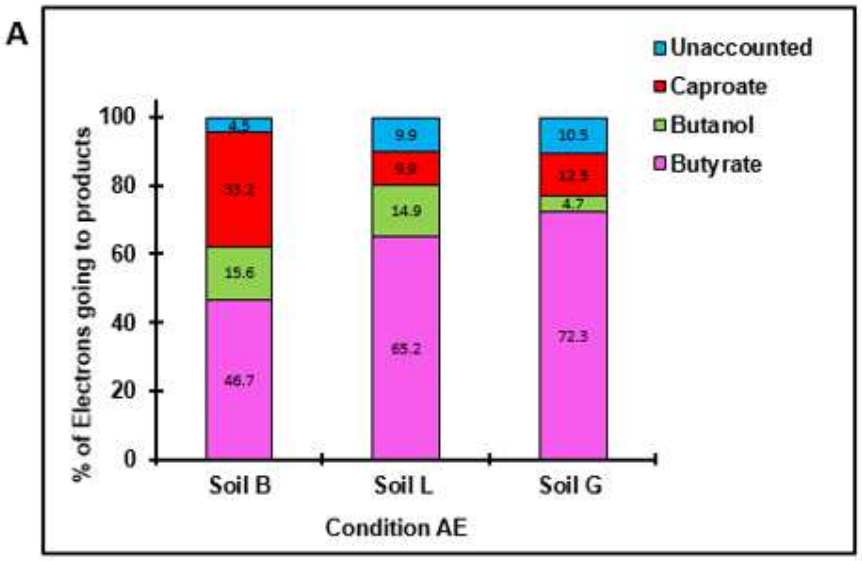
Table 3. Comparison of substrates (readily biodegradable organic compounds) added, biocatalyst used, and maximum concentration of the products formed.

Readily biodegradable feedstock	Biocatalyst	Concentration of products formed ($g\ L^{-1}$)	Reference
Ethanol + Acetate	Mixed microbial community from soils	C4: 3 ± 2, C6: 1 ± 0.5, C4 alcohol : 1 ± 0.5	This study
Ethanol + Acetate	Reactor microbiome	C6: 8.2, C8: 0.3	(Steinbusch et al. 2011)
Ethanol + Acetate	Reactor microbiome	C6: 11.1, C8: 0.6	(Grootscholten et al. 2014)
Ethanol + Acetate	Reactor microbiome	C6: 12, C8: 0.9	(Grootscholten et al. 2013b)
Ethanol + propionate	Reactor microbiome	C6: 4.9, C7: 3.2	(Grootscholten et al. 2013a)
syngas effluent	Reactor microbiome	C6: 1.7	(Vasudevan, Richter, and Angenent 2014)
Ethanol + Acetate + propionate	mixed anaerobic culture	C4: 8.3, C5: 2.2, C6: 8.6,	(Reddy, Mohan, and Young-Cheol 2017)
Ethanol + Acetate + propionate	mixed anaerobic culture	C4: 3.9, C5: 3.1, C6: 6.4	(Coma et al. 2016)
Ethanol + Acetate	pure culture of *Clostridium kluyveri*	C4: 2.6, C6: 12.8	(Weimer and Stevenson 2012)
Lactate	microbiome enriched from pit mud	C4: 2.7, C5: 0.9, C6: 10.9	(Zhu et al. 2017)
Ethanol + Acetate	pure culture of *Clostridium kluyveri*	C4: 1.5, C5: 0.3, C6: 2.9	(Yin et al. 2017)

In summary, our results demonstrate the production of longer fatty acids (butyrate, valerate, caproate, heptanoate), along with alcohol (butanol), and molecular hydrogen from this microbial chain elongation reaction in all the soils, using different combinations and concentrations of substrates, with mixed microbial culture naturally present in the soils.

3.4 Addition of substrates increased the distribution of electrons to longer fatty acids and alcohol

As substrates like ethanol, acetate, and hydrogen were added to the soil microcosms and methanogenesis was inhibited, most amount of carbon and electrons go towards the formation of fatty acids and butanol. Among the fatty acids, the majority fraction of carbon and electrons go to form butyrate in the initial phase and later to caproate. Unaccounted electrons include those for hydrogen and biomass production, caprylate (C8) and unmonitored/unknown compounds. Figure 5 provides an analysis of electron distribution from substrates to end products, taking into account the initial amount of electrons (substrate + COD) available.



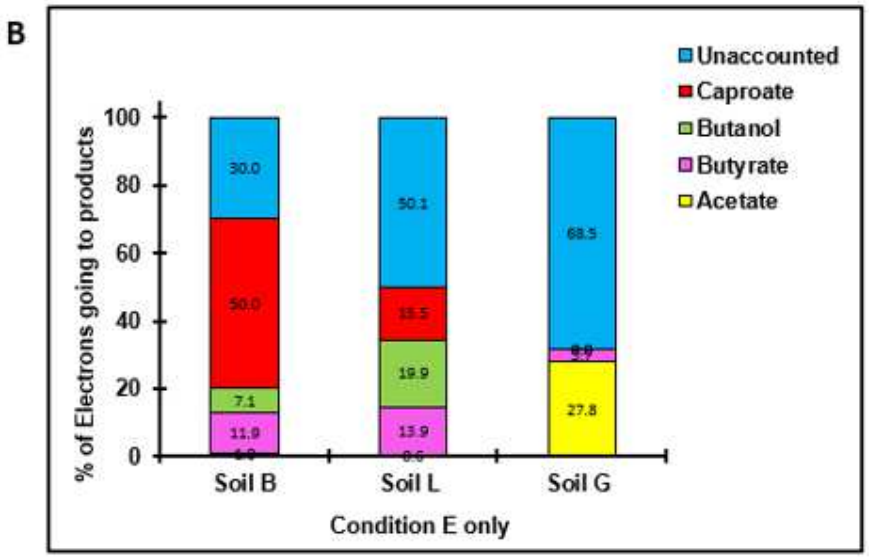


Figure 5. Electron balances in ethanol + acetate and only ethanol conditions.

Figure A. shows electron distribution from substrates (electron donor being ethanol and electron acceptor being acetate) to end products (butyrate, caproate, and butanol) corresponding to cycle 4 (time=36 to 44 days) of ethanol + acetate (AE) condition. Figure B. shows electron distribution from substrates (electron donor being ethanol) to end products (acetate, butyrate, caproate, and butanol) corresponding cycle 1 (time=1to 14 days) of only ethanol condition. As methanogenesis has been inhibited by adding BES, electrons channeled to butyrate, caproate, butanol, and hydrogen have increased depending on the presence and addition of substrates. The highest fraction electrons in the substrate were utilized for butyrate production, butyrate being a dominant chain elongation product in initial stages of ethanol + acetate, mixed culture fermentation. In ethanol only condition, fraction of electrons channeled to caproate production is more than other substrate combinations. The fermentation products remained the same, though seen in different proportions due to different substrate conditions and soil properties.

3.5 Addition of substrates selected for chain elongators within the phylum Firmicutes:

Figure 5 shows community and distribution of the major phylotypes identified (at family level) during the start and the end of microbial metabolic chain elongation semi batch experiment, from anaerobic slurries of soil, using 16S rRNA-based community analysis. The addition of substrates such as ethanol, acetate, and hydrogen have notably changed the microbial community structure. Initially the soils were dominated by phylum proteobacteria, and were considerably enriched later majorly by bacteria within order *Clostridiales* (Esquivel-Elizondo et al. 2018) and *Bacillales* (Coma et al. 2016) in phylum firmicutes and minorly within phylum bacteriodetes (represented in different shades of green in Figure 5.). Microbiota structure changed drastically (Esquivel Elizondo, Ilhan, et al. 2017) on addition of chain elongation substrates. From their 16S rRNA sequences data, relative abundance of clostridiales increased by minimum 30% to a maximum of 90% in all the different soils with ethanol + acetate condition, at the end of the experiment. Similar trends in the relative abundance of microbial communities were observed for acetate + hydrogen and ethanol + hydrogen conditions. The increased relative abundance of phylotypes within clostridiales correlate with higher distribution of electrons to fatty acids such as butyrate and caproate, observed in the presence of ethanol and acetate. Clostridiales were also reported to be abundant in chain elongation processes supplied with different combination of small chain fatty acids and alcohol (butanol) with ethanol and acetate (Coma et al. 2016). Many clostridium species are known for considerable fatty acid production such as acetate, butyrate, and caproate, (C4) alcohol butanol, and hydrogen, so usually detected or chosen for in fatty acid reactor experiments (Coma et al. 2016). Butanol is seen to be naturally formed by a number of clostridia. In the first phase of fermentation which is acidogenic, clostridia usually produce fatty acids and hydrogen, during their

exponential growth phase. The second, solventogenic phase, they mostly produce butanol (Lee et al. 2008).

Predominant phylotypes at the family level that were enriched and detected at the end of experiments were *Clostridiaceae* (Esquivel Elizondo, Delgado, et al. 2017, Steinbusch et al. 2011)*, Peptococcaceae*, *Lachnospiraceae*, (Esquivel-Elizondo et al. 2018, Esquivel Elizondo, Ilhan, et al. 2017) *Porphyromonadaceae* (Esquivel Elizondo, Delgado, et al. 2017)*, Bacillaceae,* and *Ruminococcaceae* (Steinbusch et al. 2011, Coma et al. 2016). Phylotypes in the three families *Ruminococcaceae, Clostridiaceae,* and *Lachnospiraceae* are identified for being responsible for high butyrate production observed throughout the fermentation (Esquivel Elizondo, Ilhan, et al. 2017). Certain species responsible for chain elongation, such as *Clostridium kluyveri, Megasphaera elsdenii* (Spirito et al. 2014), *Azospira oryzae* (Steinbusch et al. 2011), *Eubacterium pyruvativorans* (Angenent et al. 2016), and their relatives have dominantly seen to be proliferated and isolated from chain elongation reaction mixtures. In conclusion, high-throughput 16S rRNA gene sequencing analysis enabled the studying of microbial community adaptability to target substrates (Coma et al. 2016), and revealed that addition of substrates such as ethanol, acetate, and hydrogen have an important influence on the microbial community structure and function, specifically of microbes involved in fatty acids such as butyrate, and caproate production (Esquivel Elizondo, Ilhan, et al. 2017). From our relative abundance data, we observed that, the potential genus performing chain elongation was mainly *Clostridium*. Other genera included *Bacillus, Caloramator, Oxobacter, Coprococcus, Desulfosporosinus, Oscillospira, Ruminococcus, Syntrophomonas, and Sedimentibacter*. All these genera were anaerobic, and were mostly as gut or soil microbe.

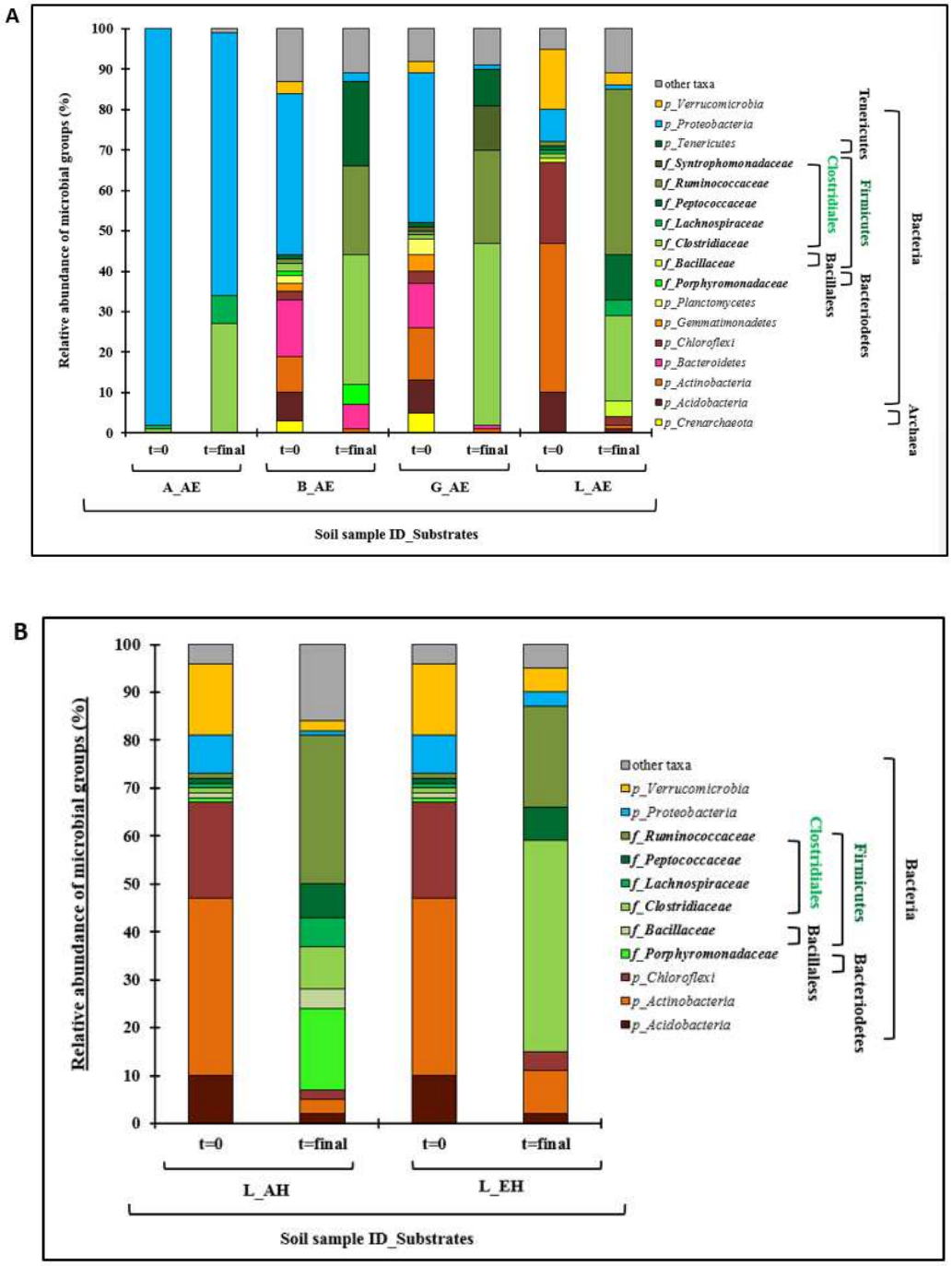


Figure 6. Relative abundance of major phylotypes present in the soil.

Main phylotypes detected, at phylum, order, and family level, during microbial chain elongation of substrates such as ethanol, acetate, and hydrogen to butyrate, caproate, butanol, and hydrogen. Relative abundances are average of duplicates. **A.** shows the

distribution of microbial community at the start and end of the ethanol + acetate conditions. **B.** shows the distribution of microbial community at the start and end of the ethanol + hydrogen and acetate + hydrogen conditions of the soil microcosms. The other taxa (grey block) stands for unidentified microorganisms within the taxonomic classification.

In conclusion, high-throughput 16S rRNA gene sequencing analysis enabled us to study the microbial community of the soils at the start and end of the experiment, and adaptability of mixed soil microbial community to target/added substrates. From this study, it has revealed how experimental conditions influence the microbial community structure and function, specifically of microbes involved in sustained fatty acids (C4 and C6) production were enriched.

3.6 Possible impact of microbial metabolic chain elongation process on soil and environment

Dynamics (fate and transport) of organic carbon in the soil, and the exchange of carbon between soil and atmosphere are complex, having implications on carbon balances and soil health (Kirkels, Cammeraat, and Kuhn 2014). Terrestrial (land based) organic matter can be found in many places, such as in the leaves that have fallen from a tree (leaf litter/foliage biomass), algae attached to rocks, animal feces rich in gut flora as part of the soil. Wetlands such as bogs, marsh, peats, and swamps are ecosystems rich in organic matter. Landscapes such as wetlands, soil associated with them is saturated with water, slowing decomposition, and causing wetlands to accumulate organic matter. It is studied that, soil organic matter comprising of soil organic carbon has a strong impact on soil quality and functionality which include retention and cycling

of nutrients, retention and transmission of water, aeration and gaseous exchange, biotransformation of compounds, buffering of soil reaction, transport of trace metals, maintenance of biodiversity by supplying food for soil microbes, and greenhouse gas emissions (Lal 2016). Hydrophobicity of soil and dynamics of soil moisture depends on accumulation of long chain organic compounds between soil particles, reducing the wettability of the soil (Doerr, Shakesby, and Walsh 2000). Microbial chain elongation can be one of the processes which contribute to the formation of long chain organics. Biological/microbial activity has a long term influence on mechanical characteristics of soil such as soil structure, texture, penetration resistance, stiffness, and shear strength (Aspiras et al. 1971). Addition of readily decomposable substrates causes rapid stimulation of soil microflora/microbial biomass, which is accompanied by a significant increase in water aggregate stability of the soil with different textural composition. In our case, addition of ethanol and acetate which are readily consumed substrates can stimulate growth of chain elongating microbial community. Due to microbial synthesis of soil binding agents, it would be a microbial based soil amendment process, having a desired effect in poorly structured and unstable soils, which are susceptible to erosion (Kiem and Kandeler 1997).

CHAPTER 4

CONCLUSIONS AND OUTLOOK

In the presented feasibility study, we observed that microbial chain elongation pathway occurred/was ubiquitous in all the four tested soils, thus it is an important event to be considered in carbon cycling. At a mildly acidic to near neutral pH, we observed a sustained, stable production of elongation product from the mixed microbial culture, enriched from soil, under anaerobic and methanogenesis suppressed conditions. It has been studied that, microbes gain energy by transferring electrons from a more negative potential to a more positive one during the chain elongation process. Thus, ethanol oxidation coupled with chain elongation is a thermodynamically favorable process.

The common even numbered carbon products of microbial chain elongation process in soil were butyrate, caproate, butanol, along with molecular hydrogen, which were produced from synthetic substrates such as acetate being electron acceptor with reduced compounds such as ethanol or hydrogen being electron donors. Uneven/odd numbered carbon chains such as valerate and heptanoate, were also formed through this elongation process when substrates such as propionate were used. However for different soils, varying trends in product concentrations and formation rates were observed due to difference in the physicochemical and microbial characteristics of the soils, and substrate combinations used. At the end of the experiment, the microbial community of soils was seen to enrich with microbes belonging from families such as *Clostridiaceae, Peptococcaceae*, *Lachnospiraceae*, *Porphyromonadaceae, Bacillaceae,* and *Ruminococcaceae.* One of the advantages of testing chain elongation with mixed culture is, they are resilient and stable, resulting in higher production of elongation products compared to using pure strains. Microbial chain elongation with enriched microbial cultures from soil can also be an important platform for biotechnological

production and bioprocessing of more stable and value added specialty medium chain fatty acids. Products such as caproate which can be used as precursors to biofuels can make this chain elongation process, one of the technology to generate renewable energy.

In mixed anaerobic systems, methanogenesis is a major electron sink. Under methane inhibiting conditions, we observed increase in the fraction of electrons directed to production of longer fatty acids and alcohols. These complex organic molecules can be dead-end products that may accumulate in deep soils and sediments, as they are more stable, and not very easily degradable, unlike the short/simple carbon compounds. In a way, it might help to sequester carbon in the soil for a longer time duration, affecting the carbon cycle. Its implication being, control of greenhouse gas production/emission, which occur due to mineralization of organic carbon to carbon dioxide and methane, thus mitigating environmental conditions and climate changes.

Due to increased microbial activity and carbon stabilization in forms of larger organic compounds, it can affect soil productivity, strength, aggregate stability, and water holding ability. As large concentrations of molecular hydrogen are produced during elongation reactions, it also shows potential as a novel bioremediation technique that rely on addition of carbon sources in the soil, for oxidized contaminants in the soil and groundwater.

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