Amino Acid and Related Catabolism of *Thermoanaerobacter* Species

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Philosophiae Doctor degree in Biology

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Abstract

The protein and amino acid metabolism of thermophilic bacteria has been a neglected aspect of their physiology despite its biotechnological potential. Under anaerobic conditions, the metabolism of many proteogenic amino acids requires the presence of an electron scavenging system in order to effectively remove reducing equivalences and make the overall reaction thermodynamically favorable. *Thermoanaerobacter* and *Caldanaerobacter* strains cultivated on branched-chain amino acids (BCAAs) in the presence of a methanogen or thiosulfate resulted in production of their corresponding branched-chain fatty acid (BCFA) or a mixture including the BCFA and the corresponding branched-chain alcohol (BCOH), respectively. Thus, these electron scavenging systems reveal a difference in the electron flow resulting in different fermentation products. Studies involving the entire genus showed differences in amino acid utilization patterns and the degree to which strains are proteolytic, although the inherent ability to degrade BCAAs to their corresponding BCFA and BCOH appears to be a universal feature. However, the ratio of BCOH/BCFA varies widely. Initially, the pathway responsible for BCOH formation was unclear; subsequent investigations using ^{13}C-labeled BCAAs, as well as exogenously added fatty acids (e.g. acetate, 3-methyl-1-butyrate) demonstrated that some *Thermoanaerobacter* and *Caldanaerobacter* strains could use the fatty acids as electron acceptors and convert them to their corresponding alcohols. Subsequent work demonstrated that *Thermoanaerobacter pseudoethanolicus* and *Thermoanaerobacter* strain AK85 are useful agents for the biotransformation of C2-C6 fatty acids to their corresponding alcohols in the presence of an electron donor.

Útdráttur

Sá hluti lífeðlisfræði hitakærra baktería sem snýr að prótein- og amínósýruefnaskiptum hefur að mestu verið vanræktur í gegnum tíðina þrátt fyrir möguleika sem í þeim felast á sviði líftækni. Við loftfirrðar aðstæður krefjast margar próteinmyndandi amínósýrur utanaðkomandi rafeindaþega til þess að úr verði orkufræðilega hagstæðar aðstæður fyrir niðurbrotið. *Thermoanaerobacter* og *Caldanaerobacter* stofnar sem ræktaðir voru á greinóttum amínósýrum (e. BCAAs) ýmist í viðurvist metanframleiðanda eða þíósúlfats framleiddu annars vegar samsvarandi greinóttar fitusýrur (e. BCFA) og hins vegar blöndu af greinóttum fitusýrum og samsvarandi greinóttum alkóhólum (e. BCOH). Þessi rafeindaþegakerfi sýna mun á rafeindaflæði sem leiðir af sér framleiðslu mismunandi lokaafurða. Rannsóknir sem framkvæmdar voru á ættkvíslinni leiddu í ljós mun á niðurbrotsmynstri amínósýra og vörpuðu ljósi á að hvaða leyti stofnarnir eru prótínsundrandi þó svo að hæfnin til að brjóta niður greinóttar amínósýrur og mynda úr þeim greinóttar fitusýrur og alkóhól virðist gilda um þá alla. Hlutföllin milli framleiddra greinóttra alkóhóla og fitusýra eru hins vegar mjög breytileg. Upphaflega var BCOH framleiðsluferlið óljóst en með notkun ^{13}C-merktra greinóttra amínósýra og viðbættra fitusýra (t.d. asetats, 3-metýl-1-bútýrats) var hægt að sýna fram á hæfni sumra *Thermoanaerobacter* og *Caldanaerobacter* stofna til þess að nýta fitusýrurnar sem elektrónuþega og breyta þeim í samsvarandi alkóhól. Rannsóknir sýndu einnig að *Thermoanaerobacter pseudoethanolicus* og *Thermoanaerobacter* stofn AK85 megi nýta til lífummyndunar á C2-C6 fitusýrum í samsvarandi alkóhólsambönd í viðurvist rafeindagjafa.

Dedicated to my offspring

May your future be bright and full of adventure

List of Papers

This work is based upon work summarized in eight papers, four of which have been previously published, one has been accepted for publication, and three are in the format of manuscripts.

Paper I - Branched-chain alcohol formation by *Thermoanaerobacter brockii* and *Thermoanaerobacter yonseiensis*. (2014). *Anaerobe*. 30, 82-84.

Paper II - Branched-chain alcohol formation by thermophilic bacteria within the genera of *Thermoanaerobacter* and *Caldanaerobacter*. *Extremophiles*. (2015). 19(4):809-818. doi: 10.1007/s00792-015-0756-z

Paper III - Amino acid metabolism of *Thermoanaerobacter* strain AK90: the role of electron scavenging systems on end product formation. (2015). *Journal of Amino Acids*. doi:10.1155/2015/410492.

Paper IV – Branched-chain amino acid catabolism of *Thermoanaerobacter* strain AK85 and the influence of culture conditions on branched-chain alcohol formation. (2019). Accepted for publication in *Amino Acids.* doi: 10.1007/s00726-019-02744-z

Paper V – Protein and amino acid metabolism of *Thermoanaerobacter pseudoethanolicus*: role of carboxylic acid reduction in branched-chain alcohol formation. Submitted to *Applied and Environmental Microbiology*.

Paper VI – Protein and amino acid metabolism of *Thermoanaerobacter* and *Caldanaerobacter* species. Unpublished manuscript.

Paper VII - Biotransformation of organic acids to their corresponding alcohols by *Thermoanaerobacter pseudoethanolicus*. (2019). *Anaerobe*, 57, 28-31.

Paper VIII - Bioreduction of organic acids to alcohols by *Thermoanaerobacter pseudoethanolicus*. Submitted to *Biotechnology and Bioengineering*.

Works not included in this thesis

Scully, S.M., Orlygsson, J. (2019). Dataset Describing the Amino Acid Catabolism of *Thermoanaerobacter* strain AK85: the Influence of Culture Conditions on End Product Formation. *Data in Brief*, 24, DOI: 10.1016/j.dib.2019.103938

Declaration of Contribution

Paper I

Scully and Örlygsson designed the experiments while Scully performed the laboratory work, performed the data analysis, and wrote a rough draft of the manuscript. Örlygsson reviewed and edited the manuscript.

Paper II

Örlygsson and Scully designed the screening and experimental work involving the culture collection. Iloranta, Myllyamaki, and Scully performed the experimental work with Scully providing supervision. Scully collected and analyzed the data and wrote a preliminary version of the manuscript. Örlygsson edited the manuscript.

Paper III

The experiments were designed by Örlygsson and Scully while Scully performed the laboratory work, data analysis, and wrote the first draft of the manuscript. Örlygsson reviewed and edited the manuscript.

Paper IV

Scully designed the experiments and performed the laboratory work, data analysis, and wrote the first draft of the manuscript. Sigríður Jónsdóttir (University of Iceland) performed the NMR analysis while Scully interpreted the spectra. Örlygsson reviewed and edited the manuscript.

Paper V

Scully designed the experiments and performed the laboratory work with assistance from Ingvadóttir. Scully performed the data analysis and wrote a first draft of the manuscript. Örlygsson reviewed and edited the manuscript. Sigríður Jónsdóttir (University of Iceland) performed the NMR analysis while Scully interpreted the spectra. Scully and Örlygsson reviewed and edited the manuscript.

Paper VI

Experiments were designed by Scully who performed most of the experimental work with assistance from Ingvadóttir and Cooney (University of Leeds). Scully performed all of the isotopically labeled experiments. NMR analysis was performed by Sigríður Jónsdóttir (University of Iceland), and Scully interpreted the NMR spectra. Scully analyzed the data and prepared the manuscript drafts. Örlygsson reviewed and edited the manuscript.

Paper VII

Scully designed the experiments and worked with Brown in the laboratory conducting the experimental work. Ross and Brown performed the GC-MS work at the University of Leeds. Scully collected the fermentation data including that with the isotopically-labeled substrates. Scully analyzed the data and prepared the manuscript draft which was critically edited by Brown. Ross and Örlygsson reviewed and edited the manuscript.

Paper VIII

Scully designed the experiments and worked with Brown in the laboratory conducting the experimental work. Scully collected and analyzed the data and prepared the manuscript draft which was critically edited by Brown. Ross and Örlygsson reviewed and edited the manuscript.

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Abbreviations

AA – Amino acid

ABC – ATP Binding Cassette

AD – Anaerobic digestion

ADH – Alcohol dehydrogenase

ADP - Adenosine diphosphate

AOR – Aldehyde oxidoreductase

ArAA – Aromatic amino acid

ATP - Adenosine triphosphate

BCAA – Branched-chain amino acid

BCFA – Branched-chain fatty acid

BCOH – Branched-chain alcohol

BES - 2-Bromoethanesulfonate

BM – Basal mineral medium

CAR – Carboxylic acid reductase

CBP – Consolidated bioprocessing

GC – Gas chromatography

CoA – Coenzyme A

DH – Degree of hydrolysis

DSMZ - Deutsche Sammlung von Mikroorganismen und Zellkulturen

KDC - 2-Keto acid decarboxylase

GABA - 4-Aminobutanoic acid

L-G – Liquid-gas phase rato

LPSN – List of Prokaryotes with Standing in Nomenclature

MS – Mass spectrometry

MW – Molecular weight

NAD^+ - Nicotinamide adenine dinucleotide

NADH - Nicotinamide adenine dinucleotide (reduced)

$NADP^+$ - Nicotinamide adenine dinucleotide phosphate

NADPH - Nicotinamide adenine dinucleotide phosphate (reduced)

NBT - Nitrotetrazolium Blue chloride

NMR – Nuclear magnetic resonance

OD – Optical density

pCO_2 – Partial pressure of carbon dioxide

PADH – Primary alcohol dehydrogenase

PDC - Pyruvate dehydrogenase complex

pH_2 – Partial pressure of hydrogen

PMF – Proton motive force

PMS - Phenazine methosulfate

SADH – Secondary alcohol dehydrogenase

SCFA – Short-chain fatty acid

SLP – Substrate level phosphorylation

SMF – Sodium motive force

TCA – Tricarboxylic acid cycle

THF - Tetrahydrofolic acid

TLC – Thin layer chromatography

TPP - Thiamine pyrophosphate

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First and foremost, I would like to express my sincere gratitude to Professor Jóhann Örlygsson for serving as my supervisor over the past years. His tremendous patience with my "difficult personality" is no small feat and should be widely acknowledged. I would also like to thank Eva María Ingvadóttir for her friendship over the years as well as her assistance in the laboratory.

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1 The Genus of *Thermoanaerobacter*

Class *Clostridia* is a highly diverse collection of strictly anaerobic, spore-forming, Gram positive rods, non-sulfate reducing bacteria with a high diversity metabolic capabilities (Cato & Stackebrandt, 1989). This includes the degradation of carbohydrates, proteins and amino acids, and purines with some species even being able to ferment organic acids and alcohols (including diols and keto alcohols). The majority of the *Clostridia* species which have received most attention are those with clear biotechnological potential, such as solvent production, or those that are disease causing agents. This, unfortunately, has created many knowledge gaps with respect to the physiological intricacies of other *Clostridia*, including those that are thermophilic such as species within the genera of *Thermoanaerobacter* and *Caldanaerobacter*.

Interest in thermophilic *Clostridia* has increased steadily since the 1920s with the description of *Clostridium thermocellum*, noteworthy for its ability to degrade crystalline cellulose (Viljoen, Fred, & Peterson, 1926). By 1979, only a handful of thermophilic *Clostridia* had been described including several species now within *Thermoanaerobacteraceae*, including *Thermoanaerobacterium* (formerly *Clostridium*) *thermosaccharolyticum* and *Thermoanaerobacter* (formerly *Clostridium*) *thermohydrosulfuricum* (now *Thermoanaerobacter*), and *Moorella* (formerly *Clostridium*) *thermoaceticum* in addition to the aforementioned *Clostridium thermocellum* (Canganella, 2013). In the decades since, the number of validly described thermophilic *Clostridia* has grown to more than one hundred individual species (Wagner & Wiegel, 2008). Much of this increased interest in the biotechnological potential of thermophilic *Clostridia* can likely be attributed to the desire to move away from petroleum-derived fuels towards renewables such as bioethanol that was popular in the 1970s particularly with respect to the fermentation of carbohydrates (Scully & Orlygsson, 2015). Furthermore, more concerted efforts to bioprospect geothermal areas such as the features of Yellowstone National Park (WY, USA) have also brought increased attention to thermoanaerobes in general.

Much of the work on the physiology of thermophilic *Clostridia* has focused almost exclusively on their fermentation of carbohydrates. While the ability to degrade proteins and amino acids has been characterized in the preliminary works reporting novel species, comprehensive studies of their ability to degrade the twenty proteogenic amino acids and perform Stickland reactions (described in further detail in Section 2.3.3) are seldom reported. This chapter will focus exclusively on the genera of *Thermoanaerobacter* and *Caldanaerobacter*.

Historically, members of *Clostridia* were largely mesophilic although several families within class *Clostridia* are exclusively thermophilic with two orders (*Thermoanaerobacteriales* and *Thermodesulfobiaceae*) and related families as shown in Figure 1. Reclassifications of clades within the genus with the increasing popularity of 16S rDNA sequencing revealed deep divisions allowing more defined taxonomic classification of interrelationships between characterized species (Cato & Stackebrandt, 1989). A number of species within the class are moderately thermophilic, with T_{opt} between 50 and 60°C, while several clades are truly thermophilic (T_{opt} > 60°C) (Brock, 1986; Sundaram, 1986).

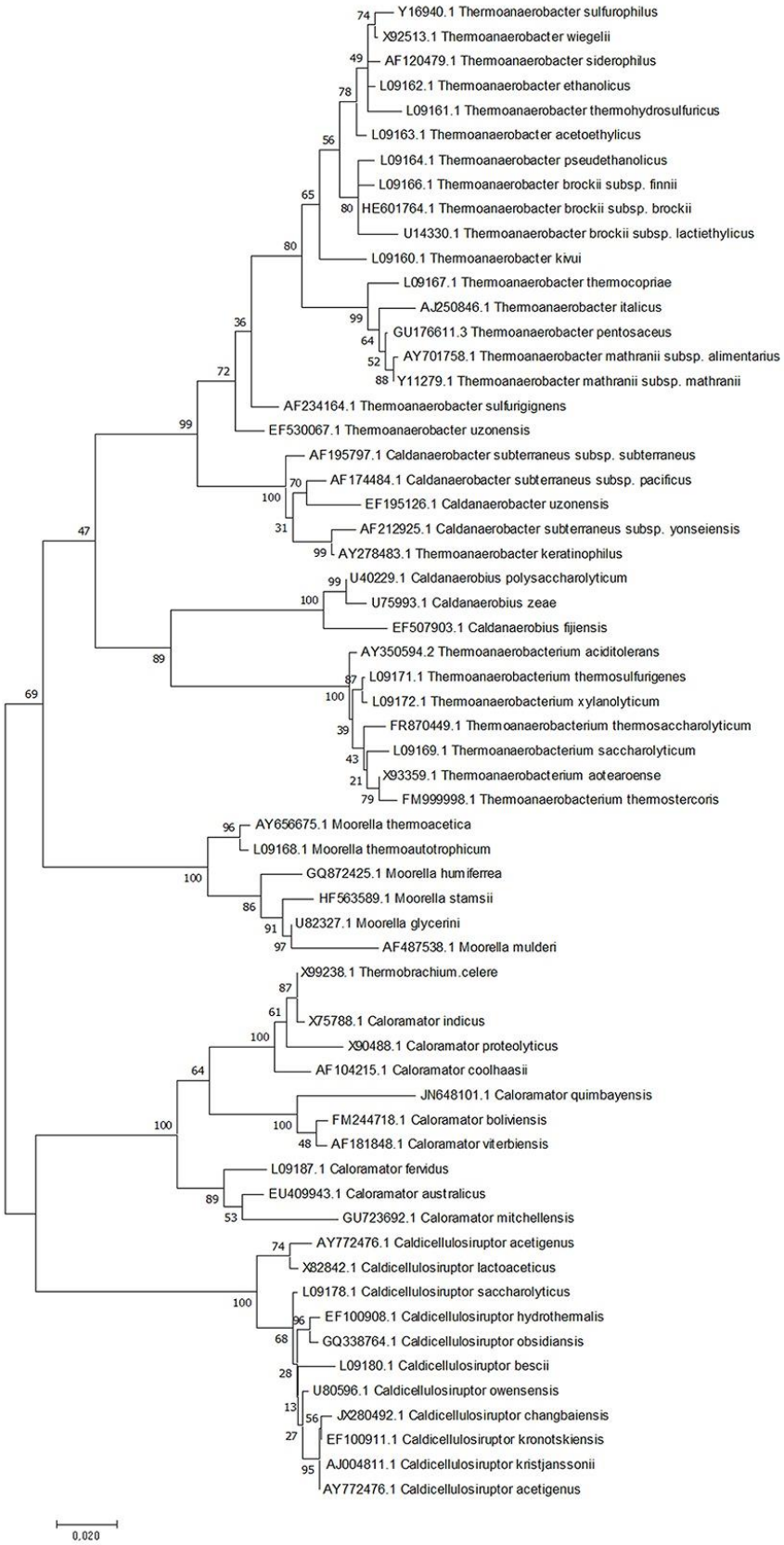

Figure 1 – Phylogeny of selected thermophilic Clostridia based upon their 16S rDNA sequences

The family *Thermoanaerobacteraceae* is a highly polyphyletic family of thermophilic anaerobes belonging to class *Clostridia*. At present, the family contains four genera: *Thermoanaerobacter*, *Caldanaerobacter*, *Thermoanaerobacterium*, and *Moorella*. At present, there are no phenotypic standards for members of class *Clostridia* despite some efforts by Holdeman to outline common taxonomic features (Stackebrandt & Hippe, 2001). As is the case with the genus of *Clostridium*, there are no standard phenotypic characteristics for inclusion of members within the genera of *Thermoanaerobacter* or *Caldanaerobacter* and recent characterization papers frequently exclude features reported for previously described species. Like other *Clostridia*, there is a general recognized need for strictly anaerobic growth, a lack of sulfate reduction, and, in some cases, spore formation. The lack of standardization has led to fragmentary descriptions of member species with serious deficiencies with respect to the structure of the cell wall, respiratory enzymes, cellular lipid composition, substrate spectra, enzymology, and physiology, although basic physiological and biochemical properties (temperature and pH growth range and optima, lipase, lecithinase, indole, acetylmethylcarbinol, urease production, esculin and gelatin hydrolysis, meat digestion, action on milk, utilization of sugars and polymers, volatile fatty acid profiles) are usually reported for many, but not all, *Clostridia*.

Much like other *Clostridia*, the majority of members within family *Thermoanaerobacteraceae* are thermophilic neutrophiles with broad substrate spectra and metabolic diversity: cellulolytic, saccharolytic, proteolytic, purinolytic, and lithotrophic (using H_2 and CO_2) although complete and systematic screening of these metabolic capabilities is seldom reported consistently or in detail with respect to features typically reported for other *Clostridia* making direct comparisons difficult (Collins et al., 1994; Wiegel, Tanner, & Rainey, 2006). As is the case with other *Clostridia*, *Thermoanaerobacter* species are obligate anaerobes and often require a low redox potential for growth. The obligate anaerobic nature of clostridia species is due to a lack of cytochrome and electron transport machinery; as such, strains rely upon the generation of ATP by substrate level phosphorylation (SLP). Many strains are inhibited by hydrogen accumulation thus causing them to rely on interspecies hydrogen transfer to remove reducing equivalence and degrade substrates as the thermodynamics are otherwise unfavorable. Many of the species validly described have not been characterized further than the work presented in the initial characterization work. As there is no standardized system for the characterization of organisms within class *Clostridia* let along specific genera, this has left a number of gaps in knowledge with respect to their carbohydrate, amino acid, and inorganic (lithotrophic) metabolism.

The following sections within this chapter focus on chemotaxonomic features of *Thermoanaerobacter* and *Caldanaerobacter*. For a more thorough review of thermophilic clostridia or members of *Thermoanaerobacteraceae*, the reader is directed to (Wagner & Wiegel, 2008). Some of the biotechnological applications of species within these genera are also briefly discussed.

The genus *Thermoanaerobacter* is within Family I *Thermoanaerobacteraceae* in Order *Thermoanaerobacteriales* within Class *Clostridia* and currently contains 19 species and 5 subspecies according to LPSN (Parte, 2014). The type strain of the genus is *T. ethanolicus*, an isolate from Yellowstone National Park notable for being highly ethanologenic (Collins et al., 1994; Wiegel & Ljungdahl, 1981). Other strains which likely represent novel species in their own right, such as "*Thermoanaerobacter keratinophilus"*, have also been described (Riessen & Antranikian, 2001), although this strain does not have standing in nomenclature (Parte, 2014) and is likely a subspecies of *Caldanaerobacter subterraneus* based upon 16S rDNA analysis. Many of the members of *Thermoanaerobacter* were originally classified as *Clostridium* or the now obsolete genus of *Thermoanaerobium* prior to their reclassification with the migration of *Thermoanaerobium brockii* to the novel genus of *Thermoanaerobacter* (Collins et al., 1994). Other members were transferred to yet other genera such as *Caldicellulosiruptor* as was the case with "*Thermoanaerobium*" *acetigenum* (Nielsen, Mathrani, & Ahring, 1993; Onyenwoke, Lee, Dabrowski, Ahring, & Wiegel, 2006). Members of the genus have received considerable attention recently due to strains being highly ethanologenic and having broad substrate spectra. It was originally reported that one of the differentiating features between *Thermoanaerobacter* and *Thermoanaerobacterium* regards the end products of thiosulfate metabolism with *Thermoanaerobacter* species producing hydrogen sulfide and *Thermoanaerobacterium* producing elemental sulfur. This feature has since been shown to not adequately differentiate between the two genera.

Thermoanaerobacter can be divided into two groups: group I (*T. brockii*), group II (*T. italicus/mathranii*). The defunct third group contains members that now belong to the genus of *Caldanaerobacter* (Subbotina, et al., 2003). Three members previously assigned to this genus, *T. subterraneus*, *T. tengcongensis* and *T. yonseiensis*, were reassigned as subspecies of *Caldanaerobacter subterraneus* (Fardeau et al., 2004). The major differentiating feature of *Caldanaerobacter* species from *Thermoanaerobacter* species is alanine formation from glucose by the former.

Thermoanaerobacter species have been isolated from a diverse range of environs ranging from geothermal features to artificially heated anthropogenic environments. A number of species, such as *T. thermohydrosulfuricus*, appear to be cosmopolitan while others are less geographically distributed (Wiegel & Ljungdahl, 1984). *Thermoanaerobacter* strains (e.g. *T. mathranii* subsp. *Alimentarius*) have been isolated from canned meat (Carlier, Bonne, & Bedora-Faure, 2006). A number of isolates have also been prospected from deep sea vents (Subbotina et al., 2003). *T. thermocopriae* is cellulolytic and has been isolated from a diverse range of environments including camel feces, compost, soil, and a hot spring in Japan (Jin, Yamasato, & Toda, 1988). *T. mathranii* was isolated from a biomat and sediment sample, slightly alkaline hot spring, in Hveragerði, Iceland (Larsen, Nielsen, & Ahring, 1997).

Thermoanaerobacter species are thermophilic neutrophilic anaerobes, Gram positive or Gram-variable rods with temperature and pH optima around 65-75°C and 7.0, respectively.

Endospore formation is frequently observed. Species within the genus are saccharolytic and most are proteolytic; all species described can utilize thiosulfate as a terminal electron acceptor. The characteristics of species currently within the genus are summarized in Table 1.

Table 1 - Growth characteristics of Thermoanaerobacter and Caldanaerobacter type strains; optimum conditions are bolded where available.

Strain	Isolation Location	Growth Conditions			Reference
		$T_{min/opt/max}$ (°C)	$pH_{min/opt/max}$	NaCl (%)	
T. acetoethylicus (DSM 2359)	Geothermal feature, Yellowstone National Park (USA)	40/**65**/80	5.5/ND/8.5	ND	(Ben-Bassat & Zeikus, 1981; Rainey & Stackebrandt, 1993)
T. brockii subsp. *brockii* (DSM 1457)	Geothermal feature, Yellowstone National Park (USA)	35/**65-70**/85	5.5/**6.5-7.5**/9.5	ND	(Zeikus, Hegge, & Anderson, 1979)
T. brockii subsp. *finnii* (DSM 3389)	Lake Kivu (East Africa)	40/**65**/75	ND/**6.5-6.8**/ND	ND	(Cayol et al., 1995; Schmid, et al., 1986)
T. brockii subsp. *lactiethylicus* (DSM 9801)	Deep subsurface French oil well (FR)	40/**55-60**/75	5.0/**7.0**/8.5	0/**1**/4	(Cayol et al., 1995; Kondratieva, et al., 1989)
T. italicus (DSM 9252)	Geothermal feature (IT)	45/**70**/78	NR/**7.0**/NR	0/NR/1	(Kozianowski, et al., 1997)
T. ethanolicus (DSM 2246)	Geothermal feature, Yellowstone National Park (USA)	37/**69**/78	4.4/**5.8-8.5**/9.8	ND	(Juergen Wiegel & Ljungdahl, 1981)
T. kivui (DSM 2030)	Lake Kivu (CD)	50/**66**/72	5.3/**6.4**/7.3	ND	(Leigh, Mayer, & Wolfe, 1981; Leigh & Wolfe, 1983)
T. mathranii subsp. *mathranii* (DSM 11426)	Geothermal feature (IS)	50/**70-75**/75	4.7/**7.0**/8.8	ND[b]	(Larsen et al., 1997)
T. mathranii subsp. *Alimentarius*	Canned meat	45/ND/70	NR	NR	(Carlier et al., 2006)
T. pentosaceusus (DSM 25963)	CSTR fed household waste (DK)	50/**70**/80	5.5/**7.0**/8.5	0/ND/4	(Tomás, Karakashev, & Angelidaki, 2013)
T. pseudoethanolicus (DSM 2355)	Geothermal feature, Yellowstone National Park (USA)	ND/**65**/ND	ND	ND	(Onyenwoke, et al., 2007; Zeikus, Ben-Bassat, & Hegge, 1980)
T. siderophilus (DSM 12299)	Geothermal feature (Kamchatka, RU)	39/**70**/78	4.8/**6.5**/8.2	0/ND/3.5	(Slobodkin et al., 1999)
T. sulfurigenens (DSM 17917)	Geothermal feature (NZ)	34/**65**/72	4.0/**5.0-6.5**/8.0	ND[a]	(Y.-J. Lee et al., 2007)
T. sulfurophilus (DSM 11584)	Geothermal feature (Kamchatka, RU)	ND/**55-60**/ND	ND	ND	(Bonch-Osmolovskaya et al., 1997)
T. thermocopriae	Geothermal feature (JP)	47/**60**/74	6.0/**6.5-7.3**/8.0	ND	(Jin et al., 1988)
T. thermohydrosulfuricus (DSM 567)	Geothermal feature, Yellowstone National Park (WY, USA)	37/**67-69**/78	5.5/**6.9-7.5**/9.2	ND	(Lee, et al., 1993)
T. uzonensis (DSM 18761)	Geothermal feature (Kamchatka, RU)	32.5/**61**/69	4.2/**7.1**/8.9	ND	(Wagner et al., 2008)
T. wiegelii (DSM 10319)	Geothermal feature (NZ)	38/**65-68**/78	5.5/**6.8**/7.2	ND	(Cook, et al., 1996)
C. subterraneus subsp. *yonseiensis* (DSM 13777)	Geothermal feature (ID)	50/**75**/85	4.5/**6.5**/9.0	NR	(Kim, et al., 2001)
C. subterraneus subsp. *subterraneus* (DSM 13054)	Oil well (FR)	40/**65**/75	6.0/**7.5**/8.5	0/**0**/3	(Fardeau et al., 2004; Fardeau et al., 2000)
C. subterraneus subsp. *pacificus* (DSM 12653)	Deep sea thermal vent (Okinawa, JP)	50/**70**/80	5.8/**6.8-7.1**/7.6	ND/**2-2.5**/ND[c]	(Sokolova et al., 2001)
C. subterraneus subsp. *tengcongensis* (DSM 15242)	Geothermal feature (Tengcong, CN)	50/**75**/80	5,5/**7.0-7.5**/9.0	0/**0.2**/2.5	(Xue, et al., 2001)
C. uzonensis (DSM 18923)	Geothermal feature (Kamchatka, RU)	50/**68-70**/75	4.8/**6.8**/8.0	0/**0.5**/2	(Kozina, et al., 2010)

[a]Strain tolerates 1 M sodium thiosulfate, [b]growth not affected at 2% w/v NaCl; [c]requires seawater for growth; ND-Not determined, NR – Not reported

Several members of *Thermoanaerobacter* are noted spore-formers with several strains producing highly heat resistant endospores. For example, *T. siderophilus* endospores survive 121°C for at least 90 minutes (Slobodkin et al., 1999). The spores of *T. thermohydrosulfuricum* have been noted to be particularly resilient to elevated temperatures. Hyun and coworkers reported D-values of *T. thermohydrosulfuricus* of 770, 123, and 11 minutes at 100, 110, and 121°C, respectively (Hyun, et al., 1983). The authors demonstrated that the heat-resistance of these endospores was due to a thicker cortex layer as compared to mesophilic endospores. *T. brockii* subsp. *finnii*, despite being closely related to *T. ethanolicus*, forms heat-resistant endospores (Schmid et al., 1986). As a result, prolonged sterilization times have been used by several groups as a precautionary measure during media preparation and culture disposal (Chades, Scully, Ingvadottir, & Orlygsson, 2018; Hyun & Zeikus, 1985a).

Like many *Clostridia*, *Thermoanaerobacter* species demonstrate diverse metabolic capabilities. Members within the genus are typically heterotrophic and saccharolytic, degrading hexoses, pentoses, and starch to a mixture of ethanol, acetate, lactate, hydrogen, and CO_2. Additionally, other strains exhibit autotrophic features such as *T. kivui*, which is acetogenic and hydrogen oxidizing (Leigh, Mayer, & Wolfe, 1981; Leigh & Wolfe, 1983), while *Caldanaerobacter subterraneus* subsp. *pacificus* can utilize CO as the sole carbon source (Fardeau et al., 2004). It should be noted that autotrophic features such as the ability to utilize $CO/CO_2/H_2$ have not been widely examined for *Thermoanaerobacter* and *Caldanaerobacter* species.

Beyond being able to utilize the common hexoses (glucose, galactose, mannose), most strains are capable of degrading pentoses, including xylose although less commonly arabinose and disaccharides, notably maltose and cellobiose. The dominant end product is typically ethanol although strains producing acetate as the dominant end product (such as *T. kivui*) have been described and lactate is often present as an end product. Ethanol production by members of the genus has been extensively studied but will not be discussed here. Interested readers are directed to recent reviews on the subject (Scully & Orlygsson, 2015b; Scully & Orlygsson, 2017, 2018, 2019; Taylor et al., 2009).

All species within the genus are capable of degrading polysaccharides which commonly include amylose, xylan, and sometime pectin but rarely cellulose. *T. thermocopriae* seems to be unique insofar as it is the only strain with truly cellulolytic capabilities (Jin et al., 1988). Interestingly, an unexplored aspect of the glycolytic capabilities of many *Clostridia* including *Thermoanaerobacter* is the ability to degrade β-1,3-glycans although many species within the genus seem to be capable of degrading laminarin and lichenin (Scully, unpublished data). The utilization of sugar alcohols has not been uniformly examined although a recent study reveals that several members of the genus, such as *T. pseudoethanolicus,* can ferment mannitol to ethanol in high yield whilst fermentation times are prolonged (Chades et al., 2018). Interestingly, Subbotina and co-workers noted that number of *Thermoanaerobacter* isolates possessed features that have not been noted in

other strains, such as agarose hydrolysis and CO utilization (Subbotina et al., 2003). This serves to highlight the broad metabolic capabilities of *Thermoanaerobacter* species beyond the well-characterized type species.

Despite several studies on the ability of *Thermoanaerobacter* species to utilize amino acids (Fardeau, Patel, Magot, & Ollivier, 1997; Faudon et al., 1995), there has been relatively little mention of the proteolytic and amino acid degrading capabilities of members of the genus aside from sporadic mentions within the initial characterization papers. Interestingly, *C. subterraneus* subsp. *yonseiensis* was noted to produce propionate, butyrate, 3-methyl-1-butyrate (*iso*-valerate), 2-propanol, and 1-pentanol in small quantities when grown on glucose (Kim et al., 2001) although these end-products could be from fermentations of amino acids present in yeast extract. The amino acid metabolism of *Thermoanaerobacter* is covered in more detail in Chapter 2.

Thermoanaerobacter species also produce several heat-stable enzymes which have found biotechnological applications, particularly glycohydrolases. Pectinases are common among members of the genus (Lee et al., 1993) which have applications in the processing of lignocellulosic biomass; the pectinate lyase from *T. italicus* has been characterized (Kozianowski et al., 1997). An area of extreme interest has been the heat-stable glucosidases, amylases, and pullulanases from multiple *Thermoanaerobacter* species, including *T. thermohydrosulfuricus* and *T. pseudoethanolicus*, which have applications in the processing of starch (Antranikian, Zablowski, & Gottschalk, 1987; Bertoldo & Antranikian, 2002; Hii, Tan, Ling, & Ariff, 2012; Hyun & Zeikus, 1985a, 1985b; Madi, Antranikian, Ohmiya, & Gottschalk, 1987; Mathupala & Zeikus, 1993; Mathupala, Saha, & Zeikus, 1990; Melasniemi, 1988; Saha, Mathupala, & Zeikus, 1988). At least one *Thermoanaerobacter* strain has been shown to produce a cyclodextrin glycotransferase (Alcalde, Plou, Pastor, & Ballesteros, 1998; Avci & Donmez, 2009; Hyun & Zeikus, 1985c; Pedersen et al., 1999; Podkovyrov & Zeikus, 1992; Saha & Zeikus, 1990) which has applications in the production of cyclodextrins (Van der Veen, Uitdehaag, Dijkstra, & Dijkhuizen, 2000). The xylanases produced by many *Thermoanaerobacter* species have applications in paper-pulping (Jessen & Orlygsson, 2012; Sonne-Hansen, Mathrani, & Ahring, 1993) as well as designing co-cultures suited for the consolidated bioprocessing (CBP) of lignocellulosic biomass (Verbeke et al., 2013). As described later (Section 2.2.1), *C. subterraneus* subsp. *yonseiensis* produces a serine protease (Jang, Kim, Pyun, & Kim, 2002) and "*T. keratinophilus*" a heat-tolerant keratinase (Kublanov et al., 2009; Riessen & Antranikian, 2001; Tsiroulnikov et al., 2004).

Multiple strains of *Thermoanaerobacter* produce several alcohol dehydrogenases (ADHs) (Zhou et al., 2017) involved in ethanol formation, including those with selectivity for primary and secondary hydroxyl groups. These ADHs have been exploited in synthetic organic chemistry for the reduction of ketones to chiral alcohols and cofactor regeneration (Bryant & Ljungdahl, 1981; Bryant, Wiegel, & Ljungdahl, 1988; Seebach, Züger, Giovannini, Sonnleitner, & Fiechter, 1984; Sonnleitner, Fiechter, & Giovannini, 1984; Sonnleitner, Giovannini, & Fiechter, 1985). While beyond the scope of this work, the use

of *Thermoanaerobacter* ADHs in synthesis has been explored in the reviews cited herein (Faber, 2011; Musa & Phillips, 2011; Nealon, Musa, Patel, & Phillips, 2015; Scully & Orlygsson, 2019; Whitesides & Wong, 1985; Wong & Whitesides, 1994).

2 Anaerobic Protein and Amino Acid Metabolism

Due to the physiological importance and relative abundance of proteins in living things and the environment, the degradation of proteinaceous materials is a non-trivial matter. Amino acids are ubiquitous building blocks that are thought to have been synthesized abiotically on primordial earth (Bada & Diego, 2016; Kitadai & Maruyama, 2018; Miller, 1953; Parker et al., 2011). Spectra of amino acids have even been detected in interstellar space (Singh, Shivani, Misra, & Tandon, 2013; Thaddeus, 2006). The function of protein and amino acid metabolism includes nitrogen assimilation (review: Reid & Stutz, 2005), energy conservation, carbon and nitrogen cycling. Amino acids and their intermediates are building blocks for anabolic reactions, and osmolytes in addition to their role in protein structure and function. From a clinical standpoint, proteolysis and the degradation of amino acids can be an important diagnostic feature. The structure of the proteogenic α-amino acids is shown in Figure 2.

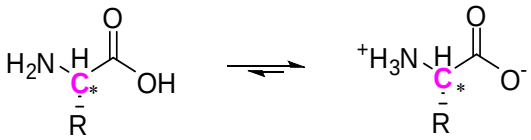

*Figure 2 - The generic structure of an α-amino acid and its rearrangement to a zwitterion; the α is highlighted pink, * denotes a chiral center*

Among the proteogenic amino acids, most have a single chiral center with the notable exceptions of glycine, which is achiral, and isoleucine and threonine which have a second chiral carbon atom on their side chains. It should be noted that the zwitterionic form dominates at and around pH 7.4. As such, the proteogenic amino acids incorporate only the L-enantiomer amino acids in proteins although D-amino acids do have biological significance (review: Radkov & Moe, 2014). The discussion herein will be limited strictly to the catabolism of L-amino acids.

The role of protein and amino acid metabolism is a vital component of the anaerobic food chain. Many amino acids can serve as the sole source of carbon, nitrogen and energy. End products of amino acid catabolism are generally a mixture of ammonium (NH_4^+), hydrogen

sulfide, thiols, disulfides, CO_2, volatile fatty acids, and alcohols (Mossel, Corry, Struijk, & Baird, 1995).

The catabolism of amino acids under anaerobic conditions is often dependent upon a terminal electron acceptor being present to drive thermodynamically unfavorable reactions; the presence of sulfate for its corresponding reduction to hydrogen sulfide is a classical example often employed by fermentative organisms in freshwater environments (Gottschalk, 1986). The energy yield of amino acid fermentation is similar to that of carbohydrate fermentation making them a potentially significant source of carbon and energy in the form of ATP (de Vladar, 2012).

It is widely understood that many bacteria require amino acids for growth and that proteinaceous materials are ubiquitous in many environments. However, the concentration of free amino acids in an environment is often difficult to ascertain. Studies of the amino acid concentration in rumen fluid found low concentrations (less that 1 mM) of amino acids such as aspartic acid, glutamic acid, alanine, glycine, valine, and leucine in addition to non-proteogenic amino acids such as gamma aminobutyric acid, and 5-aminovaleric acid (Wright & Hungate, 1967). It has long been recognized that many *Clostridia* require amino acids and vitamin supplementation for growth (Stickland, 1934) and thermophilic clostridia, such as *Thermoanaerobacter* species, require amino acid-containing yeast extract (Leigh et al., 1981; Wiegel, Tanner, & Rainey, 2006). The majority of the information on the amino acid catabolism among *Clostridia* has been gleaned from well-established proteolytic members such as *Clostridium sporogenes* (Stickland, 1935), *Clostridium botulinum* (Deklevat & Dasgupta, 1990; Peck, Stringer, & Carter, 2011; Tjaberg, 1973a, 1973b), *Clostridium sticklandii* (Elsden, Hilton, & Waller, 1976; Fonknechten et al., 2010), *Eubacterium limosum* and *Eubacterium ruminantium* (Blackburn & Hobson, 1962), and others (reviewed by McInerney, 1988). Detailed work on the enzymes involved in the amino acid metabolism of thermophilic *Clostridia* has not been reported in literature. Furthermore, other aspects of inorganic nitrogen metabolism such as nitrogen fixation have not been thoroughly investigated but have been reported among species of the closely related genera of *Thermoanaerobacterium* (Collins et al., 1994) although this is a well-established feature among other mesophilic *Clostridia* (Chen, 2005). The anaerobic degradation of proteins is important for the cycling of nitrogen and sulfur species. While the metabolism of proteins has been highly studied among anaerobic environments such as the rumen, tissue wounds, and in various food spoilage processes, the catabolism of proteins and amino acids among thermophiles has been scarcely examined despite their importance in nutrient cycling in geothermal environments.

The degradation of amino acids is a complex process involving several oxidation and reduction steps and is often only possible under specific conditions. Investigations into amino acid catabolism have primarily been restricted to mesophilic anaerobes particularly those found in ruminants such as *Cl. perfringens* and *Cl. bifermentans*, both of which are saccharolytic and strongly proteolytic. Furthermore, studies have often focused on the catabolic metabolism of protein rather than the fates of individual amino acids. While a number of studies have examined thermophilic *Clostridia*, these works have typically been limited to selective case studies rather than systematic investigations. The primary end products of amino acid fermentation include short-chain fatty acids, ammonia, CO_2, and hydrogen. The products and intermediates of amino acid catabolism have been noted to have an important role as flavor components in food and beverages. Many of the initial studies on the production of branched-chain and aromatic fatty acids and their corresponding alcohols and aldehydes have focused on microorganisms involved in fermentation. The steps of amino acid catabolism are discussed in detail in Chapter 2.3.

The key differences between aerobic and anaerobic amino acid catabolism is that in the former oxygen serves as the terminal electron acceptor, thus making the oxidation of amino acids to fully oxidized end products thermodynamically favorable. Under anaerobic conditions the oxidation of amino acids is often incomplete although through the use of alternative electron sinks, such as an inorganic electron acceptor or interspecies hydrogen transfer, catabolism of otherwise unfavorable reactions can proceed. The variables involved in the catabolism of amino acids under anaerobic conditions are discussed in Chapter 2.2.

While a number of reviews on the subject of anaerobic amino acid catabolism exists (Barker, 1981), few offer a comprehensive examination of the degradation of amino acids. This chapter seeks to outline all major aspects of anaerobic amino acid metabolism and include the small quantity of work that has been done on thermophilic *Clostridia*. Recent work using whole genome sequencing has given insights into the amino acid metabolism of *Cl. sticklandii* (Fonknechten et al., 2010). Given the paucity of data pertaining to the mechanism of amino acid catabolism of thermophilic *Clostridia*, specifically *Thermoanaerobacter* species, the protein catabolism of *Clostridia* will be covered instead. This chapter will provide an overview of proteolytic *Clostridia*, including factors and details of protein degradation.

2.1 Protein and Amino Acid Degrading *Clostridia*

Given the ubiquity of *Clostridia* in anaerobic environments, the importance of understanding their role in proteolysis is critical from the perspective of nutrient cycling, particularly carbon, sulfur, and nitrogen, as well as the influence on the overall community profile of an environ. Furthermore, the catabolism of peptides and amino acids can lead to the production of a number of potentially inhibitory metabolites including ammonium, amines, fatty acids, phenols, and indoles, and as shown by this work, short-chain fatty alcohols. Additionally, from a clinical standpoint, the tissue damage caused by assortment of proteolytic activities produced by *Cl. difficile* and other pathogenic clostridia has been implicated in the difference in strain virulence/pathogenicity (Seddon, Hemingway, & Borriello, 1990) but also play a key role in other environments.

A diverse range of environments have been found to harbor proteolytic *Clostridia*. The anoxic environment of the rumen has been extensively studied due to the obvious implications for animal husbandry (Eschenlauer et al., 2002; Firkins, Yu, & Morrison, 2007), as have those found in soil (review: Loll & Bollag, 1983), no doubt due to its implications in agriculture. The degradation of peptides and amino acids in the digestive tract by organisms which include potentially pathogenic *Clostridium* species have also been well examined (Richardson, Mckain, & Wallace, 2013). In the marine environment, obligate anaerobes have been shown to utilize sulfate reduction for amino acid utilization while other halophilic clostridia have been shown to utilize betaine, a common osmolyte (Fendrich, Hippe, & Gottschalk, 1990). Proteolytic *Clostridia* have also been isolated from both mesophilic and thermophilic bioreactors and geothermal features.

2.1.1 Categorization and classification of proteolytic *Clostridia*

The classification of proteolytic bacteria is somewhat problematic and a dated approach as being saccharolytic, proteolytic and/or lipolytic is often not a mutually exclusive taxonomic feature. Proteolytic *Clostridia* are generally regarded as those organisms which are able to utilize proteins, single amino acids or groups of amino acids when grown in media containing only proteins, amino acids and growth factors (Mead, 1977 and references therein). This definition is problematic and has likely grown out of an understanding based upon *Clostridia* that are members of complex consortia in which amino acid-utilizing species are not necessarily those responsible for protein hydrolysis. Furthermore, as explained in subsequent sections, the utilization of specific amino acids is often depending upon various environmental parameters. It has been suggested that many of the so-called “Negativicutes” should be regarded as a subclass within *Clostridia* and will thus also be considered here (Yutin & Galperin, 2013).

Historically, a number of easily distinguishable features have been used to group proteolytic *Clostridia*, namely positive indole reaction (from the degradation of tryptophan), action on milk (indicative of casein degradation), and hydrolysis of chopped meat medium. The ability to degrade specific types of proteins and polypeptides as well as the catabolism of specific amino acids or pairs of amino acids (Stickland reactions) are also important taxonomic features. For instance, the degradation of glutamate and the resulting end products is a feature that has historically been used to group members of *Clostridia* (such as glutamate degraders: *Cl. cochlearium, Cl. tetani, Cl. malenominatum, Cl. limosum, Cl. tetanomorphum*). Unfortunately, the specific conditions required (such as electron acceptors, inhibition phenomenon) can make reporting these features accurately problematic. The use of 16S rDNA sequencing reveals that proteolytic organism are often interspersed within purely saccharolytic organisms, although the ability to utilize proteins and amino acids to at least some degree seems to be a ubiquitous feature. This is evidenced by the production of protease and action in milk by traditional butanol and butyric acid producing *Clostridia* such as *Cl. beijerinckii* (ATCC 10132) and *Cl. acetobutylicum* (Uchino, Miura, & Doi, 1968). Another major taxonomic indicator used is the indole test which detects the production of the aforementioned metabolite from tryptophan which is notable among *Cl. indolis, Cl. sphenoides, Cl. clostridioforme,* and *Cl. tetanomorphum,* although negative for *Cl. pasteurianum*.

Despite calls in the 1930s to undertake the characterization of the metabolism of nitrogenous compounds with the same importance as the characterization of carbohydrate metabolism (Barker, 1939), there have been few attempts to do so. The first attempt to classify proteolytic *Clostridia* on the basis of amino acid utilization was carried out by Mead (1971) by cultivating strains in 3% casein hydrolysate. Mead divided the *Clostridia* into four groups on the basis of the utilization patterns, namely their ability to perform Stickland reactions, utilize glycine, proline, and arginine. One of the shortcomings of Mead´s classification is that it largely ignores strains capable to utilize branched-chain amino acids (BCAAs) and aromatic amino acids (ArAAs). Subsequent work by Elsden & Hilton (1979) using a two dimensional TLC, examined the utilization patterns of 9 strains of *Clostridium* in tryptophan-supplemented casamino acid medium. While this technique has the advantage of being rapid, the utilization of many amino acids is not thermodynamically favorable which may lead to incomplete utilization. Furthermore, complex patterns can emerge due to the anabolic production other amino acids or inhibition phenomenon causing incomplete utilization. Furthermore, the utilization of some amino acids may not be thermodynamically favorable under these conditions and may be missed entirely. The cultivation of microorganisms in minimal media with single amino acids or pairs of amino acids, as opposed to complex matrices, has provided additional insight into the catabolic capabilities of anaerobes. In some cases, the inclusion of an electron scavenging system, most often a hydrogenotrophic methanogen, has been used. This approach has been used in most modern characterization papers although it has the notable disadvantage of being highly laborious.

The use of modern informatics tools such as the MEROPS database, which contains information about peptidases curated from whole genome sequenced organisms as well as those of evolutionary or clinical relevance, reveals that many of the listed clostridia, including thermophilic clostridia, contain multiple proteases (Rawlings et al., 2018). Many of the listed peptidases are based on consensus sequences with very few having been purified to homogeneity and characterized. While this serves to highlight the ubiquity of clostridial peptidases, it does little to aid the classification of *Clostridia* as proteolytic.

The diversity of catabolic approaches to amino acid catabolism is not fully captured by any of the aforementioned classification schemes. A complicating factor may include inhibition phenomenon by other components in the medium. For example, Akkad and Blackburn (1963) noted that proteolytic isolates demonstrating protein degradation in liquid medium did not always show casein hydrolysis on solid medium; additionally, the presence of carbohydrates may inhibit proteolysis. These observations have potential implications for the isolation and study of proteolytic organisms in synthetic media or in complex matrices where multiple carbon and nitrogen sources are available.

Furthermore, a study of leucine-degrading enrichment cultures from various protein-containing environmental samples found that different clusters within the genus of *Clostridium* had different strategies for using reducing equivalents generated during amino acid catabolism (Ato, Ishii, & Igarashi, 2014). Strains within *Clostridium* Cluster I paired leucine catabolism to acetate reduction to yield butyrate; similarly, strains within Cluster XIV utilized acetate for leucine oxidation which resulted in the formation of propionate. Interestingly, some enrichments coupled leucine utilization to reductive butyrate synthesis from CO_2 (Ato et al., 2014). *Bacteroides ruminicola* strain B14 cannot grow on peptides alone as their utilization does not meet their maintenance energy; interestingly, the inclusion of acetate or C4-C5 carboxylic acids, such as 2-methyl-1-propionate (*iso*-butyrate), 2-methyl-butyrate, 3-methyl-1-butyrate (*iso*-valerate), allows for peptide catabolism (Russell, 1983) and ATP generation. It should be explicitly stated that 2-methyl-1-butyrate, 3-methyl-1-butyrate, and 2-methyl-1-propionate are end products of BCAA catabolism and acetate is an end product of carbohydrate metabolism as well as the catabolism of several amino acids.

2.1.2 Protein and Amino acid degrading *Clostridia*

Among *Clostridium* species, there are a diversity of behaviors associated with protein and amino acid catabolism; there are some species which are highly specialized amino acid degraders, such as *Cl. cochlearium* which degrades glutamate, and those that are more versatile with respect to their range of amino acid substrates, while others can utilize large proteins or in some cases small peptides such as *Eubacterium acidaminophilum*. *Cl. cochlearium* can only ferment L-glutamate, L-glutamine, and L-histidine whereas *Cl. tetanmorphum* can accept a broader range of substrates as a carbon source (Laanbroek, Smit, Nulend, & Veldkamp, 1979).

Individual proteolytic *Clostridia* demonstrate a wide range of behaviors ranging from being saccharolytic/asaccharolytic, degrading proteins only, peptides, amino acids, etc. A wide variety of proteolytic chemistries are apparently dictated by the nature of the proteinaceous substrate that can be degraded. For instance, the pathogenic *Cl. perfringens* is proteolytic against proteinaceous muscle tissue and is strongly activated by Ca^{2+} (Hapchuk, Perason, & Price, 1979) although being inhibited by casein and casein hydrolysates (Curran, Solberg, Blaschek, & Rosen, 1981). It is well known that casein hydrolysis can yield antimicrobial peptides which inhibit proteases.

Interestingly, the asaccharolytic *Clostridium hydroxybenzoicum* can decarboxylate *p*-hydroxybenzoates but does not utilize aromatic amino acids and the addition of ArAAs does not increase decarboxylase activity (Zhang, Mandelc, & Wiegel, 1994; Zhang & Wiegel, 1990). While this strain utilizes arginine, the majority of the carbon is directed to the formation of ornithine rather than energy forming pathways as evidenced by the low acetate yields. *Cl. neopropionicum* can utilize acetate as a sink for reducing potential for valine and leucine catabolism (Ato et al., 2014) as can *Cl. acetireducens* (Girbal, Orlygsson, Reinders, & Gottschal, 1997; Orlygsson, et al., 1996).

Several reports of acetogenic *Clostridia* have also been mentioned in the literature; the utilization of CO and CO_2 via the Wood-Ljungdahl pathway as an electron sink allowing the oxidation of numerous substrates. The versatile CO/H_2 and methanol-utilizing *Cl. methoxybenzovorans*, which also demethylates aromatic compounds, degrades mixtures of peptides and casamino acids to acetate (Mechichi et al., 1999). Other examples include *Clostridium mayombei* (Kane, Brauman, & Breznak, 1991), *Eubacterium limosum* (Genthner, Davis, & Bryant, 1981), *Natronoincoala histinovorans* (Zhilina et al., 1998), and *Sporomusa aerivorans* (Boga, Ludwig, & Brune, 2003). It has also been demonstrated that the acetogenic *Thermoanaerobacter kivui* can utilize alanine in the presence of

thiosulfate. *Acetobacterium woodii*, despite being described as not utilizing alanine (Balch, Schoberth, Tanner, & Wolfe, 1977), was found to have a gene cluster coding for alanine uptake and subsequent oxidation to pyruvate (Dönig & Müller, 2018).

Interestingly, the acetogenic *Cl. drakei*, *Cl. carboxidivorans*, and *Cl. scatologenes* utilize a number of amino acids, including glutamate, as the sole carbon source (Liou, Balkwill, Drake, & Tanner, 2005). It is worth noting that acetate, ethanol, butyrate, and butanol were noted as end products when a N_2/CO_2 atmosphere was used. If CO_2 were used as an electron sink, it is possible that this could have allowed for the utilization of these amino acids.

Very few proteolytic and amino acid-degrading thermophilic *Clostridia* have been described although several examples have been reported from a diverse range of environments. The moderately thermophilic *Thermoanaerovibrio* (formerly *Selenomonas*) *acidaminovorans,* a succinate decarboxylating anaerobe isolated from mesophilic methanogenic sludge, degrades a wide range of amino acids to organic acids (Guangsheng, Plugge, Roelofsen, Houwen, & Stams, 1992). Toda and coworkers isolated and characterized a protease-producing thermophilic anaerobic bacterium, *Thermobacteroides leptospartum* (Toda, Saiki, Uozumi, & Beppu, 1988). *Clostridium* (*Thermobacteroides*) *stercorarium* subsp. *leptospartum* (DSM 9219) isolated from cattle compost was found to produce a thermostable protease (Toda et al., 1988). The thermophilic piezophile, *Anoxybacter fermentans*, isolated from a deep-sea hydrothermal sulfide feature, demonstrates an impressive ability to utilize a broad range of carbohydrates and amino acids (Zeng et al., 2015). Work specifically on members of the genus of *Thermoanaerobacter* were performed in the mid-1990s and will be covered in more detail in subsequent sections.

Unfortunately, many of the aforementioned examples only report the utilization of these substrates qualitatively and end products are generally not reported. There are a few examples of thermophilic *Clostridia* producing specific end products from proteins and amino acids. The ability of *Coprothermobacter* (formerly *Thermobacteroides*) *proteolyticus* to utilize gelatin with and without a hydrogenotrophic methanogen was evaluated in 1986 (Ollivier & Garcia, 1986). In pure culture, *T. proteolyticus* yielded a mixture of acetate, 3-methyl-1-butyrate, H_2, and CO_2 while co-culturing with one or more methanogens resulted in a mixture of acetate, 2- and 3-methyl-1-butyrate, propionate, and CO_2. *Thermoanaerovibrio* (*Selenomonas) acidaminovorans*, a highly versatile proton-reducing anaerobe, is able to grow by decarboxylation of succinate to propionate. The moderately thermophilic and proteolytic *Clostridium* strain P2 can also degrade serine, threonine, alanine, and the BCAAs to acetate or their corresponding fatty acids,

respectively, under methanogenic conditions (Örlygsson, 1994; Orlygsson, Houwen, & Svensson, 1995).

A number of studies on the protein- and amino acid utilizing capabilities of *Thermoanaerobacter* strains have been reported. A number of *Thermoanaerobacter* species, such as *T. brockii* and *T. ethanolicus*, have shown improved ability to utilize mixtures of amino acids and peptides with the addition of thiosulfate which is reduced to hydrogen sulfide (Fardeau et al., 1997; Faudon et al., 1995). Unsurprisingly, higher OD values (more cell biomass) and end product titers were higher when thiosulfate was included in the medium as evidenced by the increased formation of 2-methyl-1-propionate, propionate, and 3-methyl-1-butyric acid (Faudon et al., 1995). Furthermore, gelatin was weakly utilized by several strains and it was noted that *T. brockii* seemed to better utilize peptides than *T. ethanolicus* based on the amount of end products formed (Faudon et al., 1995). It should be noted that the sulfur balance reported was low suggesting that not all of the electrons are accounted for in the end product analysis. Enrichment cultures from the Uzon Caldera (Russia) reveal that a number of thermophilic anaerobes that can degrade proteins, including highly recalcitrant proteins such as keratin (Kublanov et al., 2009).

2.2 Factors affecting protein and amino acid catabolism

The influence of culture parameters on protein and amino acid catabolism is of central importance. Secondarily, the influence of amino acid end products on the fermentative organisms themselves is also of importance. Unfortunately, studies on the influence of specific variables on the catabolism of proteins and amino acids are often limited to mixed consortia in anaerobic systems and few systematic studies of pure organism have been undertaken. That said, several variables seem to be important for understanding amino acid catabolism such as physical parameters, pH, the presence of ammonia, and the partial pressure of hydrogen. Some of these parameters will be discussed in detail below.

2.2.1 Ammonia accumulation

While ammonium is the preferred nitrogen source for many organisms (review: von Wirén & Merrick, 2004), high ammonium concentrations are cytotoxic. It is generally well accepted that free ammonia (NH_3) is the toxic form, depending upon the pH of the system (Bajapi & Iannotti, 1988). Ammonia/ammonium also serves as a buffer to the pH drop associated with the production of volatile fatty acids (Bajapi & Iannotti, 1988). While the transport of ammonium ions into the cell via specific transporters is widely distributed in nature, there are no specific export mechanisms for ammonium. It should be noted that ammonia (pK_a=9.24) can diffuse across the plasma membrane and become protonated leading to an increase in cytosolic pH. Additionally, NH_4^+ can leak through K^+ channels, disrupting ion gradients needed for energy transduction.

A 1976 study on the impact of ammonium concentration on the activity of enzymes involved in nitrogen assimilation and amino acid anabolism from mixed rumen bacteria found that lower NH_4^+ resulted in an increase in glutamine synthetase while the activities of asparagine synthetase and aspartate aminotransferase remained unchanged (Erfle, Sauer, & Mahadevan, 1976). Additionally, low ammonium concentration facilitated the rapid utilization of alanine and glycine while high ammonium concentrations resulted in the synthesis of alanine which could serve as an ammonia sink using glycolysis intermediates (i.e. pyruvate). At elevated NH_4^+ concentrations due to urea supplementation, ruminal fluid experienced an increase in alanine formation as well as an increase in the rates of protein degradation (Wallace, 1979).

The understanding of the nature of ammonium toxicity is mostly limited to model organisms and the impact of ammonium accumulation in mixed culture. The response of individual organisms can vary from shift in end product formation to complete inhibition of growth. Work using hydrogenotrophic methanogens, such as *Methanolobus* and *Methanohalophus* species, which are common in many anaerobic environments with

amino acid-fermenting organisms, suggests that ammonium influences the pH gradient (cystolic vs extracellular) and lowers the concentration of cytosolic K^+ (Kadam & Boone, 1996), which is normally the dominant ion in the intracellular space. Previous work suggests that acetoclastic methanogens are more sensitive than hydrogenotrophic methanogens (Koster & Lettinga, 1984). Studies on model organisms (*Bacillus subtilis*, *Escherichia coli*, and *Corynebacterium glutamicum*) has suggested that the deleterious effects of NH_4^+ accumulation are due to elevated osmolarity and increased ionic strength in the medium (Müller, Walter, Wirtz, & Burkovski, 2006). Work on *Saccharomyces cerevisiae* shows that ammonium is toxic during potassium limitation and under conditions where ammonium was inhibitory, the amount of amino acids excreted into the medium by the cells was increased (Hess, Lu, Rabinowitz, & Botstein, 2006).

To address the impact of elevated ammonium concentrations, one common strategy is to utilize pathways involved in nitrogen assimilation. The aforementioned study on *Methanolobus* and *Methanohalophus* species, the activities of enzymes involved in nitrogen assimilation (glutamine synthase, glutamate dehydrogenase, and alanine dehydrogenase) only showed activity at pH 7 or greater (Kadam & Boone, 1996) although the authors did not examine the influence of growth at high ammonium concentrations on the specific activities of these enzymes. Rumen consortia and isolates have also been noted to excrete mixtures of amino acids, including alanine, when grown on ammonium as the primary nitrogen source (Stevenson, 1977). The extremophilic archaea, *Pyrococcus furiosus*, produces L-alanine as a major fermentation product from carbohydrates which was affected by NH_4^+ concentration (Kengen & Stams, 1994). Subsequent work also showed that this feature is common among *Thermotoga* species and the authors posited that this is a remnant of ancient metabolism (Ravot et al., 1996). Work on the moderately thermophilic *Clostridium* strain P2, which was isolated from a thermophilic anaerobic digester, also demonstrated alanine formation during glucose fermentation at a high ammonia concentration of 280 mM (Orlygsson, Houwen, & Svensson, 1993).

In mixed cultures, such as anaerobic digestion (AD) systems or among rumen microbiota, changes in ammonium concentrations can cause populations shifts (review: Rajagopal, Massé, & Singh, 2013). A study examining population dynamics of an AD reactor at 35°C at various ammonium concentrations (up to 25 g/L) over 3 months found dramatic populations shifts in both the bacterial and archaeal phylotypes over time depending on ammonium concentration (Poirier, et al., 2016). It was found that members within order *Clostridiales* were associated with low ammonium loadings (10 g/L) but the population shifted dramatically at higher loadings (Poirier et al., 2016). Interestingly, ammonium loadings of 25 g/L caused the emergence of *Caldicoprobacter*, a genus typically associated with thermophily. Thermophilic AD has often shown to be difficult at high ammonia loadings. It should be noted that the accumulation of ammonium can also cause the synthesis of alanine from pyruvate (Orlygsson, Anderson, & Svensson, 1995; Ravot et al., 1996).

2.2.2 Partial pressure of hydrogen

The partial pressure of hydrogen (pH_2) is a major influence on the thermodynamic favorability of amino acid catabolism under anaerobic conditions. Hydrogen inhibition is not uniformly reported among proteolytic *Clostridia* although a variety of effects have been observed. Most commonly, the inhibition of hydrogen is evaluated qualitatively by using hydrogen as the headspace gas rather than more detailed experiments making the degree to which hydrogen causing inhibition or shifts in end product fermentation difficult to assess.

An illustrative example of the influence of pH_2 is that of *Acidaminobacter hydrogenoformans*. When the strain was grown with very little headspace, only 5 mM of the initial 20 mM of glutamate was fermented and only 4 mM (of the initial 20 mM) when the headspace consisted of H_2. The use of N_2 as a headspace gas with a larger gas phase (not defined) allowed the degradation of 9.8 mM of glutamate while the including of either a sulfate-reducing or hydrogenotrophic methanogen allowed for near complete utilization of glutamate (Stams & Hansen, 1984). Alternately, the presence of hydrogen in the headspace can cause shifts in end product formation such as those observed when *Anaeromusa* (*Selenomonas*) *acidaminophilus* is grown on glutamate during which a shift from acetate being the dominant product to propionate is observed. A similar trend was observed with aspartate (acetate/propionate to succinate) (Nanninga et al., 1987).

Acetomicrobium (formerly *Anaerobaculum*) *thermoterrenum* demonstrates a degree of hydrogen inhibition when grown on glucose under various conditions (Menes & Muxi, 2002). When the strain was cultivated at an L-G ratio of 0.56, only 18.8% of the glucose was degraded which increased to 88.2% when the L-G ratio was decreased to 0.037. Similarly, when cultivated in co-culture with a methanogen, all of the glucose was consumed. Interestingly, when *Ac. thermoterrenum* was grown on leucine, crotonate but not a hydrogen scavenging methanogen resulted in formation of 3-methyl-1-butyrate.

In the absence of an electron scavenger, the fermentation of leucine by *Caloramator proteoclasticus* ceased at pH_2 values of 7.7×10^{-2} atm while the fermentation of valine stopped at 4.0×10^{-2} atm (Tarlera, Muxí, Soubes, & Stams, 1997). Inhibition of amino acid metabolism due to hydrogen accumulation was also observed to various degrees in *Aminobacterium colombiense* (Baena et al., 1998). Alternately, in addition to not being inhibited by hydrogen accumulation, *Cl. collagenovorans* and *Cl. proteolyticum* rapidly consume hydrogen yielding acetate (Jain & Zeikus, 1988).

The importance of interspecies hydrogen transfer is evident when amino acids are degraded in mixture cultures, particularly when a hydrogenotrophic methanogen is present (Nagase, 1982). *Cl. cochlearium* is unaffected by increased partial pressure of hydrogen (pH_2) during glutamate fermentation (Laanbroek and Nanninga, unpublished data in

Nanninga & Gottschal, 1985). Inhibiting interspecies hydrogen transfer in mixed ruminal cultures with carbon monoxide *in vitro* resulted in the selective inhibition of BCAA degradation from tryptone (Russell & Jeraci, 1984), demonstrating the importance of interspecies hydrogen transfer in keeping the pH_2 low.

2.2.3 Redox balance and electron acceptors

Related to the role of pH_2 is the role of terminal electron acceptors in keeping the pH_2 low. Interestingly, the role of terminal electron acceptors may not be universal. For instance, *Acetomicrobium* (formerly *Anaerobaculum*) *mobile* can degrade leucine to isovalerate in the presence of crotonate as an electron acceptor but not when a hydrogenotrophic methanogen is used (Menes & Muxi, 2002). The use of a Fe(III) reduction by clostridia during carbohydrate and amino acid catabolism is widely known (Lovley, 1997) and the same goes for sulfate-reduction (Smith & Klug, 1981).

The redox state of the amino acid is also critical. Studies on mixed consortia from bovine rumen noted that the BCAAs and lysine liberated from casein hydrolysis were less rapidly catabolized than the other amino acids present (Mangan, 1972). While the author points to a potential anabolic source to explain this degradation, it is more likely that the poor thermodynamic favorability and the impact of the removal of reducing equivalence can explain the limited degradation of these AAs. During growth of a mixed proteolytic culture together with a hydrogenotrophic methanogen on peptone, all BCAAs, hydrolyzed from the peptone, were degraded to their corresponding BCFA (Örlygsson, 1994). Addition of BES, an inhibitor for the methanogen, resulted in incomplete BCAA degradation indicating the importance of an electron scavenging system in such a culture.

The use of inorganic electron acceptors can shift the end product profile as well as the $NADH/NAD^+$ ratio. Unsurprisingly, a sink for reducing equivalence is needed in order to degrade highly reduced amino acids. Studies on the deamination step in mixed consortia from rumen have demonstrated that the addition of NADH to glutamate dehydrogenase assays slows the rate of the reaction and that optimum activity for this step is between pH 8 and 9 (Hino & Russell, 1985). The oxidation state of the amino acid also proved critical; oxidized amino acids (i.e. arginine and proline) were more slowly deaminated than neutral amino acids (such as serine and threonine). While the deamination rates of increased $NADH/NAD^+$ ratios did not affect the oxidized amino acids, reduced amino acids demonstrated a marked decrease in the rates of deamination (Hino & Russell, 1985).

The use of acetate as an electron acceptor has not been widely investigated. It has long been known that acetate can enhance ethanol yields from hexoses and pentoses although it has been demonstrated that *Cl. butyricum* can utilize acetate and an electron sink while forming butyrate as the reduced product during mannitol fermentation (Heyndrickx, De Vos, Speybrouck, & De Ley, 2008). The use of acetic acid as an electron sink during BCAA catabolism was demonstrated with *Clostridium acetireducens* (Orlygsson, et al., 1996) and later work (Ato et al., 2014) also demonstrated that *Cl. neopropionicum* (Cluster XIVb) converted leucine and valine to their corresponding acids in the presence of acetate.

2.2.4 Other variables influencing amino acid catabolism

Generally, the concentration of protons in a fermentation system is among the most influential parameters as it can have a tremendous impact on physiological parameters such as membrane potential and proton motive force, internal pH, and the permeability of some molecular species based upon their pK_a. Given that bacteria cannot regulate their intracellular pH makes the ability of fully protonated organic acids to diffuse across the cell membrane affecting intracellular pH problematic (Durre, 2009); thus, the accumulation of these organic acids can lead to "leaky" membranes. As such, the formation of organic acids as a result of amino acid catabolism can be problematic. Anaerobes cannot maintain intracellular pH in response to pH drop associated with acid production due to the influx of fully protonated acids across the cellular membrane at low pH (Durre, 2009). The intracellular pH can, however, be kept one pH unit above the extracellular by shifting fermentation to solvent production (Durre, 2009).

In addition to the peptide bond being susceptible to base-catalyzed hydrolysis, a number of amino acids are sensitive to extreme pH values, particularly at elevated temperatures (Dawson, Elliott, Elliott, & Jones, 1986). Additionally, the influence of pH on pathways involving amino acid catabolism is also of importance. Low pH values often cause the direct decarboxylation of amino acids leading to amine formation which can serve to regulate intracellular pH (Gottschalk, 1986). The fermentative metabolism of amino acids is also subject to the influence of pH.

Many of the proteogenic amino acids are susceptible to hydrolysis at higher temperatures (Dawson et al., 1986). The factors affecting 2-methyl-1-propanol, 2-methyl-1-butanol and 3-methyl-1-butanol produced from BCAAs via the Ehrlich pathway have been long understood in organisms such as *S. cerevisiae* although information on the impact of elevated temperatures on prokaryotes is sparse. Increased sugar and aerobic conditions decrease the amount of fusel alcohols formed while increasing temperature increases the amount formed (Hough & Stevens, 1961). It has been previously demonstrated that the amount of fusel alcohols is optimum at 30°C as compared to optimum ethanol production at 25°C (Merritt, 1966).

Additionally, the influence of media composition also seems to be critical for the catabolism of amino acids. For instance, glycine requires selenium for the function of glycine reductase (Costilow, 1977). A study on the production of glycine reductase by *Cl. sporogenes* showed that cells cultivated in rich medium (2% tryptone, 1% yeast extract) with exogenously added pyruvate, proline, and hydroxyproline showed to be deficient in one or more components of the glycine reductase system (Venugopalan, 1980). Furthermore, the addition of proline and hydroxyproline affected the uptake of glycine into the cells (Venugopalan, 1980) which suggests that regulatory mechanisms are at play.

Finally, the concentration of amino acids can be inhibitory. For example, the acetogenic *Natronoincola histidinovorans* demonstrated inhibition during histidine degradation above concentrations of 12 mM (Zhilina et al., 1998). Similar phenomena are common with other substrates and have been well-studied (Bajapi & Iannotti, 1988).

2.3 Sequential metabolism of proteins and amino acids

The complete degradation of protein to amino acids and their subsequent fermentation products requires several steps. Broadly speaking, external hydrolysis of proteins to peptides is followed by the import of either oligopeptides or single amino acids into the cell, followed by further degradation by endopeptidases. Free amino acids can then be catabolized through a series of reactions to various fermentation products. Key aspects of each step of this process are described in subsequent sections.

2.3.1 Protein hydrolysis

The transformation and mineralization of proteins begins with hydrolysis of the peptide bond to yield smaller oligopeptides. The extracellular hydrolysis of proteins into oligopeptides and single amino acids is accomplished by a combination of proteases and peptidases. The complete hydrolysis of a polypeptide typically requires two or more proteases: an aminopeptidase with broad specificity and a proline-specific aminopeptidase-P (Yaron & Berger, 1970; Yaron & Mlynar, 1968). Proteases are typically categorized on the basis of their active site chemistry; major classes of proteases include acid, thiol alkaline (serine), and metallo-proteases. Proteases can be either exo- or intracellular with proteases acting on high molecular weight targets often being extracellular while enzymes performing intracellular hydrolysis on smaller molecules, such as membrane-soluble oligopeptides, are often intracellular (review: Morihara, 1974).

The primary rate-limiting factor for protein hydrolysis is solubility with highly soluble proteins being degraded more rapidly than poorly soluble proteins (Hobson & Wallace, 1982b, 1982a; Örlygsson, 1994). The degradation of fibrous proteins such as keratin, collagen, etc may require the production of a biosurfactant to aid solubility. Tertiary features, such as the number of disulfide bridges, and the *N*-terminal moiety are also important factors (McInerney, 1988). Thermal denaturation can aid peptide bond hydrolysis. The hydrolysis of peptide bonds at 37°C is generally favorable with ΔG_h values between -5.9 to -15.1 kJ/mol depending on the size of the resultant products (Martin, 1998) although the loss of secondary structure is also important for peptide bond hydrolysis.

The specificity of protease ranges from highly selective to highly promiscuous. The hydrolysis of proteins into free amino acids often requires the activity of several types of proteases. The hydrolysis of oligopeptides can either occur at the COOH terminal peptide bond or through hydrolysis of an internal peptide bond. Endopeptidases cleave non-terminal or "internal" peptide bonds of a protein yielding a shorter peptide. Specific oligopeptidases target oligopeptides rather than whole proteins (Doonan, 2002). In contrast, exopeptidases cleave terminal peptide bonds of a peptide to liberate a single amino acid; aminopeptidase or a carboxypeptidase cleave from the N-terminus or C-terminus (Doonan, 2002).

Several thermophilic *Clostridia* have been noted to produce proteases. *Caldanaerobacter subterraneus* subsp. *yonseiensis* produces "thermicin", a heat-stable subtilisin-like serine protease which is active against some forms of collagen (Jang et al., 2002). The strain expresses an inactive form of the enzyme with a N-terminal pro-sequence which is cleaved auto-catalytically during heat-treatment. Thermicin shows an unusual specificity for cleavage of the gly and pro residues which differentiates it from other subtilisin-like proteases.

Keratin, a fibrous protein notable for its high strength, is a particularly recalcitrant substrate and is not degraded by other proteases. Several microbial keratinase-producing organism have been described (reviews: Brandelli, Daroit, & Riffel, 2010; Gupta & Ramnani, 2006; Gupta, Sharma, & Beg, 2013), including a number of *Clostridia*. A strain of *Clostridium sporogens* gv. *pennavorans*, isolated from a geothermal feature, produces a low MW thermostable keratinase with a broad range of activity (Ionata, et al., 2008). A number of *Thermoanaerobacter* strains have been noted to possess keratinolytic activities such as "*Thermoanaerobacter keratinophilus*" which produces an extracellular keratinase (Riessen & Antranikian, 2001) while *Thermoanaerobacter brockii* subsp. *brockii* and subsp. *finnii*, do not have such activity (Friedricht & Antranikian, 1996). It should be noted that "*Thermoanaerobacter keratinophilus*" likely falls within the genus of *Caldanaerobacter* on the basis of its 16S rRNA genes. *Thermoanaerobacter* strain 1004-09, isolated from a hot spring in Russia, also demonstrates strong keratinolytic activity in addition to degrading gelatin, casein, and albumin (Kublanov et al., 2009).The keratinase of *Thermoanaerobacter* strain S290, isolated from a geothermal feature in Kamtchatka, was found to be useful for attacking the β-sheets of keratin in an effort to find enzymes for the removal of β-amyloid prions from infected materials (Tsiroulnikov et al., 2004).

The regulation of protease activity is of importance to maintaining the availability of carbon and nitrogen sources as well as conserving energy. Unsurprisingly, the synthesis of extracellular enzymes, including protease, is often subject to product inhibition and catabolite repression with the presence of substrates often inducing expression (general review: Glenn, 1976). While the majority of the work on this topic has focused on easily-studied facultative anaerobes, a number of studies have specifically examined protease production by *Clostridia*. The protease activity of the amino acid-fermenting *Cl. sporogenes* strain C25 was investigated in batch and continuous culture by (Allison & Macfarlane, 1990). Protease production was strongly linked to energy-limiting conditions with the presence of glucose, NH_4^+, phosphate, ATP, ADP, and some amino acids inhibiting protease formation. The authors observed that protease production coincided with the onset of the stationary phase. *Cl. perfringens* demonstrates extracellular protease production during the exponential growth phase with a higher degree of proteolysis being apparent when glucose is limited and high concentrations of peptides are provided (Allison & MacFarlane, 1989).

Regulation of protease and neurotoxin production by strains of *Clostridium botulinum* is inhibited by free amino acids, in this case tryptophan and arginine (Leyer & Johnson,

1990; Patterson-Curtis & Johnson, 1989). Another strategy to limit the activity of proteases is their synthesis in an inactive pro-form which must be activated by hydrolysis. Strains of *Cl. botulinum* (types A, B, and F) produce two proteases one of which is likely in the inactive zymogen form (Tjaberg, 1973a). *Cl. histolyticum* produces a protease near the end of the exponential phase (Takahashi & Seifter, 1972).

A study by MacFarlane & MacFarlane (1992) investigating the metalloproteases produced by *Cl. bifermentans* found that both glucose and peptone stimulated protease production in continuous culture as did phosphate up to a concentration of 5 g/L (40 mM). Interestingly, ammonium did not affect protease production. Unlike *Cl. sporogenes*, the protease production of *Cl. bifermentans* occurs during the exponential growth phase and ceases during the stationary phase much like *Cl. perfringens* (Allison & MacFarlane, 1989).

2.3.2 Amino Acid importers

The transport of amino acids has received less attention than carbohydrates although there are several studies on Firmicutes, particularly *Corynebacterium glutamicum*, with sporadic examinations of organisms within Class *Clostridia*. While beyond the scope of this work, it should be noted that amino acid transport is directly linked to the process of proteolysis and subsequent fermentation while also playing a key role in the energy metabolism of the organism.

The import of polypeptides and amino acids into the cell is a crucial step in catabolism. Unfortunately, this aspect has received relatively little attention although it is apparent that some individual amino acids or groups of amino acids have specific transporters. Protein hydrolysis does not need to be complete as some oligopeptides can readily cross the cellular membrane (Huo et al., 2011; Loll & Bollag, 1983) and are subsequently degraded by endopeptidases. The transport of molecules through the cytoplasmic membrane is often actively facilitated by ATP-linked transporters, proton motive force (PMF), or sodium motive force (SMF) using either proton- or Na-ATPases (Konings, Albers, Koning, & Driessen, 2002). The import of polypeptides and free amino acids is facilitated by specific transporters or migration across the membrane; despite this critical function, there have been very few reports on the import of amino acids by *Clostridia*. ABC transporters have been extensively reviewed elsewhere (Beek, Guskov, & Slotboom, 2014).

Generally speaking, there are five modes of carrier-mediated transport in bacteria:

1. Facilitated diffusion
2. Shock sensitive systems
3. Proton symport
4. Na^+ symport
5. Phosphoenolpyruvate phosphotransferase (PEP-PTS)

Among the examples that can be found in the literature it seems that the mechanisms used for amino acid transport by *Clostridia* are diverse with there being examples of both proton- and sodium-dependent transport, facilitated diffusion, and active transport. *Cl. acetobutylicum* has been found to use an H^+ symport mechanism (Driessen, Ubbink-Kok, & Konings, 1988) while *Cl. thermoautotrophicum* and *Cl. thermoaceticum* transport glycine, alanine, and serine using H^+ symport coupled to CO oxidation (Hugenholtz & Ljungdahl, 1989, 1990).

2.3.3 Fermentative pathways related to amino acid catabolism

The majority of information regarding amino acid catabolism comes from studies on yeast, particularly *Saccharomyces cerevisiae* although a number of studies on clostridial pathways of well-establish proteolytic clostridia have been examined in some detail. The catabolism of nitrogen-containing compounds results in the formation of three key nitrogenous compounds: ammonium, glutamate, and glutamine. It should be noted that lysine, ornithine, and proline are racemized to their corresponding D-amino acid to avoid confusion between anabolic and catabolic pathways (Andreesen, Bahl, & Gottschalk, 1989).

Overall, amino acid catabolism proceeds through several discrete steps: the deamination of amino acids yields the corresponding α-keto acid, ammonia and reducing equivalents in the form of reduced cofactors. Deamination can occur via three different methods: oxidative deamination facilitated by dehydrogenases or transaminases, deamination without oxidation/reduction via α,β-elimination yielding the corresponding keto acid, or reductive amination.

Specific aspects of each step in the catabolic process are covered in subsequent subsections. Carbon skeletons from amino acid catabolism have three major entry points into central metabolism: pyruvate, oxaloacetate, and α- ketoglutarate or acyl CoA (e.g. acetyl-CoA, succinyl CoA).

Deamination of amino acids

The first step of amino acid catabolism involves the reversible elimination of the amino group catalyzed by an aminotransferase (transaminase) by transferring the amino group of an amino acid to an α-keto acid (such as α-ketoglutarate). The resultant aminated α-keto acid (typically glutamate) can then undergo dehydrogenation to regenerate the α-keto acid and ammonium.

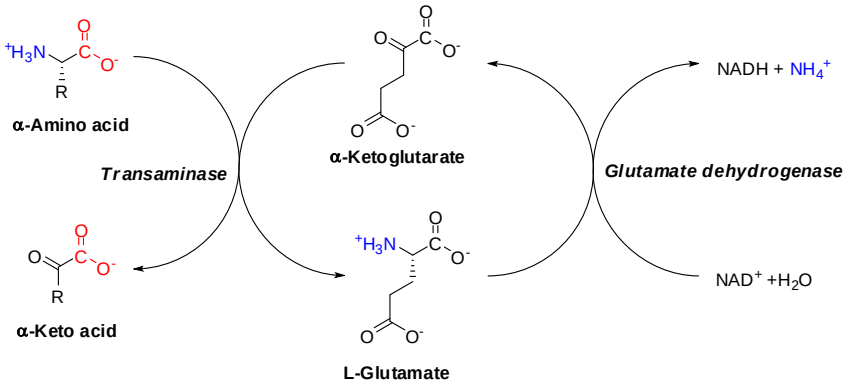

Figure 3 – Role of glutamate dehydrogenase in transamination reactions

This can be accomplished by either an α- or β-elimination depending on whether the group is a primary or secondary amine, respectively. Among *Clostridia*, two enzymes are known to catalyze the α-elimination (substitution of alpha amino group with hydrogen) of amino acids: glycine reductase and D-proline reductase (Buckel, 2005). Additionally, sarcosine reductase and betaine reductase catalyze similar reactions (Buckel, 2005).

Decarboxylation

Decarboxylation of the α-keto acids is often facilitated by a coenzyme thiamin diphosphate (thiamine pyrophosphate, TPP) which is derived from Vitamin B_1 (thiamin) via diphosphorylation via a kinase using ATP. In this case, the role of TPP is to extend the α-keto group to the electron sink (Silverman, 2002). Similar to pyruvate catabolism facilitated by pyruvate decarboxylase (PDC), 2-keto acids are one of the intermediates of both amino acid biosynthesis and catabolism. 2-keto acid decarboxylases (KDCs) convert 2-keto acids to their corresponding aldehydes. KDCs are common enzymes in plants and eukaryotes but are less common among bacteria (Konig, 1998).

Stickland reactions

A number of *Clostridia* have the unique ability to catabolize amino acids as a carbon source under anaerobic conditions without the use of an external electron acceptor such as nitrate or thiosulfate. This is facilitated via the Stickland reaction in which pairs of amino acids, one acting as an oxidizing agent (electron acceptor) and the other as a reducing agent (electron donor), are degraded as summarized below.

$R_2CHCHNH_2COOH$ (*electron donor*) + $2H_2O$ → $R_2CHCOOH + CO_2 + NH_3 + 4\,[H]$

$2\ RCH_2CHNH_2COOH$ (*electron acceptor*) + $4[H]$ → $2\ RCH_2CH_2COOH + 2\ NH_3$

Overall: $R´CHNH_2COOH + 2R”CHNH_2COOH + 2H_2O$ → $R’COOH + CO_2 + 3\ NH_3 + 2R”CH_2COOH$

The reducing equivalence produced by oxidative deamination is coupled to a reductive deamination. A number of proteolytic clostridia species utilize the Strickland reaction which involves a transamination of an amino acid, such as glycine or proline, acting as an electron acceptor. A good example of this is *Cl. sticklandii* (Fonknechten et al., 2010; Schwartz & Schäfer, 1973). A variety of amino acid pairs have been reported in the literature although glycine and proline have been most commonly noted as electron acceptors for Stickland reactions; a more comprehensive list of potential oxidation/reduction pairs are provided in the table below.

Table 2 - Amino acids involved in Stickland reactions in Clostridia (modified from de Vladar, 2012)

Electron donors (reductants)	**Donors/acceptors** (partner dependent)	**Electron acceptors** (oxidants)
Alanine	Leucine	**Glycine**
Valine	Phenylalanine	Aspartic acid
Serine	Tyrosine	**Proline**
Isoleucine	Tryptophan	
Threonine	Arginine	Sarcosine
Glutamic acid		Betaine
Histidine		Ornithine
Cysteine		
Methionine		
Lysine		

2.3.4 Catabolism of Specific Amino Acids

The majority of work done on the catabolism of specific amino acids has focused on mesophilic clostridia species and some food spoilage organisms. A notable exception includes the work done on *Saccharomyces* involving the Ehrlich pathway. It is likely that many fermentative organisms are cable of amino acid utilization, either as single substrates or under syntrophic conditions or the use of an electron scavenger. However, these capabilities are seldom examined (Schink & Stams, 2013).

Due to thermodynamic constraints, many amino acids are not degraded unless a suitable terminal electron acceptor is present. As such, there is no routine regimen of terminal electron acceptors used during the screening of organisms nor is the utilization of all amino acids routinely examined when characterizing novel species. While co-culture with a hydrogenotrophic methanogen is typically sufficient to access amino acid utilization, this also is not routinely examined. Furthermore, even where the ability to utilize single amino acids is checked, the presented results are often only qualitative with end products not being reported. As such, there are many knowledge gaps even for strains known to be proteolytic.

A comprehensive overview of the catabolism of all 20 proteogenic amino acids is beyond the scope of this work. Interested readers should refer to the reviews of Barker (1981), McInerney (1988), and Andreesen et al. (1989). However, the fermentation of selected amino acids using different mechanisms is discussed below.

Glycine

Glycine is rather unique among the proteogenic amino acids as it is the only one that is achiral. Glycine is commonly found in a high abundance in some proteins such as collagen and keratin. The catabolism of glycine has been extensively reviewed (Andreesen, 1994), in part due to its participation in the Stickland reaction as well as some of the unusual aspects of its metabolism. The catabolism of glycine has a reductive and oxidative branch requiring glycine decarboxylase and glycine reductase, respectively. Both the oxidative and reductive pathways generate ATP. Ultimately, the reductive pathway cleaves of the peptide bond yielding acetate and ammonium ions.

Given the ease with which glycine is formed abiotically, the somewhat unusual aspects of the mechanism involved in its metabolism, its participation as an oxidant in the Stickland reactions, and the similarities to the Wood-Ljundahl pathway, it may be worth speculating that glycine metabolism is an evolutionary holdover from more primitive anaerobes.

Among the best studied glycine-degraders are *Cl. sticklandii* and *Eubacterium acidaminophilum* which both belong within Cluster XI. The reduction of glycine appears to be critical for energy conservation among (*Clostridia*) as its conversion to acetyl-phosphate facilitates SLP (Andreesen, 2004). Glycine reductase activity has been best studied in *Eubacterium acidaminophilum* and *Cl. sticklandii* in the context of Stickland reactions where glycine serves as an electron acceptor. The structure and function of glycine reductase has been reviewed by (Andreesen, 2004).

Fermentations of glycine generally direct one quarter of the carbon flow to CO_2 via glycine decarboxylase while the remainder is catabolized to acetate by the selenocysteine-containing glycine reductase system (Andreesen et al., 1989). Recent insights from the whole genome sequence of *Cl. sticklandii* reveal that the genes responsible for coding the glycine synthase/reductase pathway coexist with genes that code for a complete Wood-Ljungdahl pathway (Fonknechten et al., 2010). The authors posited that the presence of a complete set of genes associated with the Wood-Ljungdahl pathway for acetate production from CO_2 provide methylene-THF for use by glycine synthase/reductase albeit without net ATP production since ATP is consumed during formate activation. It is important to note that the Wood-Ljungdahl pathway is suppressed under normal fermentative conditions. It should also be noted that one of the proposed physiological functions of amino acid decarboxylases is to regulate intracellular pCO_2 (Silverman, 2002).

Alanine

Alanine is usually oxidatively deaminated to pyruvate and pyruvate decarboxylated to give acetate, CO_2 and hydrogen according to the following equation:

$$\text{Alanine} + 3H_2O \rightarrow \text{acetate}^- + HCO_3^- + NH_4^+ + 2H_2 + H^+$$

Due to the thermodynamic nature of the reaction, alanine cannot usually be degraded fermentatively unless the electrons produced are scavenged, usually by co-cultivating the amino acid degrading bacterium in a co-culture with a hydrogenotrophic methanogen or by adding thiosulfate. Although the ΔG for the overall reaction is only slightly positive, the bottleneck is the oxidative deamination step ($\Delta G^{\circ\prime} = 0$ to + 50 kJ/mol). Other pathways known for degrading alanine is the acrylate pathway, performed by *Clostridium propionicum* (Figure 4) also giving pyruvate as the intermediate. The oxidative and ATP-yielding branch yields acetate as the end product while the reductive branch produces 1-propionate. In the case of *Cl. propionicum*, one third of the pyruvate is then oxidized to acetate and CO_2 generating ATP via SLP while the remaining two thirds is reduced to *R*-lactate and subsequently to propionate through acyryl-CoA (Gottschalk, 1986) according to the following equation:

$$3\ \text{L-Alanine} + 2H_2O \rightarrow 3\ NH_4^+ + CO_2 + \text{Acetate}^- + 2\ \text{Propionate}^-$$

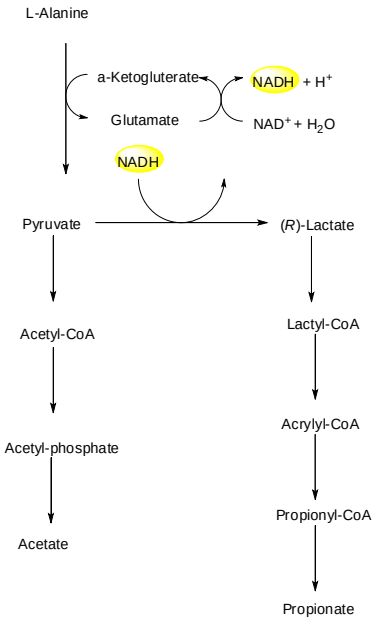

Figure 4 - Fermentation of L-Alanine via the Acrylate Pathway (modified from Gottschalk, 1986 and references therein)

Serine and Threonine

Serine and threonine are often degraded to acetate and propionate, respectively. The overall ΔG for the catabolism of threonine and serine are favourable and thus, there is no need for external electron scavenging systems for their fermentation. The direct deamination is via a specific dehydratase to the corresponding α-keto acid and ammonium. Prokaryotes typically contain separate threonine dehydratases for catabolic and anabolic pathways although the structural differences are not fully understood (Yu, Li, & Wang, 2013). There are more pathways known for threonine degradation than mentioned above and end-products from threonine catabolism may include not only propionate but also acetate, and occasionally butyric acid (McInerney, 1988).

Glutamate

The metabolism of glutamate is of central importance due to its role in other aspects of metabolism and has attracted a lot of attention due to a number of unusual features such as the use of radical intermediates. It is worth noting that glutamate biosynthesis is of high importance as it is the only route for the incorporation of inorganic nitrogen into biomolecules (White, Drummond, & Fuqua, 2012; White, 1995). Glutamate also servers a critical role in transamination reactions which is the preliminary step in the catabolism of all twenty proteogenic amino acids.

The fermentative pathways involving glutamate catabolism can potentially lead to a diverse milieu of end products depending on which metabolic pathways are involved. Remarkably, at least five distinct pathways for glutamate fermentation have been noted in the literature (Buckel, 2001). Ultimately, the anaerobic catabolic pathways involving glutamate degradation typically yield a mixture of SCFAs (e.g. acetate, propionate, butyrate) as well as CO_2, ammonia, and hydrogen (McInerney, 1988).

In some organisms, glutamate can be directly decarboxylated via glutamate decarboxylase to form gamma-aminobutyric acid (GABA). This can serve two functions, namely to counter the generation of protons from other metabolic processes (Castanie-Cornet & Foster, 2001) or to conserve energy via generation of a proton gradient (Molenaar, et al., 1993). The glutamate dehydrogenase from *Cl. perfringens* is even commercially available.

Two classical pathways for glutamate degradation have been described: the 3-methylaspartate pathway observed in *Cl. tetanomorphum*, and hydroxyglutarate pathway observed in *Peptococcus aerogenes* and *Acidaminococcus fermentans* (McInerney, 1988). An overview of these two pathways is shown in Figure 5. The other pathways have been widely investigated and are beyond the scope of this work.

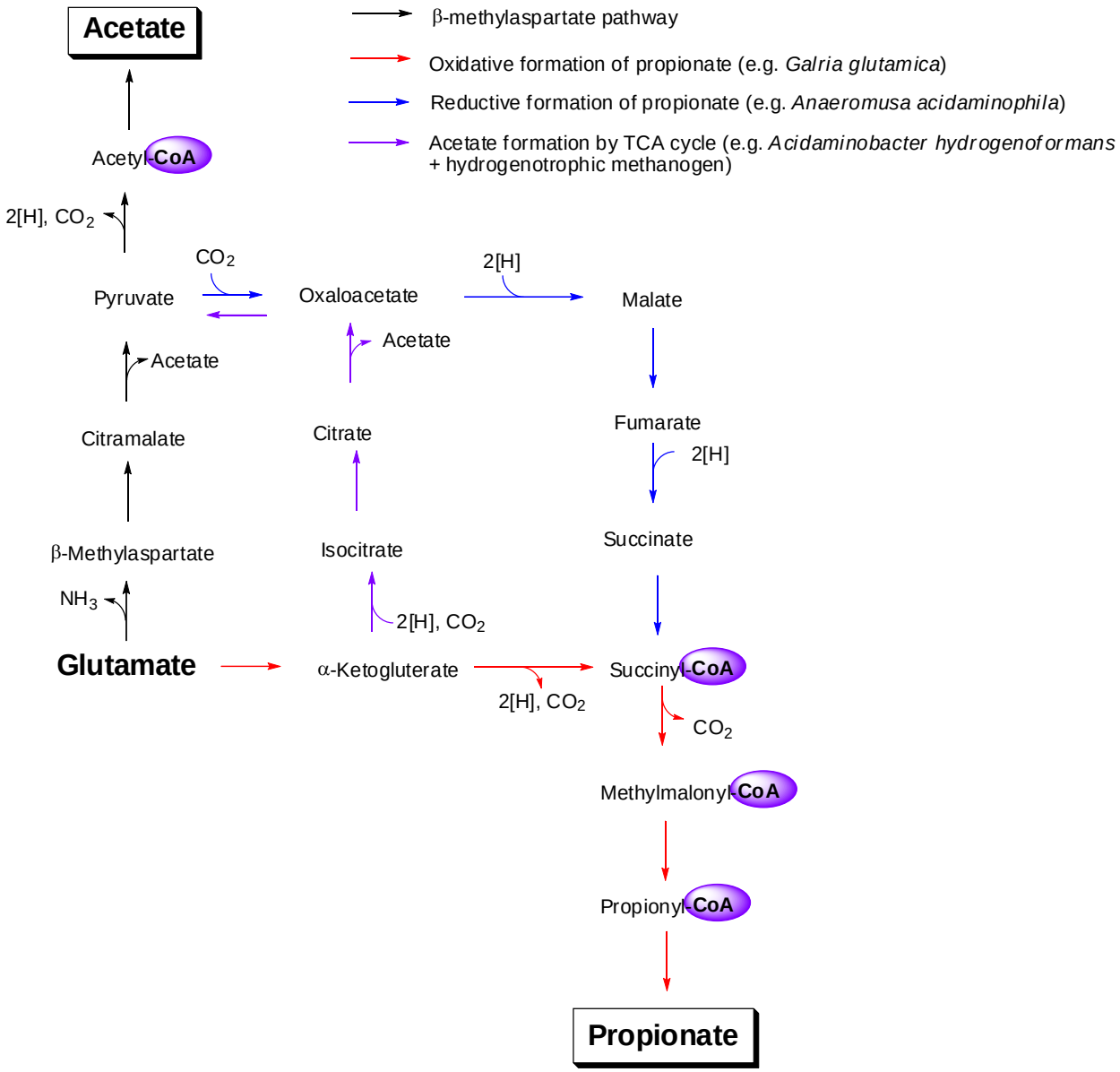

Figure 5 - Pathways involved in glutamate fermentation (modified from Schink & Stams, 2006)

Branched-chain Amino Acids

The branched-chain amino acids are typically degraded to their corresponding fatty acid as generalized below in which the dominant end product is a carboxylic acid that is one carbon atom shorter. The free energy changes for the catabolism of valine, leucine and isoleucine are slightly endergonic (ΔG from +4 to +10 kJ/mol) without the presence of an exergonic electron acceptor. The degradation of BCAAs has been linked to fatty acid biosynthesis (Stadtman 1950), as a mechanism for starvation, as well as energy conservation via SLP.

Elsden & Hilton (1978) studied the BCAA catabolism of 27 mesophilic clostridia. The majority of the strains followed the above conversions. A number of strains, including *Cl. difficile* and *Cl. sporogenes*, also produced 4-methyl-valeric acid (isocaproic acid) from leucine and 3-methyl-valeric acid from isoleucine, supporting the idea that isoleucine and leucine can also act as an electron acceptor during Stickland reactions. After deamination, the further oxidation of the 2-oxo acid (α-keto acid) leads to a CoA intermediate and CO_2, the former of which yields the fatty acid with ATP formed by SLP (Kim, Darley, Selmer, & Buckel, 2006). Similar to the action of acetate kinase, the branched chain amino acids yield ATP via SLP. Additionally, a reductive branched metabolism yielding more complex metabolic end products patterns has also been reported. The first report of carboxylic acids with the same number of carbon atoms (i.e. 4-methylpentanoate from leucine) as the parent BCAA were reported by Cohen-Bazire (1948) in studies on *Cl. sporogenes*. Further studies using ^{14}C- labeled leucine examining the catabolism by *Cl. sporogenes* gave a mixture of isobutyric acid which implicates its formation via beta-leucine, catalyzed by leucine 2,3-aminomutase, and beta-ketoisocaproic acid (Poston, 1976). *Cl. difficile* also converts L-leucine to ammonia, CO_2, 3-methyl-butanoate, and 4-methylpentanoate.

The reductive branch of L-leucine metabolism requires multiple steps, as shown in Figure 6, that results in the six carbon carboxylic acid, 4-methylpentanoate. This proceeds through a CoA intermediate via a 2-hydroxyacyl-CoA dehydratase using a radical mechanism (Kim, Darley, Buckel, & Pierik, 2008).

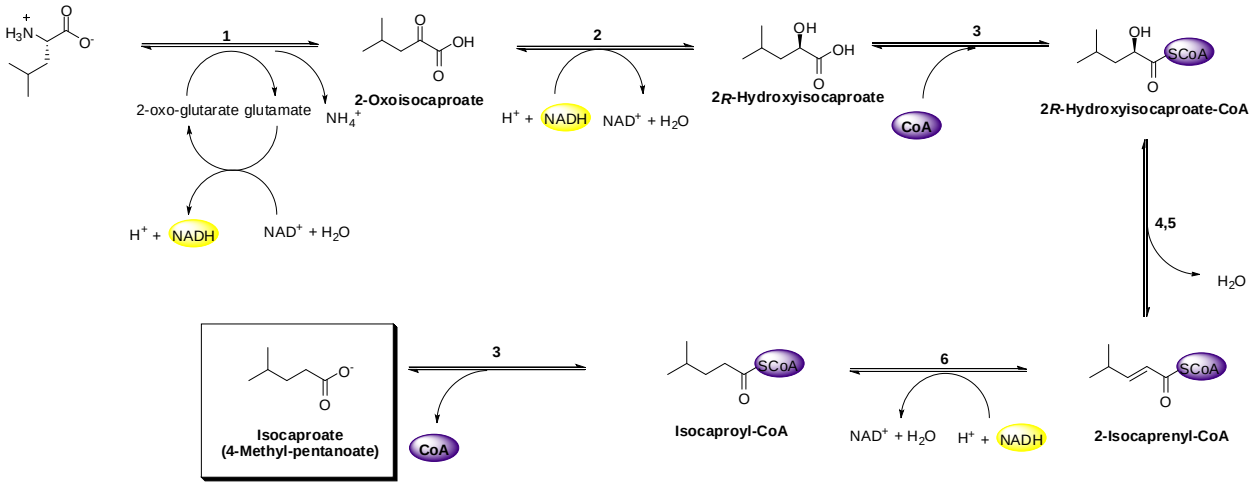

Figure 6 - Reductive branch of L-leucine fermentation by Cl. difficile (modified from Kim, Darley, Selmer, & Buckel, 2006). 1- Leucine aminotransferase and glutamate dehydrogenase, 2- (R)-2-hydroxyisocaproate dehydrogenase, 3- 2-hydroxyisocaproate CoA transferase, 4 – activator of dehydratase, 5 – 2-hydroxyisocaproyl-CoA dehydratase, 6- acyl-CoA dehydrogenase and electron transfer proteins

Aromatic Amino Acids

The catabolism of aromatic amino acids (ArAAs) has not been as well characterized as the amino acids discussed thus far. Unlike aerobic catabolism of aromatic compounds, the aromatic ring of aromatic amino acids is unaltered; this is due to the thermodynamic challenge associated with the further mineralization of aromatic carboxylic acids and alcohols under anaerobic conditions. As is the case for BCAAs, the degradation of ArAAs has both an oxidative and reductive branch. For instance, the oxidative branch of phenylalanine catabolism leads to the formation of phenylacetate and yields ATP via SLP while the reductive branched can act as an electron sink to maintain redox balance while generating 3- phenylpropionate.

The catabolism of aromatic amino acids yields a mixture of aromatic end products. The major end products that have been reported during an examination of 23 strains of mesophilic clostridia include either the corresponding fatty acid, a shortened fatty acid, or in some cases complete removal of the side chain from the aromatic ring. Thus, potential end products from phenylacetic acid, phenyl propionic acid, and phenyllactic acid from phenylalanine while tyrosine gives hydroxyphenylacetic acid, hydroxyphenyllactic acid, hydroxyphenylpropionic acid, phenol, and *p*-cresol (Elsden et al., 1976). Likewise, tryptophan gives indole, indole-3-acetic acid, and indole-3-propionic acid (Elsden et al., 1976)

3 Aims

Compared to the degradation and catabolism of carbohydrates, protein and amino acid catabolism by anaerobes has received considerably less attention. Aspects of protein and amino acid catabolism have been limited to the role of food spoilage organisms and the generation of flavor components in food while the roles of protein and amino acid degrading organisms from environments with elevated temperatures (i.e. geothermal areas) have received very little attention. While there have been several several studies on the amino acid catabolism of thermophilic clostridia in the 1990s, no systematic studies of the protein metabolism of *Thermoanaerobacter* and *Caldanaerobacter*, aside from limited work on *T. brockii* and closely-related species, has been undertaken.

The major aim of this work to to systematically explore the catabolism of the 20 proteogenic amino acids by the type strains of *Thermoanaerobacter* and *Caldanaerobacter* with a particular emphasis on understanding the nature of BCOH formation from the cooresponding BCAAs. The proposed pathway for *Thermoanaerobacter* species, for which a suggestion is given in *Figure 7*, is a focus point for this work.

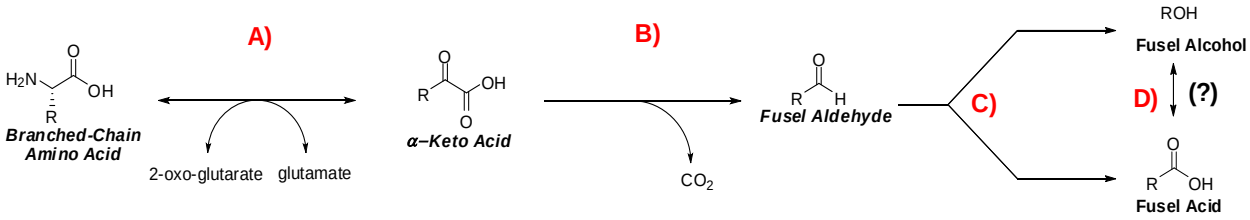

Figure 7 – Proposed pathway for branched-chain amino acids to their corresponding alcohols and acids; A – deaminase, B – decarboxylase, C – alcohol/aldehyde dehydrogenase, D – carboxylic acid reductase

The main objectives of this thesis are as follows:

- Gain better understanding of the degradation of proteins and amino acids of *Thermoanaerobacter* and *Caldanaerobacter* species
- Investigate the role of electron acceptor for the degradation of amino acids, with emphasis on BCAAs
- Gain deeper understanding of the physiology of amino acid metabolism of *Thermoanaerobacter* and *Caldanaerobacter* species, particularly the role of carboxylic acid reduction in BCOH formation

- Investigate the production of “higher order” alcohols from conversion of short-chain fatty acids

4 Results and Discussion

The following chapter summarizes and highlights key points from the work presented in **Papers I-VIII**. Where appropriate, data not included in published papers or the presented manuscripts will be mentioned. The initial observations relating to BCOH formation as communicated in **Papers I** and **II** are summarized in Section 4.1 while more detailed studies of the amino acid metabolism of *Thermoanaerobacter* strains (**Papers III-V**) and studies on the genera of *Thermoanaerobacter* and *Caldanaerobacter* (**Paper VI**) are presented in Section 4.2. The work describing the conversion of carboxylic acids to their corresponding alcohols using *Thermoanaerobacter pseudoethanolicus* (**Paper VII** and **VIII**) is summarized in Section 4.3.

4.1 Preliminary observations of branched-chain alcohol formation from branched-chain amino acids

The appearance of unknown peaks using gas chromatograph with column aimed for detecting volatile fatty acids and alcohols was observed during studies on the amino acid catabolism of several *Thermoanaerobacter* and *Caldanaerobacter* strains, notably strain AK68, AK85, and AK 90. This led to efforts to identify these peaks and explain their metabolic origin. Further investigations led to identification of BCOH (2-methyl-1-propanol, 2-methyl-1-butanol, and 3-methyl-1-butanol) in the medium of these bacteria.

Subsequent studies on the BCAA metabolism were repeated with several type strains within the genera of *Thermoanaerobacter* and *Caldanaerobacter* (**Paper I**). It should be pointed out to the reader that while the title of the paper refers to "*Thermoanaerobacter yonseiensis",* which does have standing in the genus of *Thermoanaerobacter* at this time (Parte, 2014) despite it being moved to *Caldanaerobacter* (Fardeau et al., 2004), this species is best placed phylogenetically and physiologically within the genus of *Caldanaerobacter* as a subspecies of *C. subterraneus* as it is refered to elsewhere throughout this work.

In this study, *T. brockii* and *C. subterraneus* subsp. *yonseiensis* were cultivated on all three BCAAs as single substrates with and without thiosulfate as well as in a co-culture with a thermophilic hydrogenotrophic methanogen as exemplified by the end products for each respective strain on leucine as shown in Figure 8. Without any addition of an electron scavenging system, the BCAAs were only fractionally degraded. However, when the

strains were cultivated with the hydrogenotrophic methanogen, the BCAAs were degraded to their corresponding, one carbon shorter, BCFA. Thus, valine was catabolized to 2-methyl-1-propionate, leucine to 3-methyl-1-butyrate, and isoleucine to 2-methyl-1-butyrate. However, when the selected strains of *Thermoanaerobacter* and *Caldanaerobacter* were cultivated using the BCAAs as single substrate but together with thiosulfate as an electron acceptor, the strains produced a mixture of both the BCFA and BCOH in addition to hydrogen sulfide. For example, under these conditions valine was converted to a mixture of 2-methyl-1-propionate and 2-methyl-1-propanol. Further work on *C. subterraneus* subsp. *yonseiensis* showed that increased thiosulfate concentrations could enhance both amino acid degradation and alcohol formation. It was also clear that the partial pressure of hydrogen was of great importance for the ratio of the alcohol over the acid; at high L-G phase ratio, approximately five times higher concentrations of BCOH formed as compared with low
L-G phase ratios.

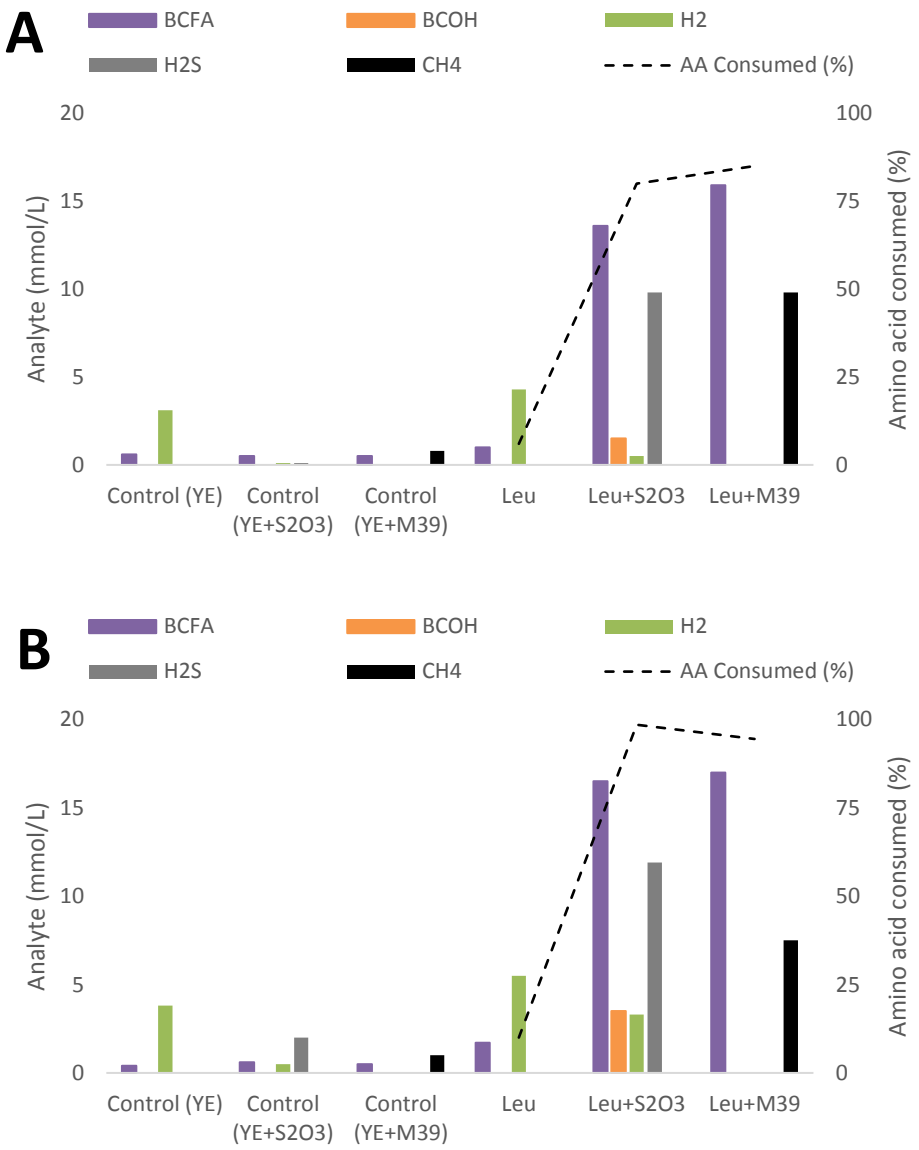

Figure 8 - Catabolism of leucine under thiosulfate or in co-culture with a methanogen (M39) by A- Thermoanaerobacter brockii subsp. brockii and B- Caldanaerobacter subterraneus subsp. yonseiensis (Data from Paper I, Scully & Orlygsson, 2014).

To further explore the ability of thermophilic anaerobes to produce BCFAs and BCOHs from BCAAs in the presence of thiosulfate, 48 strains belonging to various genera of thermophilic anaerobes, both strains from culture collections as well as bacteria isolated from Icelandic geothermal hot springs (**Paper II**) in addition to several type strains acquired from culture collections were examined for their ability to produce BCOHs from BCAAs. The culture collection contained thermophilic anaerobes isolated from geothermal areas around Iceland with strains belonging to genera *Caldanaerobacter*, *Caldicellulosiruptor*, *Caloramator*, *Caldanaerobious*, *Paenibacillus Thermoanaerobacterium*, and *Thermoanaerobacter*. The main outcome from this

screening experiment was that the ability to degrade BCAAs was only found within genera of *Thermoanaerobacter* and *Caldanaerobacter* with one exception in which *Caloramator viterbiensis* produced BCFAs from BCAAs but not the corresponding alcohol as summarized in Figure 9. The lack of BCOH formation from other genera does not necessarily preclude the possibility that they are capable of this metabolism.

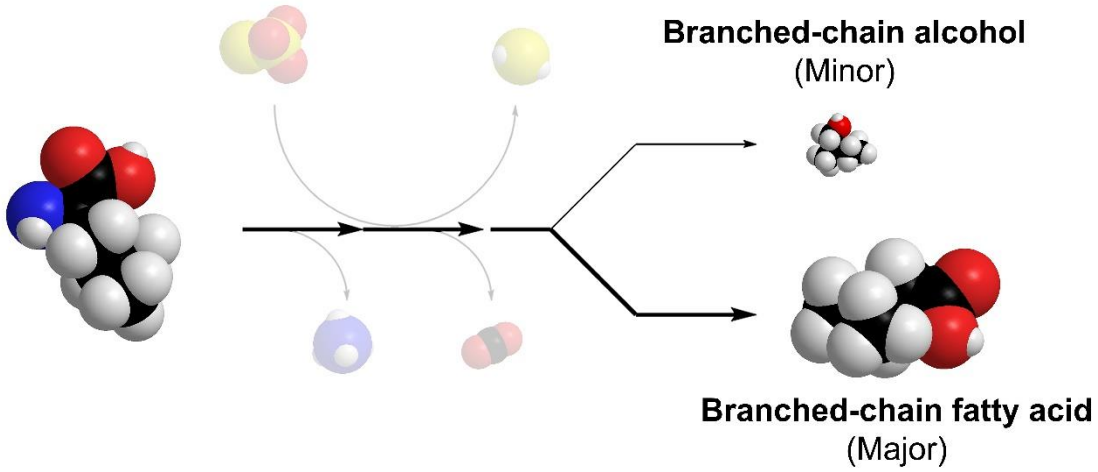

Figure 9 – The fermentation of branched-chain amino acids in the presence of thiosulfate to mixed end products

As observed previously, the BCFA was always the dominant product with the corresponding BCOH representing only a small portion of the carbon balance. Additionally, the amount of hydrogen sulfide produced was lower than the theoretical yield and the remainder of the sulfur could not be accounted for which suggests that the sulfur metabolism of these strains is more complex than is currently known.

In order to better examine BCAA metabolism, six strains were cultivated on all three BCAAs as single substrates to demonstrate yields and fermentation characteristics of their degradation. The *Thermoanaerobacter* and *Caldanaerobacter* strains examined differed considerable concerning how much of the BCAAs were degraded and also, interestingly, in how much H_2S (end product from thiosulfate) was produced. In all cases, excess of the electron donor (thiosulfate) was used and completely degraded but this ended up in various concentration of H_2S and H_2. Thus, it seems that the sulfur metabolism is very different in the species selected and may be of importance in amino acid metabolism. Also, the role of pH_2 or at least the manner in which reducing equivalence is removed seems to be of importance as the use of a highly efficient system, such as a hydrogenotrophic methanogen, leads to only the corresponding fatty acid, while the reduction of thiosulfate to hydrogen sulfide results in a mixture of both the BCFA and the BCOH.

While not strictly part of this work, it should be noted that the type strains of several *Thermoanaerobacterium* and *Caldanaerobius* strains evaluated for their ability to degrade mannitol showed detectable quantities of BCFAs and trace quantities of the corresponding alcohols during glucose fermentation which is likely the result of limited catabolism of the BCAAs present (Chades et al., 2018). Yeast extract and glucose fermentation of the members of the genus of *Caldicellulosiruptor*, however, do not show traces of either BCFAs or BCOHs (Chades et al., 2018). This suggests that the ability to degrade BCAAs to their corresponding BCFAs and BCOHs may not be restricted to the genera of *Thermoanaerobacter* and *Caldanaerobacter* while formation of the BCOH may be a more unique feature or dependent upon specific culture conditions.

4.2 Influence of culture conditions on protein and BCAA catabolism on selected *Thermoanaerobacter* strains

To better investigate the physiology of BCAA catabolism, the influence of culture conditions and the enzymes involved was investigated for selected strains. Work on the influence of culture conditions on BCAA catabolism focused primarily on *Thermoanaerobacter* strain AK90 (**Paper III**), *Thermoanaerobacter* strain AK85; (**Manuscript IV**), and *Thermoanaerobacter pseudoethanolicus* (**Manuscript V**). The BCAA metabolism of other type strains of *Thermoanaerobacter* and *Caldanaerobacter* were also performed but will be communicated at a later time. Finally, a detailed analysis of protein and amino acid metabolism of most of the type strains within both genera of *Thermoanaerobacter* and *Caldanaerobacter* were performed (**Manuscript VI**).

Paper III examined the amino acid catabolism of *Thermoanaerobacter* strain AK 90, a isolate from a geothermal area in Iceland (Sveinsdottir, Baldursson, & Orlygsson, 2009). The strain is most closely related to *T. thermohydrosulfuricus* (100% similarity according to its 16S rDNA). The strain was cultivated on all proteogenic amino acids either as single substrates with and without thiosulfate or in the presence of a hydrogenotrophic methanogen. The main outcome of this investigation was that the only amino acid degraded as single substrate was serine. The spectrum of amino acids degraded under other condition was much broader; alanine, cysteine, serine, threonine, phenylalanine and tyrosine together with the BCAA were degraded under both electron scavenging systems. Thus, the importance of an electron scavenging system for amino acid metabolism was clear from this study. Further studies in this investigation were towards BCAA metabolism of *T. brockii* and *T. ethanolicus*, the type species of the genus. The main focus of these experiments were on the role of initial thiosulfate concentration on end-product profiles from BCAA metabolism. In both strains, it was clear that higher concentrations of thiosulfate lead to more profound amounts of the BCFA compared with the BCOH and that, when thiosulfate was not in excess, proportionally more of the BCOH was produced.

To better understand the role of culture conditions on BCOH formation, several studies were undertaken using several strains displaying different behavior with respect to their ability to produce BCOHs from their corresponding BCAAs under thiosulfate conditions. It has been noted with other C4 and higher alcohol producing organisms, such as yeast and butanologenic *Clostridia* that several culture parameters are essential, namely pH and temperature (Hazelwood, Daran, van Maris, Pronk, & Dickinson, 2008; Hough & Stevens, 1961; Jones, 2001; Ni & Sun, 2009; Xin et al., 2017; Zheng, Tashiro, Wang, & Sonomoto, 2015). It is also known from the author´s previous work on ethanologenic *Thermoanaerobacter* strains that the pH_2 of the system, which can be influenced through manipulating L-G ratios, can also be a crucial factor in directing reducing potential and thus the flow of electrons to more reduced products such as ethanol.

To this end, work on *Thermoanaerobacter* strain AK85, which was also isolated from a geothermal area in Iceland (Sveinsdottir et al., 2009) (**Manuscript IV**), investigated its BCAA metabolism in more detail. This strain is most closely related to *Thermoanaerobacter uzonensis* (97.7% homology) according to 16S rDNA analysis and has previously demonstrated that it is highly ethanologenic (Jessen & Orlygsson, 2012). As before, the amino acid utilization profile was performed without an electron scavenger, with thiosulfate, and in a co-culture with a methanogen. There was an emphasis placed on the role of culture conditions on the production of BCOHs and BCFAs from the BCAAs, particularly variables that might influence the partial pressure of hydrogen in the system (e.g. liquid-gas phase ratio and the concentration of thiosulfate). Unsurprisingly, the concentration of thiosulfate was of critical importance for the BCFA and BCOH ratio produced while the L-G ratio had little impact on the BCOH/BCFA ratio although very little hydrogen sulfide was present at the end of the fermentation. Furthermore, temperature and pH had almost no influence on the ratio of BCOH to BCFA produced from isoleucine beyond the established range of pH/temperature at which strain AK85 will grow.

Interestingly, when culture experiments were left for a longer period of time, in this case 14 days, the corresponding BCOH was the dominant product from BCAA degradation. This strongly suggests that the flow of electrons under these conditions does not rapidly reach equilibrium. To better understand this phenomenon, kinetic experiments were performed using the BCAAs under thiosulfate conditions.

Ultimately, the kinetic studies show that BCAA fermentation is relatively slow with the concentrations of the alcohol increasing over time with the concentration of BCOHs slowly increasing after BCFAs begin to accumulate. To understand the flow of electrons and to investigate if the BCFA was being converted to BCOH, enzyme assays and fermentation studies using ^{13}C2-labeled leucine in the presence of thiosulfate (Figure 10) and ^{13}C1-labeled 3-methyl-1-butyrate using thiosulfate plus leucine as a source of reducing potential (Figure 11) were performed which indeed suggest reduction of carboxylic acid as a source of BCOH with thiosulfate. This is further supported by the presence of aldehyde oxidoreductase activity in leucine-grown cells.

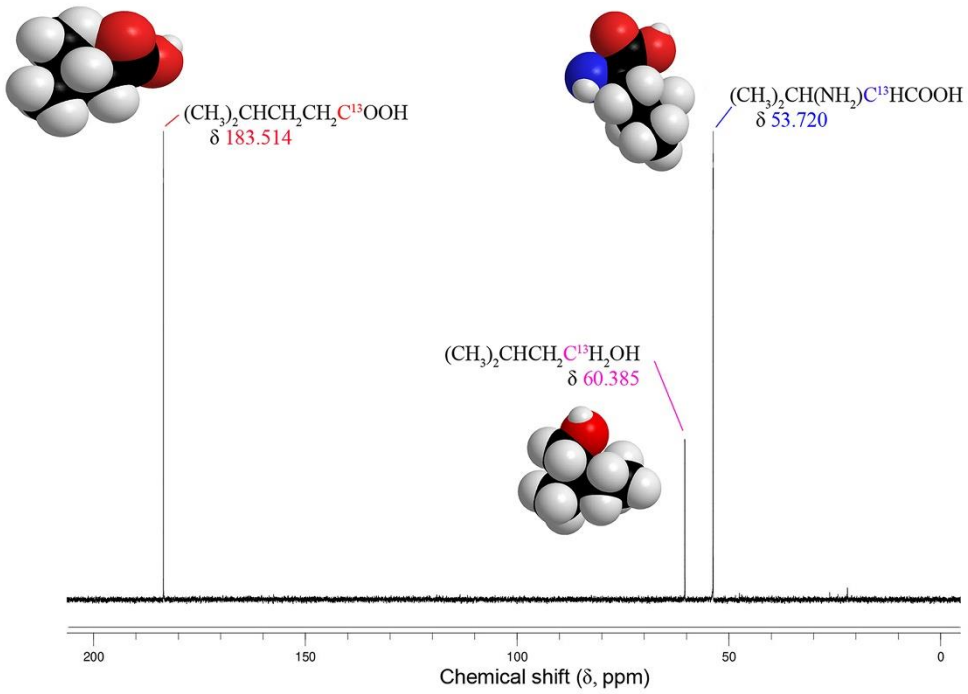

Figure 10 – ^{13}C NMR spectra of $^{13}C2$-labeled leucine fermented by Thermoanaerobacter strain AK85 in the presence of thiosulfate (20 mM)

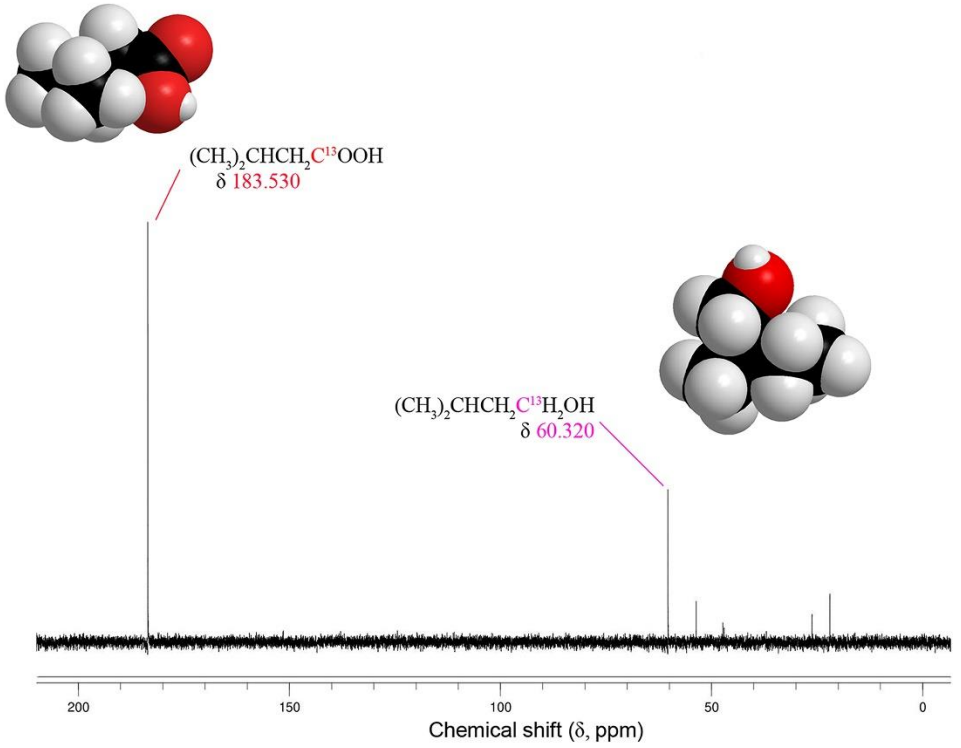

Figure 11 - ^{13}C NMR spectra of $^{13}C1$-labeled 3-methyl-1-butyrate (20 mM) in the presence of leucine (20 mM) and thiosulfate (20 mM) fermented by Thermoanaerobacter strain AK85

Similarly, a study of *Thermoanaerobacter pseudethanolicus* on BCAA metabolism was performed (**Manuscript V**). *T. pseudoethanolicus* has been extensively studied for its carbohydrate metabolism. As before, the BCAAs were not degraded to any extent by *T. pseudoethanolicus* without an electron scavenging system, but were degraded to a mixture of their corresponding BCFA and BCOH when thiosulfate was added to the culture. As has been observed with other strains, the amount of the acid formed was always considerable higher than the alcohol. Various environmental parameters on BCAA metabolism were investigated (pH, liquid-gas phase ratio, and initial thiosulfate concentrations) and as was the case with *Thermoanaerobacter* strain AK85, increased thiosulfate concentration increased the amount of the BCFA and decreased the amount of the BCOH. Interestingly, the same 14 day incubation period that resulted in a higher BCOH to BCFA ratio for strain AK85 was not observed with *T. pseudoethanolicus* as the dominant end product was always the BCFA. In stark contrast to the results with strain AK85, the observed concentrations of hydrogen sulfide were much higher at the end of the fermentation period suggesting that it was the preferred terminal electron sink instead of having electrons directed to the BCOH via BCFA reduction.

To better understand the impact of the carboxylic acid produced during BCAA fermentation as a potential electron sink, *T. pseudoethanolicus* was cultivated on $^{13}C1$ 3-methyl-1-butyrate with other electron donors (glucose or leucine) as show in in Figure 12.

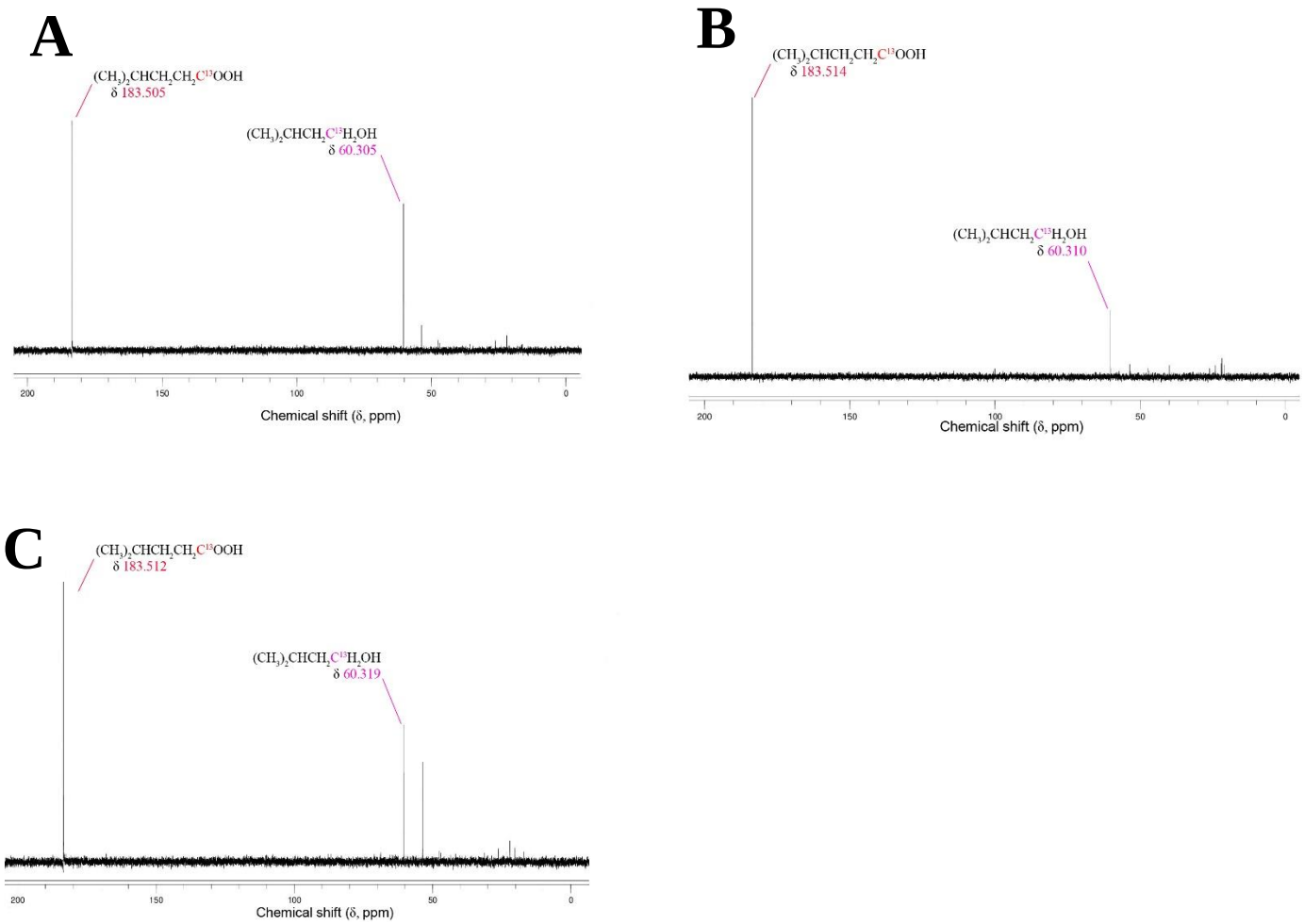

Figure 12 - Fermentation of glucose with ^{13}C1 3-methyl-1-butyrate (A), leucine with ^{13}C1 3-methyl-1-butyrate (B) and leucine in presence of thiosulfate with ^{13}C1 3-methyl-1-butyrate (C).

Based upon the enzymatic assays performed on leucine-grown cells of both *Thermoanaerobacter* strain AK85 and *T. pseudoethanolicus*, particularly in light of the carboxylic acid reductase activity, and the isotopically labeled experiments with both strains, a pathway (Figure 13) for BCAA degradation was proposed. The nature of the aldehyde intermediate is unclear and requires further investigation.

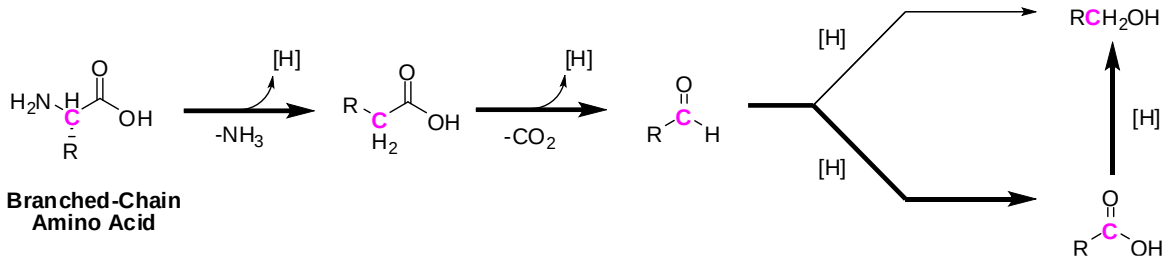

Figure 13 – Proposed pathway for branched-chain amino acid catabolism to both branched-chain fatty acid and alcohol via reduction of the fatty acid to the alcohol

As before, the ADH and AOR activities as well as the fermentation of isotopically labeled leucine and 3-methyl-1-butyrate support the notion that carboxylic acid reduction is at least in part responsible for BCOH formation, even though in the case of *T. pseudoethanolicus* the BCOH titers were much lower. Kinetic monitoring of BCAA degradation demonstrates that BCAA degradation is slow with the BCOHs only forming days into the fermentation. ^{13}C2-labeled studies of leucine confirmed the production of a mixture of BCFA and BCOH from leucine in the presence of thiosulfate. Additionally, experiments using ^{13}C1 3-methyl-1-butyrate also showed that leucine could be degraded without addition of thiosulfate if a volatile fatty acid was present to act as an electron sink. The use of other electron donors for the reduction of 3-methyl-1-butyrate presents a potential route for the reduction of carboxylic acids using inexpensive sources of reducing potential which is explored in greater detail in Section 4.3.

In order to evaluate the ability of *Thermoanaerobacter* and *Caldanaerobacter* species to utilize proteins and amino acids, the type strains of each species within the genus were cultivated on a several different types of protein substrates in different degrees of hydrolysis as described in **Manuscript VI**. Two strains, *T. mathranii* subsp. *mathranii* and *T. sulfurigenens*, show clear indications of trypsin activity while *C. subterraneus* subsp. *yonseiensis* demonstrates distinct chymotrypsin activity. While many species demonstrated some ability to hydrolyze proteins with enhanced action with the addition of thiosulfate. *T. brockii* subsp. *brockii*, *T. brockii* subsp. *finnii*, *C. subterraneus* subsp. *subterraneus*, and *C. subterraneus* subsp. *yonseiensis* showed particularly strong action on cooked meat medium and hydrolysis of other proteins such as collagen and gelatin. This suggests that several *Thermoanaerobacter* and *Caldanaerobacter* species are at least mildly proteolytic which others, such as the two afore mentioned *Caldanaerobacter* species, are more active in this regard and may play a role in nutrient cycling via the break down of proteins in the environment.

The ability of the two genera to utilize single amino acids were also evaluated and revealed that alanine, threonine, and serine could be utilized without the addition of thiosulfate by some strains. The ability to ferment BCAAs was a common feature among the genera although the ability to degrade aromatic amino acids was limited to *C. subterraneus* subsp. *subterraneus* and *C. subterraneus* subsp. *yonseiensis*. These two strains demonstrated a robust ability to ferment many amino acids as single substrates.

Comparisons of the ability of *Thermoanaerobacter* and *Caldanaerobacter* strains to degrade BCAAs was also evaluated. Like many mesophilic *Clostridia*, *Thermoanaerobacter* and *Caldanaerobacter* strains form a SCFAs that are one carbon atom shorter than the parent BCAA. Additionally, a BCOH is formed. Thus, valine yields a mixture of 2-methyl-1-butyrate and 2-methyl-1-butanol, leucine yields 3-methyl-1-butyrate and 3-methyl-1-butanol, and isoleucine gives 2-methyl-1-butyrate and 2-methyl-1-butanol. Unlike mesophilic *Clostridia*, carboxylic acids formed from the reductive branch that are the same number of carbon atoms as the parent BCAA, such as 4-methyl-pentanoic (isocaproic) acid from leucine, were not observed. The ratio of BCOH to BCFA formed vary but never exceed a 1:1 molar ratio as shown in Figure 14 for the catabolism of leucine in the presence of thiosulfate.

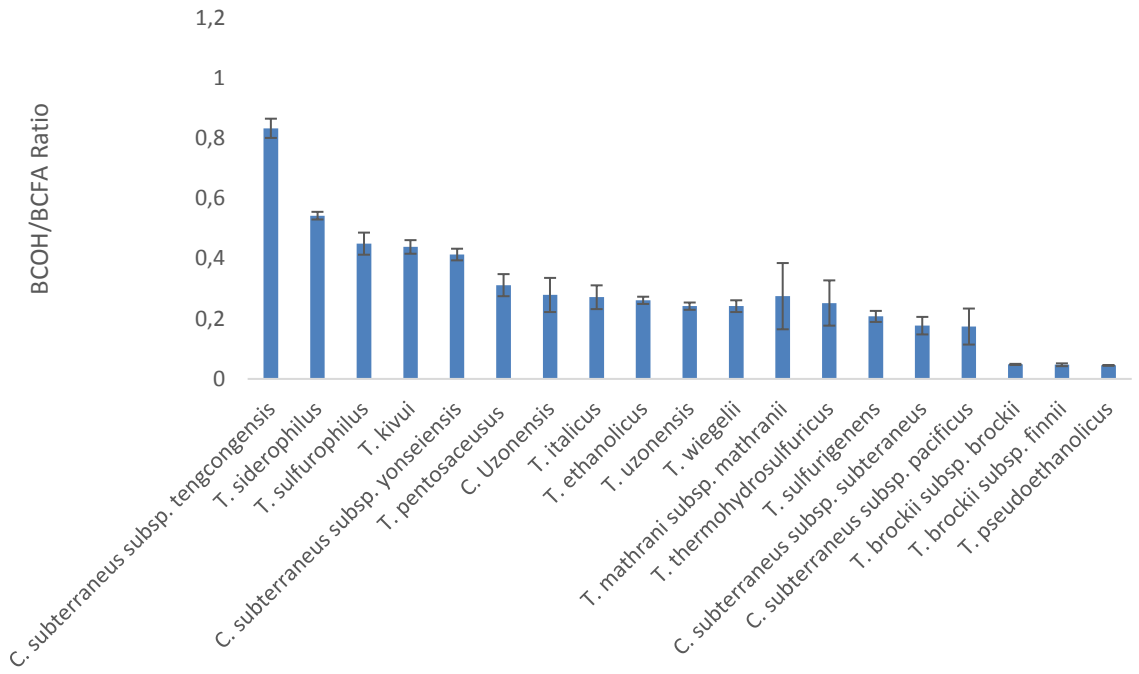

Figure 14 – Ratio of branched-chain alcohol (3-methyl-1-1-butanol) to branched-chain fatty acid (3-methyl-1-butyrate) resulting from leucine catabolism by the type strains within the genera of Thermoanaerobacter and Caldanaerobacter

The differences in the ability of strains to produce a BCOH from the corresponding BCAA differed by BCAA and by strain. These differences are likely attributable to slight differences in the substrate specificity of the enzymes involved as well as variation in how individual strains direct the reducing equilvalence relative to their ability to utilize thiosulfate as an electron sink. Interestingly, *T. pseudoethanolicus*, despite being good at reducing carboxylic acids in the presence of glucose (**Paper VII** and **VIII**), did not

produce high BCOH titers during BCAA catabolism. The kinetics of BCAA catabolism were investigated for several strains which reveals that BCAA catabolism and BCOH formation are very slow as previously observed for AK85 and *T. pseudoethanolicus*. The use of isotopically labeled glycine and leucine was also examined for selected strains although this data was not included in **Manuscript VI**.

Of particular interest was that *C. subterraneus* subsp. *tengcongensis* and *C. subterraneus* subsp. *yonseiensis* were capable of degrading the aromatic amino acids to one or more carboxylic acid products although the corresponding alcohol was not detected. This, coupled with the proteolytic nature of these strains, might make them potentially useful bioprocessing organisms for biomass containg large protein fractions. Based on analysis of the end products by TLC, it appeared that multiple carboxylic acid aromatic products were present for several of the aromatic amino acids. It is likely that these products are the corresponding fatty acids that are one and two carbons shorter than the parent amino acid. The catabolism of aromatic amino acids by these strains warrants further investigation much in the same manner as the fermentation of the BCAAs was scruitinized in **Papers IV** and **V**.

4.3 Conversion of carboxylic acids to alcohols

Unrelated work examining the inhibitory effects of neutral salts of acetate, propionate, and butyrate using *Thermoanaerobacter* strains demonstrated the conversion of short-chain fatty acids to their corresponding alcohols in the presence of a source of reducing potential, in this case, the formation of NADH from the entry of glucose into glycolysis. This suggests that *Thermoanaerobacter* and *Caldanaerobacter* strains are capable of using organic acids as an electron sink to regenerate $NAD(P)^+$. Subsequent work on the biotransformation of organic acids, ranging from 1 to 8 carbons, demonstrated that this does in fact occur and was further supported with the use of isotopically labeled substrates (**Paper VII**, **Manuscript VIII**).

The ability of *T. pseudoethanolius* to convert fatty acids to alcohols is presented in a short communication in **Paper VII**. This work demonstrates the conversion of C2 to C6 carboxylic acids to their corresponding alcohols in the presence of glucose with conversion yields between 21.0 to 61%. To conclusively demonstrate the exogenously added carboxylic acids were indeed being reduced, isotopically labeled short-chain fatty acids were added to cultures containing glucose as a source of reducing potential. Indeed, ^{13}C1-labeled short-chain alcohol was a major end product of these fermentations as evidenced by the high relative peak intensity for the resultant ^{13}C NMR spectra.

It should be noted that the reduction of acetate to ethanol may explain the highly ethanologenic nature of some, but not all, *Thermoanaerobacter* strains. The author originally postulated that the bifunctional nature of the alcohol/aldehyde dehydrogenases or a carboxylic acid reductase or some combination thereof may be responsible for the conversion of carboxylic acids to their corresponding alcohol. Work by another group independently published around the same time that this work was being written up postulates that the presence (or absence) of aldehyde:ferredoxin oxidoreductase may be in part responsible (Hitschler, Kuntz, Langschied, & Basen, 2018). The ^{13}C-labeled work in **Paper VII** supports this as does the presence of aldehyde reductase activity in **Manuscript IV** and **V**.

Other strains of *Thermoanaerobacter* demonstrated similar capabilities with apparent differences in substrate preference (as evidenced by higher or lower conversion of the carboxylic acid) which will be the subject of future communications. Further experiments using *T. pseudoethanolicus* investigated the role of various environmental factors on this conversion and are described in **Manuscript VIII**. In all cases, the conversion of carboxylic acids using glucose as a source of reducing potential showed that carboxylic acid conversion occurs rapidly and is concurrent with the exponential growth phase.

5 Future Prospects

This study demonstrates the ability of thermophilic bacteria within the genera of *Thermoanaerobacter* and *Caldanaerobacter* to degrade various amino acids in the absence and presence of an electron scavenger. The role of using various electron acceptors for degradation of BCAAs to a mixture of their BCFAs and BCOHs opens up a new way of producing high-value compounds from these amino acids. Further investigations towards maximizing the formation of the BCOH over the BCFA would be the next appropriate step. However, using amino acids would always include the cost of using expensive substrates. Therefore, the observation of the ability of the tested bacteria to reduce fatty acids to their corresponding alcohols opens up a more feasible way to produce valuable alcohols, both to be used as low-value, high-volume biofuels or high-value, low-volume fine chemicals. Instead of using expensive substrates, low cost lignocellulose and agricultural residues may open a new way of producing energy high biofuels from low value fatty acids. Thus, directions towards the use of lignocellulose would be appropriate as grounds for further study.

5.1 Pan-genus comparison to fill knowledge gaps in physiology of *Thermoanaerobacter*

Further genus-wide studies are needed to fill some of the knowledge gaps left by inconsistent characterization work. Of particular interest would be an examination of the ability of *Thermoanaerobacter* and *Caldanaerobacter* to grow autotrophically on CO and CO_2 as well as their ability to utilize carbohydrates. The latter has already been particularly undertaken by the author for hexoses, pentoses, deoxyhexoses, disaccharides, and some polymers although further work is needed. Studies using isotopically-labeled glucose (^{13}C1 and ^{13}C2) have been performed although one outstanding question that needs to be addressed is the role of acetate re-assimilation in ethanol formation. Perhaps the largest knowledge gap, partially exposed by this body of work is the differences in sulfurous end products from thiosulfate metabolism. Indeed, the ability of the genus to utilize sulfur compounds as well as other inorganic components (i.e. Fe, Mn, U species, etc) present in geothermal and other environments might answer questions on the role of these species in particular environmental niches.

To better understand the role of *Thermoanaerobacter* and *Caldanaerobacter* strains in nitrogen cycling, the ability of these strains to utilize a variety of inorganic and organic nitrogen soucres such as urea, thiourea, nitrate, nitrite, molecular nitrogen should be investigated. Although not explored here, a major feature of the genus of *Caldanaerobacter* is its ability to produce alanine as a major end product from glucose;

thus, the role of glucose intermediates as a sink for nitrogen could be an interesting area of exploration for both genera. Of particular interest is the ArAAs and the carboxylic acid products arising thereof highlighted in **Paper VI**. An experiment using an isotopically-labeled substrates to clarify the specific end products (*Figure 15*) could easily be performed.

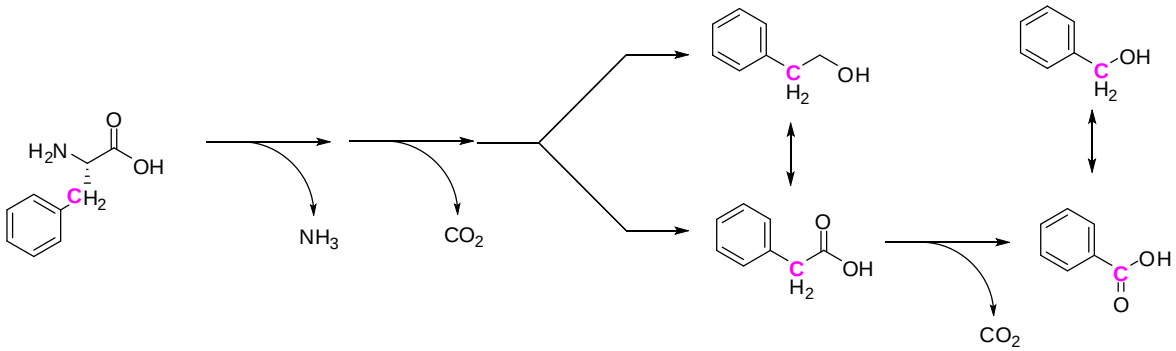

Figure 15 – Proposed experiment using ^{13}C3-phenylalanine to determine end products produced from aromatic amino acid metabolism by Caldanaerobacter spp.

Given the importance of amino acid and protein metabolism in the biosphere, a number of recommendations for the characterizations of strains yet to be described seems appropriate. Given the ease with which API ZYM assays can be performed, this would make an easy addition to the characterization of novel thermophilic *Clostridia*. Furthermore, while screening the ability to utilize multiple proteins and the 20 proteogenic amino acids with and without the electron acceptors needed to achieve thermodynamic favorability, several model amino acids or mixtures of amino acid by class should be examined using a suitable electrons scavenger.

Finally, the strains of *Thermoanaerobacter* and *Caldanaerobacter* isolated from Iceland's geothermal areas which were screened in **Paper II** highlight a number of strains, such as AK96, AK85, and AK104, which show relatively high BCOH/BCFA ratios. These strains should be re-screened for their ability to convert carboxylic acids to their corresponding alcohols as they may have unique selectivities which could be useful for transforming larger carboxylic acids to their corresponding alcohols. Furthermore, few of the strains explored in **Paper II** have undergone full characterization. Additionally, the esterases revealed in **Paper VI** could have selectivities that could be of use in synthetic chemistry, for example the enantioselective of low molecular weight polyalcohols such as 1,2-propanediol using ester-protected derivatives. Other enzyme chemistries of potential biotechnological importance, such as glycotransferases and alcohol dehydrogenases should be systematically screened for.

5.2 Amino acid physiology and protein bioprocessing

A more detailed examination on the specific pathways involved in amino acid degradation could also answer questions relating to the generation of BCOHs during fermentation as well as ArAA catabolism. Of particular use would be the whole genome sequences that are available for a number of strains within the genus. Further work on the specific gene knockouts to evaluate, for example, the importance of carboxylic acid reduction on yielding alcohols as a fermentation end product from amino acids and even carbohydrates. As multiple acidic and alcoholic end products are possible from several of the aromatic amino acids, the further application of ^{13}C2-labeled ArAAs could assist in working out pathway details. Furthermore, the physiological role that amino acid catabolism plays requires additional scrutiny as well as any regulatory mechanisms that could, for instance, explain the delayed onset of BCAA catabolism and the slow formation of BCOHs from BCFAs.

With respect to BCAA catabolism, the nature of the aldehyde (or equivalent) intermediate preceding the last fermentative step as well as the intermediate involved in carboxylic acid reduction is of interest. This intermediate may be an acyl-CoA or could proceed via an unactivated aldehyde intermediate. The substrate specificity of the enzymes involved as well as any stereopreference of the required hydride transfer reactions could be of great interest.

One aspect of the amino acid catabolism of *Thermoanaerobacter* and *Caldanaerobacter* strains in this work was the nature of the early steps in the process, namely the import of the amino acids and their subsequent deamination and decarboxylation. Further work is required to determine the exact nature of these steps. Of particular interest is the deamination reaction; glutamate dehydrogenase, if it is involved, has been shown to have a cofactor preference for either NAD or NADP in other *Clostridia* which would make it a useful tool in cofactor cycling in synthesis.

A number of *Thermoanaerobacter* and *Caldanaerobacter* species show strong proteolytic character. The thermostable proteases should be isolated and characterized. While some isolates have been characterized, the other highly proteolytic strains within the genus could potentially harbor proteases of biotechnological potential. For example, the use of protein-rich biomass such as macro algae, fish processing waste, or invasive plants such as lupine, could provide a sustainable route to the production of other biomolecules. As a number of these strains are proteolytic, these may make promising CBP organisms for the utilization of protein-rich biomass and its conversion into potentially useful biomolecules. Further work on strain adaptation and process design will need to accompany work on the fermentation of protein-rich complex biomass. The specific extracellular proteases

produced by *T. sulfurophilus* and *C. subterraneus* subsp. *yoinseiensis* may be potentially useful thermostable enzymes in their own right necessitating a closer examination of their enzymology.

Further work on the ability of *Caldanaerobacter* species to utilize ArAAs requires additional investigation. Furthermore, the ability of strains to reduce aromatic carboxylic acids to their corresponding alcohol could be a potentially useful route given the premium placed on biologically produced 1- and 2-phenylethyl alcohol since chemically produced aromatic alcohols have difficult to remove impurities. The enzymes involved in ArAA metabolism may have substrate specificity that allows for larger aromatic carboxylic acids to undergo bioreductions.

5.3 Applications of carboxylic acid reduction

While **Paper VIII** explores the impact of some culture variables on the ability of *T. pseudoethanolicus* to reduce carboxylic acids to their corresponding alcohols, other aspects remain unexplored. For instance, the impact of high partial pressures of hydrogen, which could be accomplished using hydrogen in the headspace, could further drive the reductions by increasing the NAD(P)H/NAD(P)$^+$ ratios. The use of other sources of reducing potential, such as polyalcohols or biomass hydrolysates, could prove to be an interesting avenue for future work. Additionally, the continuous *in situ* removal of alcohol products using biphasic cultivation with a non-polar organic phase such as fatty acid methyl esters (biodiesel) or a high-boiling solvent such as oleyl alcohol as has been explored with 1-butanol production, could boost carboxylic acid conversion. The use of ionic liquids and selective membranes may also be promising techniques for improving the conversions of carboxylic acids to their corresponding alcohols. Furthermore, the use of other fermentation modes such as fed-batch or continuous culture could prove to be better suited for carboxylic acid conversion. The exploration of waste materials rich in short-chain fatty acids, such as aquaculture runoff, AD waste or hydrothermally-treated process waters, could be an interesting and inexpensive source of low-cost acids which could then be upgraded to higher-value alcohols.

Conversion of carboxylic acids to alcohols is useful from a synthetic stand point. The ability of strains and their enzymes to utilize other carboxylic acid substrates, such as aromatic acids, dicarboxylic acids, carboxylic acid analogs containing N and S, and lactones and lactides could be potentially useful (Figure 16). Furthermore, mapping of the selectivies of the primary and secondary ADHs as well as the aldehyde oxidoreductases could provide novel enzymes with unusual properties which could be further improved by rational design as has been done with the secondary alcohol dehydrogenases of *T. pseudoethanolicus*.

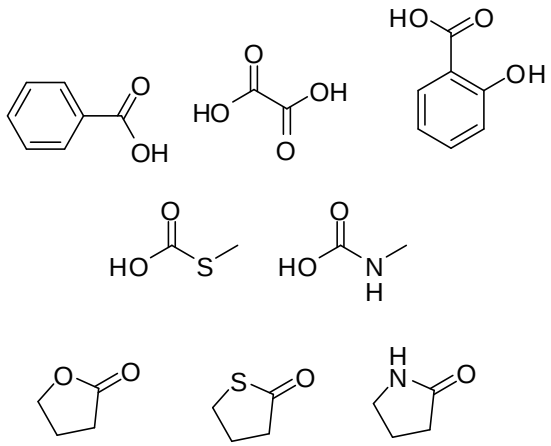

Figure 16 - Potential targets for carboxylic acid reductions catalyzed by Thermoanaerobacter sp.

6 Conclusions

The results herein highlight the importance of specific culture parameters with respect to understanding the flow of electrons during branched-chain amino acid metabolism as well as examining the protein and amino-acid degrading capability of *Thermoanaerobacter* and *Caldanaerobacter* strains with a specific emphasis on determining the origin of branched-chain alcohol formation. The major findings are listed below:

- The role of removal of electrons produced in the deamination of amino acids in general yield different end product spectra was observed with different electron accepting system; when amino acid degrading bacteria was co-cultivated with a hydrogen consuming methanogen the BCAAs were degraded to their one carbon less BCFA. When thiosulfate was added in excess to the amino acid degrading bacteria, a mixture of BCFA and BCOH was produced.
- Culture parameters such as temperature and pH have negligible impact on the production of BCOHs during BCAA degradation while the overall catabolism of BCAAs is much slower than the utilization of other substrates such as carbohydrates.
- The origin of BCOHs produced by BCAAs degraded under thiosulfate conditions is, at least in part, due to the reduction of the BCFA as confirmed by ^{13}C NMR.
- Thermophilic, anaerobic bacteria within the genera of *Thermoanaerobacter* and *Caldanaerobacter* are proteolytic to some extent and all strains are capable of BCAA fermentation with the utilization of serine and threonine also being common.
- The differences in the ratio of BCOH to BCFA produced from amino acids under thiosulfate conditions vary widely throughout the members of both genera with strains such as *T. pseudoethanolicus* producing very low BCOH titers while other strains, such as *C. subterraneus* subsp. *yoinseiensis* produce higher BCOH titers.
- *C. subterraneus* subsp. *subterraneus and C. subterraneus* subsp. *yonseiensis* are robust with respect to their utilization of proteins and amino acids and seem to be uniquely capable of ArAA fermentation.
- *Thermoanaerobacter pseudoethanolicus* can reduce C2-C6 carboxylic acids to the corresponding alcohol in the presence of a source of reducing potential such as glucose.

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Manuscripts I-VIII

Please find the following papers (I-VIII) as presented by their roman numerals.

Paper I

Branched-chain alcohol formation by *Thermoanaerobacter brockii* and *Thermoanaerobacter yonseiensis*.

Scully, S.M. and Orlygsson, J.

Anaerobe **2014**, 30, 82-84.

Paper II

Branched-chain alcohol formation by thermophilic bacteria within the genera of *Thermoanaerobacter* and *Caldanaerobacter*.

Scully, S.M., Iloranta, P., Myllymaki, P., and Orlygsson, J.

Extremophiles **2015**, 19(4):809-818. doi: 10.1007/s00792-015-0756-z

Paper III

Amino acid metabolism of *Thermoanaerobacter* strain AK90: the role of electron scavenging systems on end product formation.

Scully, S.M. and Orlygsson, J.

Journal of Amino Acids **2015**, 2015, 1-10. doi:10.1155/2015/410492.

Paper IV

Branched-chain amino acid catabolism of *Thermoanaerobacter* strain AK85 and the influence of culture conditions on branched-chain alcohol formation.

Scully, S.M. and Orlygsson, J.

Amino Acids **2019**. Accepted for publication. DOI: 10.1007/s00726-019-02744-z

Paper V

Protein and amino acid metabolism of *Thermoanaerobacter pseudoethanolicus*: role of carboxylic acid reduction in branched-chain alcohol formation.

Scully, S.M. and Orlygsson, J.

Submitted to *Biotechnology and Bioengineering*.

Paper VI

Protein and amino acid metabolism of *Thermoanaerobacter* and *Caldanaerobacter* species.

Scully, S.M., Ingvadottir, E.M., and Orlygsson, J.

Unpublished manuscript.

Paper VII

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

Anaerobe **2019**, 57, 28-31. https://doi.org/10.1016/j.anaerobe.2019.03.004

Scully, S.M., Brown, A., Ross, A.B., and Orlygsson, J.

Paper VIII

Bioreduction of organic acids to alcohols by *Thermoanaerobacter pseudoethanolicus*.

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Submitted to *Biotechnology and Bioengineering*.

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Note

Branched-chain alcohol formation from branched-chain amino acids by *Thermoanaerobacter brockii* and *Thermoanaerobacter yonseiensis*

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ABSTRACT

Thermoanaerobacter species degrade branched-chain amino acids to a mixture of their corresponding branched-chain fatty acids and alcohols in the presence of thiosulfate; only acid formation occurred when *Thermoanaerobacter* strains were cultivated in co-culture with a hydrogenotrophic methanogen. Increased pH_2 at high liquid–gas phase ratios increases the relative concentration of branched-chain alcohol.

The importance of different electron scavenging systems for the degradation of reduced amino acids, such as branched-chain amino acids (BCAAs), is well established [1–3]. The oxidation of BCAAs is thermodynamically unfavorable ($\Delta G^{\circ\prime}$ between +4.2 and +9.7 kJ/mol) but becomes thermodynamically feasible with the inclusion of an electron scavenger [2]. *Thermoanaerobacter brockii* can only degrade leucine, isoleucine, and valine in co-cultures with a hydrogen-scavenging organism, such as hydrogenotrophic methanogens [2] or sulfate reducers [3] or by the inclusion of thiosulfate which *Thermoanaerobacter* species can reduce to sulfur or hydrogen sulfide [4].

The present investigation examines the BCAA catabolism by *T. brockii* (DSM 1457) and *Thermoanaerobacter yonseiensis* (DSM 13777) under methanogenic or thiosulfate conditions. The *Thermoanaerobacter* strains were cultivated in anaerobic medium [5] in 117 mL serum bottles containing 58.5 mL of medium and 20 mM of single BCAA in either the presence or absence of thiosulfate (40 mM). The strains were cultivated without agitation and at 65 °C. When the *Thermoanaerobacter* strains were co-cultivated with *Methanothermobacter* M39, the methanogen was pre-cultivated with a H_2/CO_2 (80/20 v/v) gas phase for one week; thereafter the bottles were flushed with nitrogen and inoculated (2% v/v) with the *Thermoanaerobacter* strains. Strain M39 was isolated as described by Brynjarsdottir et al. [6] and analyzed for 16S rDNA by DSMZ using conventional methods; it has 99.7% similarity with *Methanothermobacter marburgensis* Marburg^T.

Volatile end products, hydrogen, and methane were analyzed by gas chromatography as previously described [5]. Hydrogen sulfide was analyzed according to Cord-Ruwisch [7]. Thiosulfate was analyzed according to Westley [8]. Amino acids were analyzed using the ninhydrin method. Briefly, the analysis was conducted by the addition of 100 μL of sample and 100 μL of 1% (w/v) ninhydrin reagent (60% v/v 2-propanol and 40 mM acetate buffer, pH 5.5) in a microtiter plate and incubated at 100 °C for 20 min; relevant amino acids (isoleucine, leucine, or valine) were used as standards. After cooling, 200 μL of 50% (v/v) 2-propanol was added and the absorbance read at 580 nm on a Bioscreen C (Oy Growth Curves Ab, Finland). Since the ninhydrin method is not selective for specific amino acid, values presented have background values from control bottles subtracted (yeast extract, 2 g/L). All experiments were performed in duplicate.

When the two *Thermoanaerobacter* strains were cultivated on single BCAAs, only a small portion of the BCAA was degraded to the corresponding BCFA and hydrogen (Table 1). When the *Thermoanaerobacter* strains were co-cultivated with *Methanothermobacter* strain M39, the BCAAs were almost completely degraded leading to the production of their corresponding BCFAs

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Table 1
End product formation from branched-chain amino acids under three different conditions by *T. brockii* and *T. yonseiensis*; a) single substrates b) co-culture with *Methanothermobacter* strain M39 and c) with 40 mM of S_2O_3. Values are average of two replicates ± standard deviation. Controls are experimental bottles with only yeast extract (2 g/L).

Strain	Substrate	Products (mmol L^{-1})					Amino acid (mM)	Carbon balance (%)
		BCFA	BCOH	H_2	H_2S	CH_4		
T. brockii	Control (YE)	0.6 ± 0.0	<0.1	3.1 ± 0.1	ND	ND	<0.1	ND
	Control (YE + S_2O_3)	0.5 ± 0.1	<0.1	0.1 ± 0.0	0.1 ± 0.0	ND	<0.1	ND
	Control (YE + M39)	0.5 ± 0.0	<0.1	<0.1	ND	0.8 ± 0.1	<0.1	ND
	Leu	1.0 ± 0.2	<0.1	4.3 ± 0.1	ND	ND	18.8 ± 1.3	83.3
	Leu + S_2O_3	13.6 ± 1.1	1.5 ± 0.1	0.5 ± 0.0	9.8 ± 1.1	ND	4.0 ± 0.3	94.4
	Leu + M39	15.9 ± 2.3	<0.1	<0.1	ND	9.3 ± 0.8	3.0 ± 0.5	93.5
	Ile	1.1 ± 0.1	<0.1	4.2 ± 0.0	ND	ND	18.7 ± 0.9	91.7
	Ile + S_2O_3	14.4 ± 1.3	1.2 ± 0.1	0.2 ± 0.0	12.6 ± 1.7	ND	3.6 ± 0.6	95.1
	Ile + M39	15.4 ± 1.3	<0.1	<0.1	ND	8.3 ± 0.8	14.0 ± 1.1	96.2
	Val	1.9 ± 0.2	<0.1	7.0 ± 0.3	ND	ND	17.8 ± 1.2	86.3
	Val + S_2O_3	11.0 ± 1.5	1.3 ± 0.1	0.2 ± 0.0	5.0 ± 0.5	ND	4.5 ± 0.2	80.6
	Val + M39	14.8 ± 0.9	<0.1	<0.1	ND	7.6 ± 1.0	4.4 ± 0.4	94.9
T. yonseiensis	Control (YE)	0.4 ± 0.0	<0.1	3.8 ± 0.3	ND	ND	<0.1	ND
	Control (YE + S_2O_3)	0.6 ± 0.0	<0.1	0.5 ± 0.2	2.0 ± 0.2	ND	<0.1	ND
	Control (YE + M39)	0.5 ± 0.1	<0.1	<0.1	ND	1.0 ± 0.0	<0.1	ND
	Leu	1.7 ± 0.2	<0.1	5.5 ± 0.3	ND	ND	18.0 ± 0.4	85.0
	Leu + S_2O_3	16.5 ± 1.1	3.5 ± 0.3	3.3 ± 0.2	11.9 ± 1.6	ND	0.3 ± 0.0	101.5
	Leu + M39	17.0 ± 0.8	<0.1	<0.1	ND	7.5 ± 0.5	1.1 ± 0.1	90.0
	Ile	2.0 ± 0.1	<0.1	2.8 ± 0.4	ND	ND	17.6 ± 0.9	83.3
	Ile + S_2O_3	17.4 ± 0.8	4.0 ± 0.4	11.5 ± 0.2	17.0 ± 0.8	ND	0.0 ± 0.0	107.0
	Ile + M39	16.9 ± 0.7	<0.1	<0.1	ND	8.0 ± 0.6	1.0 ± 0.1	88.9
	Val	1.6 ± 0.1	<0.1	2.1 ± 0.3	ND	ND	18.0 ± 1.0	80.0
	Val + S_2O_3	17.4 ± 1.0	2.1 ± 0.3	5.5 ± 0.1	16.7 ± 0.7	ND	0.0 ± 0.2	97.5
	Val + M39	17.9 ± 0.9	<0.1	<0.1	ND	9.3 ± 0.9	1.1 ± 0.1	94.7

ND = not determined.

and methane. Cultivations including thiosulfate (40 mM) also resulted in near complete amino acid degradation and production of a mixture of the corresponding BCFAs, branched-chain alcohols (BCOHs), and H_2S. Thus, leucine was degraded to a mixture of 3-methyl-butyrate and 3-methyl-butanol, isoleucine to 2-methyl-butyrate and 2-methyl-butanol, and valine to 2-methyl-propionate and 2-methyl-propanol. From the degradation of 20 mM of a BCAA, a maximum of 20 mM of the combined BCFA and BCOH may be formed. Since a fraction of the substrate is used for biomass production the carbon balance of 85–95% is reasonable. This is true in most cases observed by *T. yonseiensis* and *T. brockii* (Table 1).

It is well-known that most *Thermoanaerobacter* species can use thiosulfate as electron acceptor [4]. *Thermoanaerobacter brockii* has been shown to degrade the BCAAs to the corresponding BCFAs in the presence of thiosulfate but not BCOHs [2]. To investigate the role of thiosulfate and role of hydrogen concentrations in BCOH production in more detail, *T. yonseiensis* was cultivated on isoleucine (20 mM) at liquid–gas ratios (L-G) of 0.05, 1.00, and 5.88 and five thiosulfate concentrations (0, 5, 10, 20, 40 mM). At a low L-G ratio (0.05), only low concentrations of the BCOH was produced and BCFA production increased by increasing initial thiosulfate concentrations (Fig. 1A). By increasing the L-G ratio to 1.00 and 5.88, the BCOH formation increased more than 5-fold (with thiosulfate present) and less BCFA were produced (Fig. 1B and C). The ratio between the BCFA/BCOH is shown for all three L-G phases at all thiosulfate concentrations (Fig. 2) for *T. yonseiensis* showing that at low L-G phases most of the amino acids are degraded to their corresponding BCFAs. By increasing the L-G phase to 1.00 and 5.88 leads to much more branched-chain alcohol formation compared to branched-chain acid formation.

Thus, the concentrations of hydrogen seem to be of importance for BCOH formation. At low L-G ratios, the hydrogen concentrations were always below 0.6 mmol/L and very low BCOH concentrations were observed. At higher L-G ratios, hydrogen concentrations were higher, usually between 2 and 4 mmol/L and higher concentrations of BCOH formation occurred. A more detailed study of branched-chain amino acid catabolism, for instance using a genomic and proteomic approach coupled with enzymatic assays and isotopic labeling studies, could add important insights into the metabolic pathways involved in the degradation BCAAs with different hydrogen-scavenging systems. Even at high initial thiosulfate concentrations, there was considerable hydrogen accumulation observed at the end of cultivation. H_2S concentrations were usually in similar or slightly lower concentrations as the total concentrations of BCOHs and BCFAs. At low thiosulfate concentrations (5 and 10 mM), lower quantity of the amino acid was catabolized to end products due to insufficient thiosulfate to scavenge electrons. However, all thiosulfate was completely utilized at all initial thiosulfate concentrations suggesting production of other sulfur species as supported by a strong yellow color in bottles containing high initial thiosulfate concentrations. Sulfur was indeed visually observed in these cultures but not quantified.

The production of BCFAs and BCOHs from amino acids has been well established in *Saccharomyces cerevisiae* via the Ehrlich pathway [9] and trace quantities of the BCOH have been reported by organisms involved in fermentation in food products [10]. Here we report the production of BCOHs using species of *Thermoanaerobacter*. To our knowledge, the production of BCOHs from BCAAs by members of class *Clostridia* have not previously reported. Further investigation into this phenomenon among members of *Thermoanaerobacter* species and other genera needs to be done especially

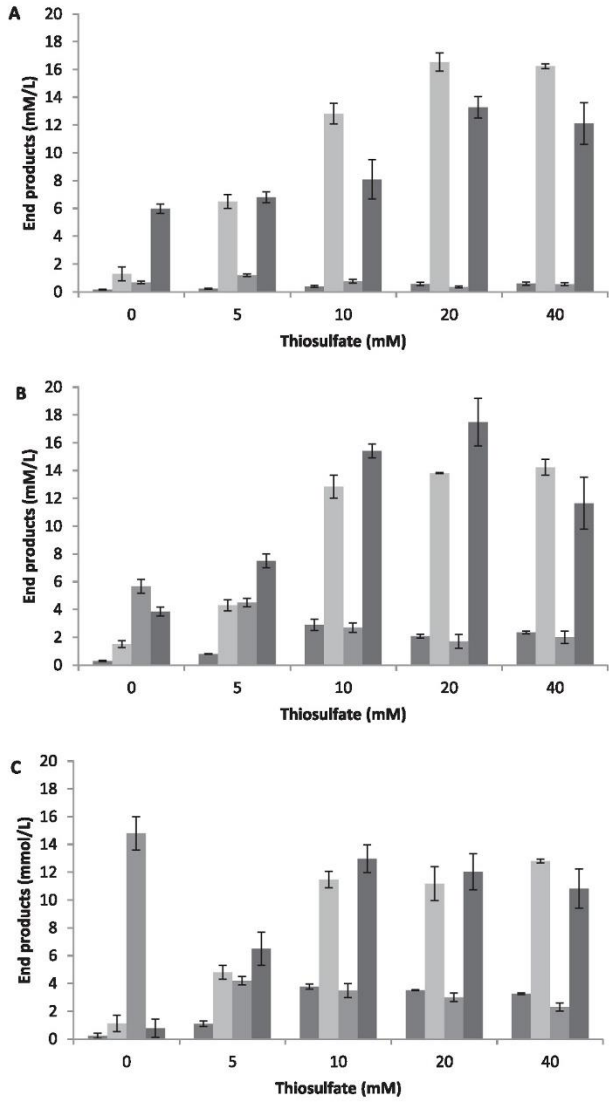

Fig. 1. A–C. End-product formation from isoleucine by *T. yonseiensis* at different initial thiosulfate concentrations. Standard deviations are shown as error bars. Columns from left to right: 2-methyl-butyrate, 2-methyl-butanol, H_2, H_2S.

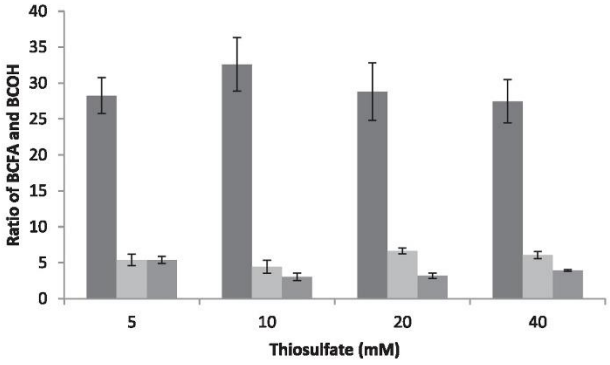

Fig. 2. Ratio of 2-methyl-butyrate to 2-methyl-butanol at different thiosulfate concentrations at three different liquid–gas phases by *T. yonseiensis*. Standard deviations are shown as error bars. Columns from left to right show different liquid–gas phases: 0.05, 1.00, 5.88.

towards the role of the pH_2 on the formation of BCOHs and pathway elucidation.

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II

Extremophiles
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ORIGINAL PAPER

Branched-chain alcohol formation by thermophilic bacteria within the genera of *Thermoanaerobacter* and *Caldanaerobacter*

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Abstract Fifty-six thermophilic strains including members of *Caldanaerobacter*, *Caldicellulosiruptor*, *Caloramator*, *Clostridium*, *Thermoanaerobacter*, and *Thermoanaerobacterium*, were investigated for branched-chain amino acid degradation in the presence of thiosulfate in batch culture. All of the *Thermoanaerobacter* and *Caldanaerobacter* strains (24) degraded the branched-chain amino acids (leucine, isoleucine, and valine) to a mixture of their corresponding branched-chain fatty acids and branched-chain alcohols. Only one *Caloramator* strain degraded the branched-chain amino acids to the corresponding branched-chain fatty acids. The ratio of branched-chain fatty acid production over branched-chain alcohol production for *Thermoanaerobacter* was 7.15, 6.61, and 11.53 for leucine, isoleucine, and valine, respectively. These values for *Caldanaerobacter* were 3.49, 4.13, and 7.31, respectively. This indicates that members within *Caldanaerobacter* produce proportionally more of the alcohols as compared with *Thermoanaerobacter*. No species within other genera investigated produced branched-chain alcohols from branched-chain amino acids in the presence of thiosulfate.

Keywords Branched-chain amino acids · Thermophiles · Thermoanaerobes · Amino acid catabolism · Biofuel

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Introduction

Thermophilic species within Class *Clostridia* from the genera of *Caldicellulosiruptor*, *Caldanaerobacter*, *Clostridium*, *Thermoanaerobacter*, and *Thermoanaerobacterium* have been intensively investigated in the context of biofuel production due to their broad substrate spectrum, especially among the sugars present in lignocellulosic biomass, and because of their high ethanol and hydrogen yields (Taylor et al. 2009; Ren et al. 2009; Chang and Yao 2011; Scully and Orlygsson 2015). The protein and amino acid catabolism of thermophilic *Clostridia* has received considerably less attention likely due to the abundance of lignocellulosic biomass. However, several investigations have been performed mainly focusing upon the thermodynamics of the catabolism and end-product formation from specific amino acids (Elsden and Hilton 1978; Barker 1981; McInerney 1988; Orlygsson et al. 1994; 1995; Faudon et al. 1995; Fardeau et al. 1997). Thermoanaerobes can catabolize amino acids by oxidative and/or reductive deamination and decarboxylation (Elsden and Hilton 1978; Andreesen et al. 1989; Orlygsson et al. 1995). Thus, the oxidative fermentation of amino acids yields the corresponding α-keto acid in the first step which is thereafter decarboxylated to one carbon shorter volatile fatty acid. For instance, alanine is deaminated to pyruvate, which is then decarboxylated to acetate.

The amino acids that undergo oxidative deamination usually have reduced oxidation states, including the branched-chain amino acids (BCAAs), alanine, and glutamate (Andreesen et al. 1989; Fardeau et al. 1997). These amino acids can only be fermented if the electrons produced from the initial deamination step are scavenged due to the unfavorable thermodynamics involved (Orlygsson

et al. 1995; Fardeau et al. 1997). For instance, the $\Delta G^{\circ\prime}$ for the degradation of the BCAAs is between +4.2 and +9.7 kJ/mol (Fardeau et al. 1997). The addition of thiosulfate (which can be reduced to H_2S or S^0) to the medium or co-cultivating the amino acid degrader with hydrogen scavenging bacteria, such as a methanogen or sulfate reducer, drives the reaction forward resulting in the amino acids being degraded to their corresponding branched-chain fatty acids (BCFA) in addition to CO_2 and ammonium (Fardeau et al. 1997).

The degradation of BCAAs to their corresponding BCFAs by *Thermoanaerobacter brockii* has been investigated in some detail (Fardeau et al. 1997). Under hydrogen scavenging conditions, leucine, isoleucine, and valine are degraded to 3-methylbutyrate, 2-methylbutyrate, and 2-methylpropionate, respectively. A recent investigation in our laboratory demonstrated that *T. brockii* (DSM 1457) and *Caldanaerobacter subterraneus* subsp. *yonseiensis* (DSM 13777) degrade BCAAs to a mixture of the corresponding BCFAs and branched chain alcohols (BCOHs) when cultivated in the presence of thiosulfate but only to the corresponding BCFAs when co-cultured with a hydrogenotrophic methanogen (Scully and Orlygsson 2014).

The majority of studies on the catabolism of the BCAAs have focused on aerobic bacteria such as species of *Staphylococcus* and *Enterococcus* (Beck et al. 2004; Ward et al. 1999), aerotolerant anaerobes including *Lactobacillus sakei* (Gutsche et al. 2012), or yeasts that use the so-called Ehrlich pathway (Ehrlich 1907; Hazelwood et al. 2008). These studies have often focused on the formation of compounds that contribute to the flavor profile of foods and beverages (branched-chain and aromatic aldehydes, alcohols, and acids) (Smit et al. 2005, 2009). Recently, some studies have focused on the production of branched-chain alcohols from protein-rich waste using genetically engineered *Escherichia coli* and *Bacillus subtilis* with the main focus being that BCOHs are promising biofuel candidates (Huo et al. 2011; Choi et al. 2014). Additionally, BCOHs can be used as building blocks for the production of higher chemicals (Sakuragi et al. 2011).

In this study, the production of BCOHs from the BCAAs was investigated within various genera of thermophilic bacteria to gain a broader knowledge whether this phenomenon is genus specific within the genera of *Thermoanaerobacter* and *Caldanaerobacter*. Thus, the present investigation examines the ability of thermophilic anaerobes from several genera to produce BCOHs from BCAAs. Forty-eight (48) thermophilic anaerobic bacteria within Class *Clostridia* isolated from various hot springs in Iceland were investigated. Additionally, eight type strains from other thermophilic genera within Class *Clostridia* were used for comparison.

Materials and methods

Medium and cultivation

The BM medium consisted of (per liter): NaH_2PO_4 2.34 g, Na_2HPO_4 3.33 g, NH_4Cl 2.2 g, NaCl 3.0 g, $CaCl_2$ 8.8 g, $MgCl_2{\cdot}6H_2O$ 0.8 g, yeast extract 2.0 g, resazurin 1 mg, trace element solution 1 ml, vitamin solution (DSM141) 1 ml, and $NaHCO_3$ 0.8 g. The trace element solution consisted of the following on a per liter basis: $FeCl_2{\cdot}4H_2O$ 2.0 g, EDTA 0.5 g, $CuCl_2$ 0.03 g, H_3BO_3, $ZnCl_2$, $MnCl_2{\cdot}4H_2O$, $(NH_4)Mo_7O_{24}$, $AlCl_3$, $CoCl_2{\cdot}6H_2O$, $NiCl_2$, and 0.05 g, $Na_2S{\cdot}9H_2O$ 0.3 g, and 1 mL of concentrated HCl. Carbon and energy sources were 20 mM. The medium was prepared by adding the buffer to distilled water containing resazurin and then boiled for 10 min and cooled under nitrogen flushing. The mixture was then transferred to serum bottles using the Hungate technique (Hungate 1969) and autoclaved for 60 min. All other components of the medium were added separately through filter (0.45 µm) sterilized solutions. All experiments were conducted at 65 °C and at pH 7.0. In all cases, experiments were performed in duplicate.

Bacterial strains

Forty-eight (48) strains from our culture collection (hereafter called AK strains) used in this investigation were isolated as previously described on various carbon sources (Orlygsson and Baldursson 2007; Orlygsson et al. 2010). Eleven strains belong to the genus *Thermoanaerobacter*, 9 to *Caldanaerobacter*, 14 to *Thermoanaerobacterium*, 7 to *Clostridium*, 4 to *Caloramator* and 3 to *Caldicellulosiruptor*. Additionally, the following strains were obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ): *Thermoanaerobacter wiegelii* (DSM 10319), *Thermoanaerobacter thermohydrosulfuricus* (DSM 567), *Thermoanaerobacter ethanolicus* (DSM 2246), *Caldanaerobacter subterraneus* subspecies *subterraneus* (DSM 13054), *Caldicellulosiruptor saccharolyticum* (DSM 8903), *Caldicellulosiruptor owensis* (DSM 13100), *Thermoanaerobacterium saccharolyticum* (DSM 571), and *Caloramator viterbiensis* (DSM 13723).

Screening for branched-chain fatty acid and branched-chain alcohol formation

All strains (AK and DSM) were screened for branched-chain amino acid degradation. The strains were inoculated from frozen (−20 °C) cultures stored in 30 % (v/v) glycerol and reactivated on BM medium containing glucose (20 mM). Reactivated cultures were inoculated (2 % v/v)

from exponential growth phase to 25 mL serum bottles (liquid–gas ratio was 1:1) containing a mixture of leucine, isoleucine, and valine (9 mM each) in the presence of thiosulfate (20 mM). Cultures were grown for 5 days and thereafter 1 mL of liquid and 0.2 mL of gases were removed for analysis of end-products.

Production of branched-chain fatty acids and branched-chain alcohols from individual branched-chain amino acids in the presence of thiosulfate

All strains showing positive growth on BCAAs in the presence of thiosulfate from the initial screening experiment (mixture of BCAAs) were cultivated in 25 mL serum bottles (L–G ratio 1:1) on 20 mM of individual BCAAs (leucine, isoleucine, and valine) containing 20 mM of thiosulfate.

Analytical methods

Hydrogen was analyzed using a Perkin Elmer gas chromatograph equipped with a thermo conductivity detector. Nitrogen was used as carrier gas at a rate of 3 ml/min, with another 10 ml/min as make-up gas in the detectors. The separation was performed on a Supelco 1010 Carboxen CC Plot Capillary Column. The oven temperature was 80 °C and the injector and detector temperatures were 200 °C. Volatile fatty acids and alcohols were analyzed by gas chromatograph (Perkin Elmer Clarus 580) using a FID detector with 30 m DB-FFAP capillary column (Agilent Industries Inc, Palo Alto, CA, US). Samples (1 mL) were centrifuged for 20 min at 6000*g*. The supernatants were acidified with 25 % formic acid, and crotonic acid was used as the internal standard. Thiosulfate was analyzed by the method of Westley (1987) with modifications as described by Scully and Orlygsson (2014); the background interference was measured by adding 10 µL of 0.25 M KCN and 10 µL of dH_2O to 180 µL of sample; after mixing at 1000 rpm for 20 s, 100 µL of Sorbo's ferric nitrate reagent (10 % w/v $Fe(NO_3)_3{\cdot}9H_2O$ and 20 % v/v HNO_3) was added. Samples were allowed to clarify and the absorbance read at 460 nm against a water blank. The analyte was then determined by repeating the procedure and substituting 10 µL of 0.20 M $CuSO_4$ for dH_2O. Thiosulfate concentrations were calculated as the background absorbance–analyte against a standard curve generated using thiosulfate concentrations between 0.1 and 10 mM. Amino acids were analyzed using the ninhydrin method by adding 100 µL of a sample and 100 µL of 1 % (w/v) ninhydrin reagent (60 % v/v 2-propanol and 40 mM acetate buffer, pH 5.5) in a microtiter plate and incubated at 100 °C for 20 min; relevant amino acids (leucine, isoleucine, and valine) were used as standards. After cooling to ambient temperature, 200 µL of 50 % (v/v) 2-propanol was added and the absorbance read at 580 nm on a Bioscreen C (Oy Growth Curves Ab, Finland). Hydrogen sulfide was quantified as described by Cord-Ruwisch (1985). Briefly, the pH of the medium was adjusted to 10 with the addition of 6 M NaOH; samples were then allowed to stand for 60 min with periodic mixing. 50 µL of sample was then transferred into a cuvette containing 1.95 mL of 5 mM $CuSO_4$ in 50 mM HCl under rapid stirring. After 5 s of stirring, the cuvette was transferred to a Perkin Elmer Lambda 25 UV–Vis spectrophotometer and the absorbance read at a wavelength of 480 nm against a dH_2O blank. Other sulfur species were not analyzed in the present investigation.

Molecular methods

DNA was extracted from the strains and used as templates in 16S rRNA PCR reactions. 16S rRNA genes were amplified with primers F9 and R1544, which are specific for bacterial genes (Skirnisdottir et al. 2000). The PCR products were sequenced with the universal 16S rRNA primers F9, F515, F1392, R357, R805, R1195, and R1544 (Skirnisdottir et al. 2000) using a Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems). Subsequently, the DNA was analyzed with a 3730 DNA analyzer from Applied Biosystems. The nucleotide sequence was displayed and analyzed with Sequencer (Gene Code Corporation). Sequences from 16S rRNA analysis were submitted to the NCBI database (National Center for Biotechnology Information) using the nucleotide–nucleotide BLAST tool (Altschul et al. 1997). The most similar sequences obtained were aligned with sequencing results in MEGA6 (Tamura et al. 2013) and the maximum likelihood method based on the Tamura–Nei model was used to generate a phylogenetic tree (Tamura and Nei 1993). Evolutionary analyses were conducted in MEGA6 (Tamura et al. 2013).

Results

Screening of the culture collection for branched-chain amino acid degradation

Fifty-six strains of thermophilic bacteria, either isolated from various hot springs in Iceland (48) or obtained from culture collections (8) were investigated in present study. The origin of AK strains showing temperature and pH at site of isolation, together with end product formation from glucose is showed in Supplementary Table 1. Additionally, substrate spectra of strains are presented. All of the *Thermoanaerobacter* (14) and *Caldanaerobacter* (10) strains were positive for BCFA and BCOH formation (Table 1). Only

Table 1 Relative production of BCOH and BCFA from BCAA by positive strains. All strains were cultivated in the presence of a mixture of leucine (9 mM), isoleucine (9 mM), and valine (9 mM) in the presence of thiosulfate (20 mM). Closest similarity of strains from partial 16S rRNA is shown within *brackets* as well as the length of the 16S rRNA used for phylogenetic analysis. NCBI accession numbers for all AK strains are also shown

Strain	Accession numbers	16S rRNA length (nucleotides)	End products	
			BCOH	BCFA
T. wiegelii (DSM 10319)		1.464	++	+++
T. thermohydrosulfuricus (DSM 567)		1.768	+	++
T. ethanolicus (DSM 2246)		1.740	++	++
AK15 *T. uzonensis* (98.6 %)	EU262599	1.469	++	+++
AK46 *T. uzonensis* (96.9 %)	KR007643	478	++	++
AK62 *T. ethanolicus* (99.7 %)	KR007645	586	++	+++
AK68 *T. thermohydrosulfuricus* (99.7 %)	KR007668	1.081	+	++
AK85 *T. uzonensis* (98.3 %)	KR007650	1.479	++	+++
AK90 *T. thermohydrosulfuricus* (100 %)	KR007667	774	+++	++++
AK98 *T. ethanolicus* (99.6 %)	KR007653	569	++	+++
AK106 *T. ethanolicus* (99.7 %)	KR007657	620	++	+++
AK107 *T. ethanolicus* (99.5 %)	KR007658	560	++	++
AK110 *T. thermohydrosulfuricus* (99.4 %)	KR007659	543	+	++
AK152 *T. thermohydrosulfuricus* (100 %)	KR007666	716	+++	++++
C. subterraneus subsp. *subterraneus* (DSM 13054)		1.372	+++	++++
AK70 *C. subterraneus* subsp. *subterraneus* (98.2 %)	KR007646	568	++	++++
AK72 *C. subterraneus* subsp. *subterraneus* (99.9 %)	KR007647	561	++++	++++
AK76 *C. subterraneus* subsp. *subterraneus* (97.8 %)	KR007648	588	++++	++++
AK77 *C. subterraneus* subsp. *subterraneus* (98.7 %)	KR007649	481	++++	++++
AK101 *C. subterraneus* subsp. *subterraneus* (99.0 %)	KR007654	578	++	++
AK102 *C. subterraneus* subsp. *subterraneus* (98.9 %)	KR007655	539	+++	+++
AK112 *C. subterraneus* subsp. *yonseiensis* (99.0 %)	KR007660	495	+++	++++
AK113 *C. subterraneus* subsp. *yonseiensis* (90.5 %)	KR007661	406	++++	++++
AK131 *C. subterraneus* subsp. *subterraneus* (98.6 %)	KR007664	519	++	+++
AK49 *Caloramator australicus* (98.3 %)	KR007644	483	–	++

For the BCOH formation: − = <0.1 mM, + = 0.1–1.0 mM, ++ = 1.0–3.0 mM, +++ = 3.0–5.0 mM; ++++ = >5.0 mM

For BCFA formation: + = 0.1–5.0 mM; ++ = 5.1–13.0 mM, +++ = 13.1–20.0 mM, ++++ = >20.0 mM

one other strain was positive for BCFA formation, *Caloramator* strain AK49, but it did not produce BCOHs. Other strains from the genera *Clostridium*, *Caldicellulosiruptor* and *Thermoanaerobacterium* did not degrade the BCAAs and thus did not produce BCFAs or BCOHs (are thus not included in Table 1). The relative amount of BCFAs and BCOHs produced by the 25 strains that were positive on BCAA degradation is shown in Table 1 together with phylogenetic data showing the closest relative of each strain. In general, the highest amounts of BCFAs (>20 mM of all three fatty acids) and BCOHs (>3 mM of all three alcohols) were produced by the genera of *Caldanaerobacter*. Most of the *Thermoanaerobacter* species produced between 5 and 13 mM of the BCFA and between 1 and 3 mM of the BCOH. The best alcohol producer, *Caldanaerobacter* strain AK72, most closely related to *C. subterraneus* subsp. *yonseiensis*, produced 24.5 ± 1.3 mM of the BCFAs (sum of 3-methylbutyrate, 2-methylbutyrate, and 2-methylpropionate) and 5.7 ± 0.3 mM of BCOH (summary of 3-methylbutanol, 2-methylbutanol, and 2-methylpropanol).

Phylogenetic relationship of selected *Caldanaerobacter* and *Thermoanaerobacter* species

Figure 1 shows the phylogenetic relationship between all *Thermoanaerobacter* and *Caldanaerobacter* species with standing in nomenclature as well as the twenty AK strains belonging to these two genera. Eleven (11) of the AK strains belong to the genus *Thermoanaerobacter* and nine (9) belong to *Caldanaerobacter*. Most of the *Thermoanaerobacter* AK strains were most closely related to *T. thermohydrosulfuricus* and *T. ethanolicus* (8 strains) and *T. uzonensis* (3 strains). Six of the *Caldanaerobacter* strains were most closely related to *Caldanaerobacter*

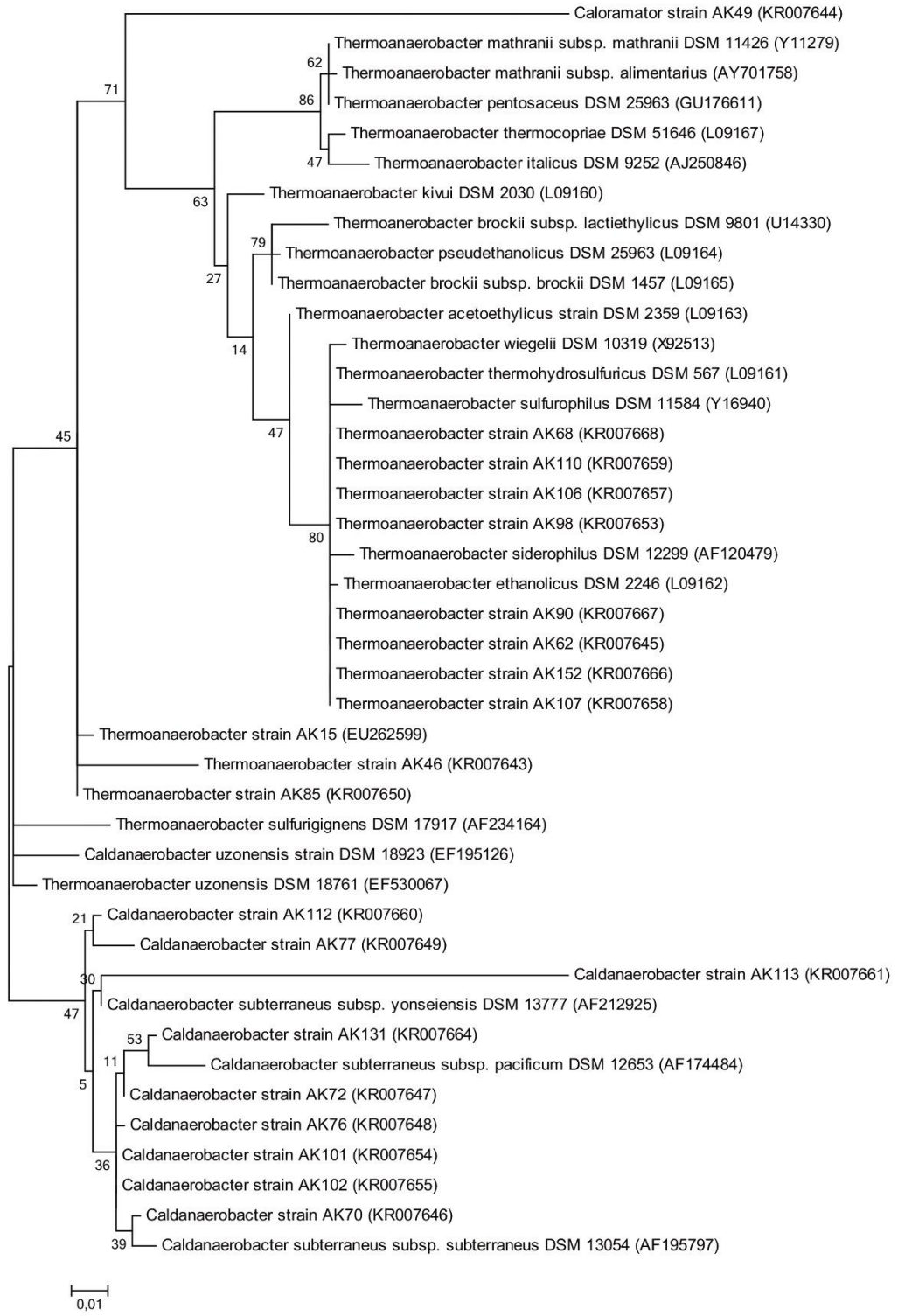

Fig. 1 Maximum likelihood phylogenetic dendrogram based on 16S rRNA gene sequences showing the position of BCOH-forming thermophilic strains within the class *Clostridia*. Bootstrap values (>50 %) based on 1000 replicates are shown at branch nodes. *Bar* represents 1 substitution per 100 nucleotides

subterraneus subsp. *subterraneus* and three to *Caldanaerobacter subterraneus* subsp. *yonseiensis*. It is noteworthy that *Caldanaerobacter* AK113 is phylogenetically distant from its nearest neighbor *Caldanaerobacter subterraneus* subsp. *yonseiensis*. The reason could be that this strain has a relatively short 16S sequenced (406 nt). Table 1 shows the most closely related species assigned for each AK strain together with accession numbers obtained from NCBI.

End product formation from individual branched-chain amino acids

All strains positive for growth on a mixture of BCAAs were tested on for growth and end product formation in the presence of thiosulfate on individual BCAAs. Data showing end product formation from these experiments are shown for *Thermoanaerobacter* and *Caldanaerobacter* species in Supplementary Tables 2 and 3, respectively. In all cases, thiosulfate was nearly completely utilized (data not shown). All strains were positive for BCAA degradation although some variations in amounts of end products were observed. For simplicity, data is presented for three representative *Thermoanaerobacter* strains (*T. wiegelii*, AK15, and AK68; Fig. 2a–c) and three *Caldanaerobacter* strains (*C. subterraneus* subsp. *subterraneus*, AK102, and AK112; Fig. 3a–c).

Thermoanaerobacter wiegelii (DSM 10319) produced between 12.0 and 14.0 mM of the BCFAs and 1.8 to 3.0 mM of the BCOHs (Fig. 2a). Hydrogen concentrations were from 5.3 to 7.8 mmol/L for the three amino acids and hydrogen sulfide from 7.8 to 12.6 mmol/L. In all cases, more than 90 % of the BCAAs were degraded and carbon balances for leucine, isoleucine, and valine were 80.5, 88.5, and 84.0 %, respectively (Fig. 2a; Supplementary Table 2). *Thermoanaerobacter* strain AK 68 (most closely related to *T. uzonensis*) almost completely degraded all three amino acids and produced similar amounts of BCFA (12.2 to 13.0 mM) and BCOH (2.3 to 4.0 mM) (Fig. 2b). Carbon balances ranged from 83.3 to 93.8 % (Supplementary Table 2). Hydrogen concentrations were very low or between 0.2 and 0.6 mmol/L and more than 11 mmol/L of H_2S were produced in all three cases. *Thermoanaerobacter* strain AK15 (most closely related to *T. uzonensis*) also almost completely degraded all three amino acids with carbon recoveries ranging from 82.0 % (isoleucine) to 88.4 % (leucine) as summarized in Supplementary Table 2. Similar amounts of BCFAs and BCOHs were observed as for *T. wiegelii*, except for BCOH formation from isoleucine which was considerable lower (Fig. 2c). Hydrogen was between 0.8 and 1.8 mmol/L and H_2S produced between 11.2 and 12.1 mmol/L.

Caldanaerobacter subterraneus subsp. *subterraneus* (DSM 13054) showed similar fermentation spectrum on all three amino acids (Fig. 3a); with the production of BCFAs

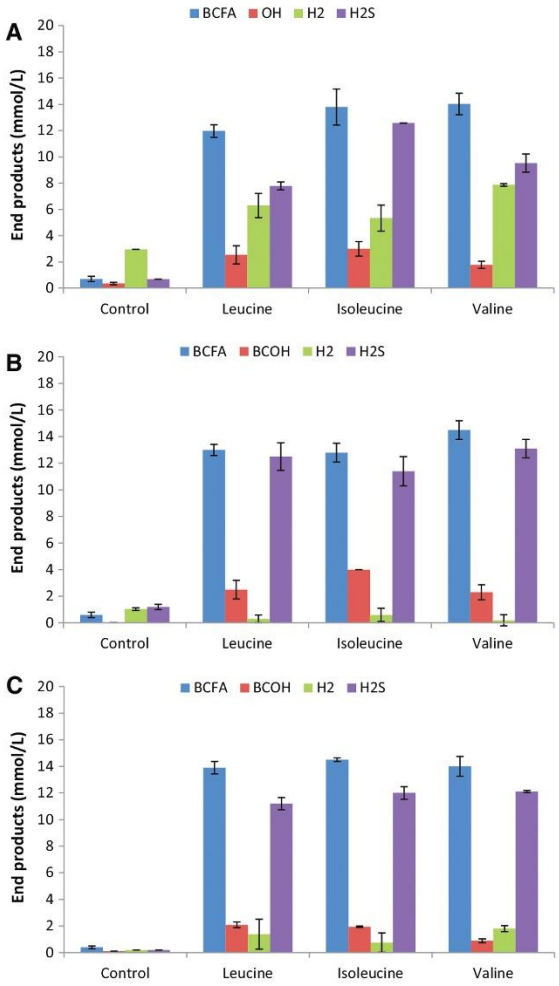

Fig. 2 Production profile from BCAA by **a** *Thermoanaerobacter wiegelii* (DSM 10319) of 20 mM thiosulfate. **b** *Thermoanaerobacter* strain AK68, and **c** *Thermoanaerobacter* strain AK15

between 14.0 and 15.6 mM and the BCOHs formation between 3.4 and 5.7 mM with carbon recoveries between 91.1 to 99.0 % (Supplementary Table 3). Very low concentrations of both H_2 (<1 mmol/L) and H_2S (<1.3 mmol/L) were observed. Figure 3b shows end product formation for *Caldanaerobacter* strain AK102 (most closely related to *Caldanaerobacter subterraneus* subsp. *subterraneus*). This strain produces lower amounts of both BCFAs and BCOHs as compared to *Caldanaerobacter subterraneus* subsp. *subterraneus* (DSM 13054) but between 5.7 and 11.0 mM of the amino acids was not degraded by this strain but carbon balances were between 85.5 and 88.5 % (Supplementary Table 3). Hydrogen concentrations were between 4.1 and 8.4 mmol/L and H_2S concentrations were between 5.8 and 8.0 mmol/L. *Caldanaerobacter* strain AK112 is most

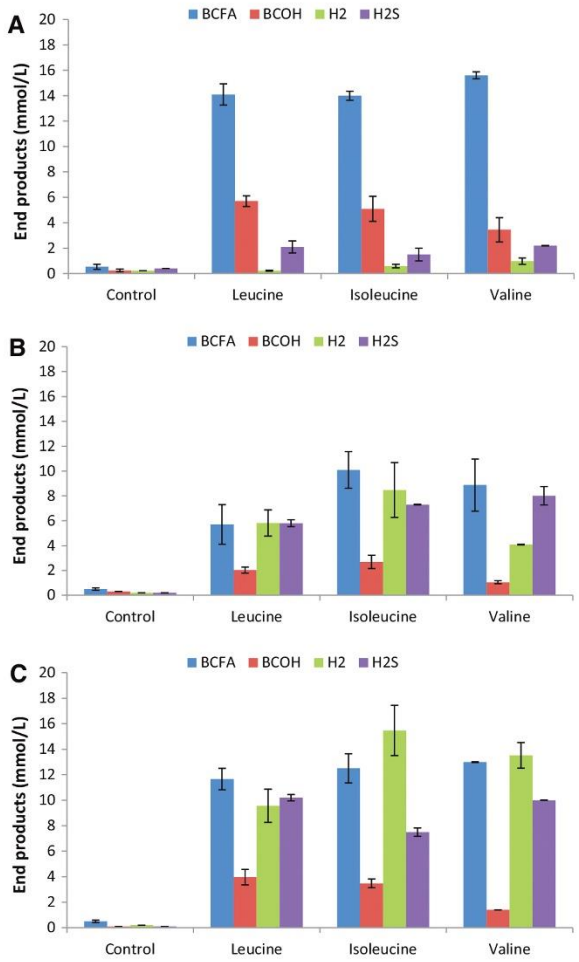

Fig. 3 Production profile from BCAA by **a** *Caldanaerobacter subterraneus* subsp. *subterraneus* in presence the of 20 mM thiosulfate. **b** strain *Caldanaerobacter* strain AK102, and **c** *Caldanaerobacter* strain AK112

closely related to *C. subterraneus* subsp. *yonseiensis* produced between 11.7 and 12.9 mM and between 1.4 and 4.0 of the BCFA and BCOHs, respectively (Fig. 3C) and carbon balances were between 82.8 and 89.3 (Supplementary Table 3). Hydrogen and H_2S concentrations ranged from 9.6 to 15.4 mmol/L and 7.5 to 10.2 mmol/L, respectively.

Statistical tests were done to reveal the difference between the ratio of BCFA production versus the BCOH production for all alcohol forming species of *Thermoanaerobacter* and *Caldanaerobacter*. The difference proved statistically significant. The data points used for the test are presented in Supplementary Tables 2 and 3. A preliminary test for equality of variances for the ratio of 3-methylbutanol and 3-methylbutanol (products from leucine degradation) indicates that there is a clear difference between the strains within the two genera ($F = 4.11$, $p = 0.015$). Therefore, a two-sample t test (95 % confidence interval) was performed that does not assume equal variances. The mean score for *Thermoanaerobacter* ($M = 7.15$, SD $= 2.31$, $N = 14$) was significantly higher than the scores for *Caldanaerobacter* ($M = 3.28$, SD $= 1.14$, $N = 11$). Similarly, t test showed a clear difference between the ratio of 2-methylbutyrate and 2-methylbutanol (from isoleucine) and the ratio of 2-methylpropionate and 2-methylpropanol (from valine).

Discussion

The present investigation examines the distribution of BCAA metabolism among selected thermophilic *Clostridia* with the main emphasis of the genera *Thermoanaerobacter* and *Caldanaerobacter*. The degradation of the BCAAs to their corresponding BCFAs been shown to be possible by *Thermoanaerobacter* species when grown under electron scavenging systems (Fardeau et al. 1997; Scully and Orlygsson 2014). Although bacteria have the enzymes responsible for the degradation of these amino acids, hydrogen accumulation from initial deamination, and decarboxylation steps inhibits further degradation. By adding thiosulfate to the cultures, *Thermoanaerobacter* and *Caldanaerobacter* species can use it as an electron acceptor producing elemental S, H_2S, or possibly other sulfur species (Lee et al. 1993; Cann et al. 2001; Kozianowski et al. 1997). Other means of scavenging electrons is by co-cultivating the amino acid degraders either with hydrogenotrophic methanogens (Fardeau et al. 1997; Scully and Orlygsson 2014) and sulfate reducers (Stams and Hansen 1984).

A recent investigation in our laboratory demonstrated that *Thermoanarobacter brockii and Caldanaerobacter subterraneus* subsp. *yonseiensis* produce a mixture of BCFAs and BCOHs from BCAAs in the presence of thiosulfate (Scully and Orlygsson 2014). Thus, these strains produced a mixture of 3-methylbutyrate and 3-methylbutanol from leucine, 2-methylbutyrate and 2-methylbutanol from isoleucine and 2-methyl-propionate and 2-methyl-propanol from valine. No BCOH formation occurred when the strains were co-cultivated with the hydrogenotrophic methanogen *Methanothermobacter* strain M39. The present investigation focused on other well-known species within these two genera as well as with other species within other genera to see if this ability of BCOH was genus specific or not.

Phylogenetic considerations

Recently, two species within *Thermoanaerobacter* were moved to a new genus, *Caldanaerobacter*. Thus, currently, the *Thermoanaerobacter* genus has 15 species and

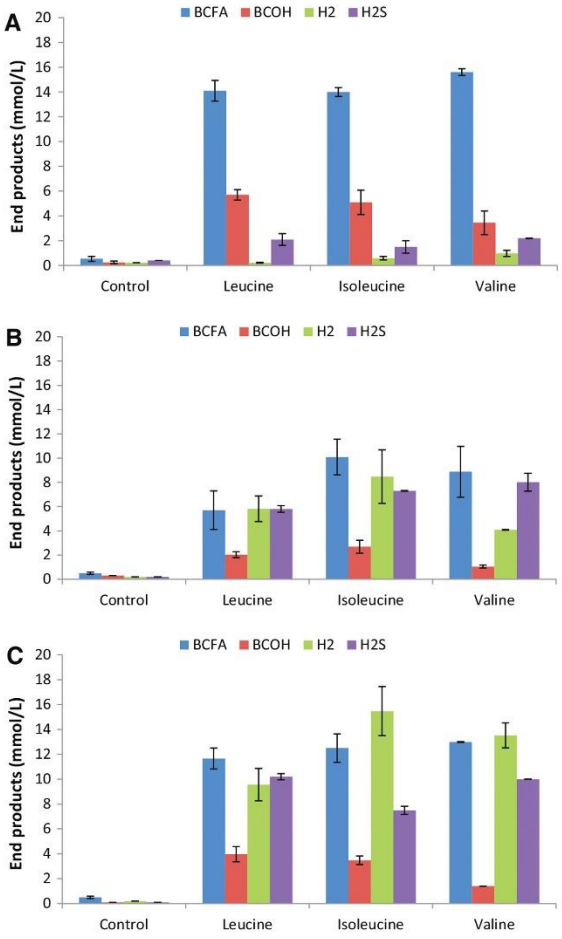

Fig. 3 Production profile from BCAA by **a** *Caldanaerobacter subterraneus* subsp. *subterraneus* in presence the of 20 mM thiosulfate. **b** strain *Caldanaerobacter* strain AK102, and **c** *Caldanaerobacter* strain AK112

closely related to *C. subterraneus* subsp. *yonseiensis* produced between 11.7 and 12.9 mM and between 1.4 and 4.0 of the BCFA and BCOHs, respectively (Fig. 3C) and carbon balances were between 82.8 and 89.3 (Supplementary Table 3). Hydrogen and H_2S concentrations ranged from 9.6 to 15.4 mmol/L and 7.5 to 10.2 mmol/L, respectively.

Statistical tests were done to reveal the difference between the ratio of BCFA production versus the BCOH production for all alcohol forming species of *Thermoanaerobacter* and *Caldanaerobacter.* The difference proved statistically significant. The data points used for the test are presented in Supplementary Tables 2 and 3. A preliminary test for equality of variances for the ratio of 3-methylbutanol and 3-methylbutanol (products from leucine degradation) indicates that there is a clear difference between the strains within the two genera ($F = 4.11$, $p = 0.015$). Therefore, a two-sample t test (95 % confidence interval) was performed that does not assume equal variances. The mean score for *Thermoanaerobacter* ($M = 7.15$, SD $= 2.31$, $N = 14$) was significantly higher than the scores for *Caldanaerobacter* ($M = 3.28$, SD $= 1.14$, $N = 11$). Similarly, t test showed a clear difference between the ratio of 2-methylbutyrate and 2-methylbutanol (from isoleucine) and the ratio of 2-methylpropionate and 2-methylpropanol (from valine).

Discussion

The present investigation examines the distribution of BCAA metabolism among selected thermophilic *Clostridia* with the main emphasis of the genera *Thermoanaerobacter* and *Caldanaerobacter*. The degradation of the BCAAs to their corresponding BCFAs been shown to be possible by *Thermoanaerobacter* species when grown under electron scavenging systems (Fardeau et al. 1997; Scully and Orlygsson 2014). Although bacteria have the enzymes responsible for the degradation of these amino acids, hydrogen accumulation from initial deamination, and decarboxylation steps inhibits further degradation. By adding thiosulfate to the cultures, *Thermoanaerobacter* and *Caldanaerobacter* species can use it as an electron acceptor producing elemental S, H_2S, or possibly other sulfur species (Lee et al. 1993; Cann et al. 2001; Kozianowski et al. 1997). Other means of scavenging electrons is by co-cultivating the amino acid degraders either with hydrogenotrophic methanogens (Fardeau et al. 1997; Scully and Orlygsson 2014) and sulfate reducers (Stams and Hansen 1984).

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Phylogenetic considerations

Recently, two species within *Thermoanaerobacter* were moved to a new genus, *Caldanaerobacter*. Thus, currently, the *Thermoanaerobacter* genus has 15 species and

five sub-species (Euzéby 1997; Parte 2014) and *Caldanaerobacter* (Fardeau et al. 2004) has two species and 4 sub-species. The phylogenetic tree of most of the type species within *Thermoanaerobacter* and *Caldanaerobacter* together with all AK strains positive for BCAA degradation is presented in Fig. 1. Many of the *Thermoanaerobacter* AK strains were closely related with *T. thermohydrosulfuricus*, *T. ethanolicus*, and *T. pseudoethanolicus*. Comparing data from Table 1 with phylogenetic relationship of good BCOH producers the only four strains producing more than 20 mM of BCFA and more than 5 mM of BCOH belong to the genus *Caldanaerobacter*. Additionally, only three strains that produced less than 1 mM of BCOH all belong to the genus *Thermoanaerobacter*. Thus, in general species within *Caldanaerobacter* produce higher concentrations of the alcohol as compared to the *Thermoanaerobacter* species investigated. This was further analyzed statistically with a *t* test in present study. Interestingly, there was also a clear difference between the ratio of BCFAs over BCOHs from different amino acids degraded. Thus, from leucine and isoleucine, this ratio was between 7.15 ± 2.31 and 6.61 ± 2.77 for *Thermoanaerobacter*, but 3.49 ± 1.18 to 4.13 ± 1.30 for *Caldanaerobacter*. This value for valine was, however, much higher (11.53 ± 4.65 and 7.31 ± 1.74 for *Thermoanaerobacter* and *Caldanaerobacter*, respectively) indicating proportionally more alcohol formation from the 6 carbon amino acids (leucine and isoleucine) as compared to valine (5 carbons). This could be due to the enzyme specificity of the different enzymes used for the degradation of the BCAAs and needs further investigation.

Degradation on single branched-chain amino acids

A great variation was observed on end product formation between strains using single BCAAs as substrates with 20 mM of thiosulfate (Supplementary Tables 2 and 3). Selected data are presented for only six strains to emphasize this variation (Figs. 2, 3). For instance, *Caldanaerobacter subterraneus* subsp. *subterraneus* showed good yields in BCFAs and BCOHs (between 91.1 and 99.0 in carbon balances) and most of the hydrogen was scavenged by thiosulfate reduction. However, the balances for sulfur species are incomplete since very little H_2S was detected at the end of cultivation. Lee and co-workers stated that *Thermoanaerobacter* species reduce thiosulfate to hydrogen sulfide, whereas *Thermoanaerobacterium* reduce it to elementary sulfur (Lee et al. 1993). Later, it became clear that this is not a unified characteristic of these genera when two species within *Thermoanaerobacterium* were shown to be unable to reduce thiosulfate to sulfur (Cann et al. 2001). Additionally, it is known that *Thermoanaerobacter italicus* produces a mixture of sulfur and hydrogen sulfide from thiosulfate (Kozianowski et al. 1997) while *Thermoanaerobacter sulfurigignens* produces only sulfur (Lee et al. 2007). In all cases, only trace amounts of thiosulfate were detected after end of cultivation in present study suggesting that all thiosulfate was reduced to other sulfur species. Sulfur was not analyzed but a strong yellow color and microscopic observation showed the presence of sulfur in some of the culture bottles, especially when H_2S was found in low concentrations. Theoretically, 20 mM of a BCAA should yield 40 mmol/L of hydrogen and if thiosulfate (20 mM) is the only electron acceptor it should produce 20 mmol/L of hydrogen sulfide. These yields are in good correlation for most of the strains tested; usually the H_2S was little lower as compared with the amount of BCFA produced (Figs. 2a–c, 3b, c; Supplementary Tables 2 and 3). However, in some cases this was not the case and very low H_2S concentrations were indeed observed. Clearly, the different species use different sulfur metabolism for scavenging the electrons and this can also been seen by different hydrogen concentrations observed. Earlier data from *Caldanaerobacter subterraneus* subsp. *yonseiensis* and *Thermoanarobacter brockii* showed H_2S yields varying from 11.9 to 17.0 mmol/L from 20 mM of BCAA in the presence of 40 mM of thiosulfate and from 7.5 to 9.3 mmol/L for *C. subterraneus* subsp. *yonseiensis* and *T. brockii*, respectively (Scully and Orlygsson 2014). Clearly, there is a need for further studies to better explore the differences in the sulfur metabolism of these two genera.

Most *Thermoanaerobacter* and *Caldanaerobacter* strains grow optimally at neutral pH and between 65 and 70 °C. Thus, for simplicity reasons, in present investigation, all strains belonging to these two genera were cultivated at the same pH and temperature (pH 7.0 and at 65 °C). This may, however, not be their optimum growth conditions and results should be taken with caution concerning yields end product formation. For the *Thermoanaerobacter* and *Caldanaerobacter* strains, a more detailed study using not only variation in the liquid–gas phase ratios and the concentration of thiosulfate, but also an investigation into the role of culture conditions such as temperature and pH is needed to better understand conditions leading to the production of BCOHs rather than the corresponding BCFAs. From the study of *Caldanaerobacter subterraneus* subsp. *yonseiensis*, it was clear that both the concentration of thiosulfate as well as the ratio of liquid gas phases was of great importance for BCOH formation (Scully and Orlygsson 2014). Thus, using a high liquid–gas ratios increases the relative amount of the BCOH over the BCFA for this strain.

The majority of studies on the catabolism of the BCAAs have focused on aerobic bacteria such as species of *Staphylococcus* and *Enterococcus* (Beck et al. 2004; Ward et al. 1999), aerotolerant anaerobes including *Lactobacillus sakei*

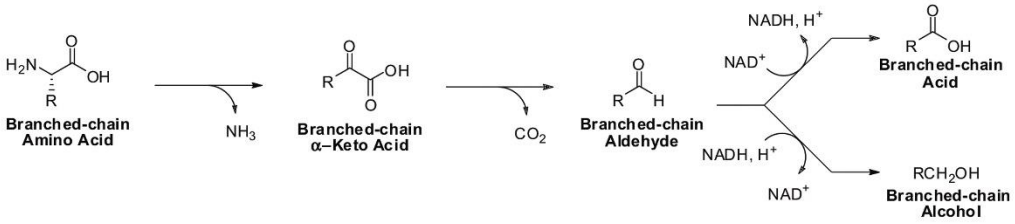

Fig. 4 Proposed pathway of BCFA and BCOH formation for *Thermoanaerobacter* and *Caldanaerobacter* species

(Gutsche et al. 2012), or yeasts that use the Ehrlich pathway as shown in Fig. 4 (Ehrlich 1907; Hazelwood et al. 2008). Here, we report the production of these products using species of *Thermoanaerobacter*. The phenomenon may be common among other members of class *Clostridia*, although the production of BCOHs may have escaped detection, as culture conditions require electron scavenging systems and low pH_2. The degradation of amino acids presents a renewable route to potentially important feedstock chemicals. The interest in the production of BCOHs has often been directed to the formation of flavor compounds (branched- and aromatic chain aldehydes, alcohols, and acids) in food and beverage products (Hazelwood et al. 2008). Additionally, (*S*)-2-methylbutanol is a potential biofuel (Peralta-Yahya and Keasling 2010) and some of the BCOHs may serve as building blocks (Sakuragi et al. 2011). A crude protein extract from fish waste or whey protein might be used as a good source of BCAAs for the production of these compounds.

Conclusions

The production of BCOHs during the fermentation of BCAAs by thermophilic *Clostridia* appears to be limited to the genera of *Thermoanaerobacter* and *Caldanaerobacter*. The amount of BCOH was found to be proportionally higher within the genus *Caldanaerobacter* compared with *Thermoanaerobacter* and more profound from leucine and isoleucine as compared with valine. Clearly, there is a difference in thiosulfate reduction between investigated species. The role of pH_2 and the enzymes involved requires further investigations to elucidate the pathway these bacteria use for BCOH formation.

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Conflict of interest The authors declare that they have no competing interests.

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Supplementary Table 1. Strains from the culture collection used in present study and the temperature and the pH of the hot springs they originate from. All strains originate from Grensdalur, South West Iceland, except for AK1, AK14, AK15 and AK17 which were isolated from the Krafla area (North East Iceland). . End product formation (mol/mol glucose) from 20 mM of glucose is shown and substrates degraded for strains when known. Abbreviations: Et = ethanol, Ac = acetate, La = lactate, H_2 = hydrogen, x = xylose, a = arabinose, ga = galactose, m = mannose, f = fructose, mt = maltose, ce = cellobiose, l= lactose, s = sucrose, rh = rhamnose, tr = trehalose, st = starch, xy = xylan, p = pyruvate, se = serine, th = threonine. ND = not determined.

Strain	**Hot spring** (Temp/pH)	**End products from glucose** (mol/mol)	**Other substrates used**
Thermoanaerobacter strain AK 15	60.4/8.6	Et (0.83), Ac (0.41), La (0.18), H_2 (0.55)	x, ga, m, f, s, p, xy
Thermoanaerobacter strain AK 46	60.0/6.7	Et (1.25), Ac (0.25), H_2 (0.45)	ND
Thermoanaerobacter strain AK 62	60.7/7.1	Et (1.33), Ac (0.30), H_2 (0.41)	ND
Thermoanaerobacter strain AK 68	64.6/8.2	Et (0.20), Ac (1.34), H_2 (1.94)	x, ga, m, f, mt, ce, s, l, t, ra, xy, p, se, th
Thermoanaerobacter strain AK 85	69.0/7.5	Et (1.70), Ac (1.34), H_2 (1.94)	x, a, ga, m, f, rh, mt, ce, s, l, t, rf, st, p, se, xy
Thermoanaerobacter strain AK 90	70.1/7.0	Et (0.80), Ac (0.9), H_2 (1.50)	x, ga, m, f, mt, ce, st, p, se,
Thermoanaerobacter strain AK 98	69.7/8.8	Et (0.75), Ac (1.11), H_2 (1.14)	ND
Thermoanaerobacter strain AK 106	69.7/8.8	Et (0.85), Ac (0.76), H_2 (1.34)	ND
Thermoanaerobacter strain AK 107	69.7/8.8	Et (0.88), Ac (0.65), H_2 (1.04)	ND
Thermoanaerobacter strain AK 110	70.4/8.2	Et (0.95), Ac (0.55), H_2 (0.99)	ND
Thermoanaerobacter strain AK 152	68.5/7.3	Et (1.45), Ac (0.30), H_2 (0.45)	X, ga, m, f, rh, mt, ce, l, st, p, s
Caldanaerobacter strain AK 70	70.9/5.4	Et (1.22), Ac (0.45), H_2 (0.87)	ND
Caldanaerobacter strain AK 72	70.9/5.4	Et (1.05), Ac (0.35), H_2 (0.67)	ND
Caldanaerobacter strain AK 76	82.0/5.7	Et (0.27), Ac (1.06), H_2 (1.25)	ND
Caldanaerobacter strain AK 77	82.0/5.7	Et (1.41), Ac (0.33), H_2 (0.40)	ND
Caldanaerobacter strain AK 101	71.3/9.7	Et (0.33), Ac (1.26), H_2 (1.40)	ND
Caldanaerobacter strain AK 102	71.3/9.7	Et (0.73), Ac (0.46), H_2 (0.60)	ND
Caldanaerobacter strain AK 112	76.3/4.7	Et (0.23), Ac (1.36), H_2 (2.20)	ND
Caldanaerobacter strain AK 113	76.3/4.7	Et (0.13), Ac (1.40), H_2 (2.45)	ND
Caldanaerobacter strain AK 131	74.8/8.5	Et (0.78), Ac (0.65), H_2 (1.01)	ND

Supplementary Table 1 (continued) Strains from the culture collection used in present study and the temperature and the pH of the hot springs they originate from. All strains originate from Grensdalur, South West Iceland, except for AK1, AK14, AK15 and AK17 which were isolated from the Krafla area (North East Iceland). . End product formation (mol/mol glucose) from 20 mM of glucose is shown and substrates degraded for strains when known. Abbreviations: Et = ethanol, Ac = acetate, La = lactate, H_2 = hydrogen, x = xylose, a = arabinose, ga = galactose, m = mannose, f = fructose, mt = maltose, ce = cellobiose, l= lactose, s = sucrose, rh = rhamnose, tr = trehalose, st = starch, xy = xylan, p = pyruvate, se = serine, th = threonine. ND = not determined.

Strain	**Hot spring** (Temp/pH)	**End products from glucose** (mol/mol)	**Other substrates used**
Thermoanerobacterium strain AK 17	65.0/6.5	Et (1.50), Ac (0.38), H_2 (0.59)	a, x, ga, m, f, l, s, py, se, th
Thermoanerobacterium strain AK 22	56.0/5.5	Et (1.25), Ac (0.63), H_2 (0.98)	ND
Thermoanerobacterium strain AK 25	53.0/6.2	Et (1.00), Ac (0.60), H_2 (1.00)	ND
Thermoanerobacterium strain AK 27	50.0/5.4	Et (1.05), Ac (0.70), H_2 (0.93)	ND
Thermoanerobacterium strain AK 39	50.0/7.7	Et (0.15), Ac (0.51), But (0.59), H_2 (0.75)	ND
Thermoanerobacterium strain AK 40	49.0/6.7	Et (1.23), Ac (0.33), H_2 (0.55)	ND
Thermoanerobacterium strain AK 42	58.0/6.2	Et (1.05), Ac (0.55), H_2 (0.85)	ND
Thermoanerobacterium strain AK 43	66.0/5.3	Et (0.85), Ac (0.15), H_2 (0.25)	ND
Thermoanerobacterium strain AK 44	66.0/5.3	Et (1.20), Ac (0.50), H_2 (0.63)	ND
Thermoanerobacterium strain AK 45	60.0/6.7	Et (1.25), Ac (0.46), H_2 (0.77)	ND
Thermoanerobacterium strain AK 58	59.0/7.0	Et (0.71), Ac (0.70), H_2 (0.93)	ND
Thermoanerobacterium strain AK 59	62.0/7.4	Et (0.98), Ac (0.60), H_2 (0.90)	ND
Thermoanerobacterium strain AK 60	62.0/7.4	Et (1.25), Ac (0.50), H_2 (0.74)	ND
Thermoanerobacterium strain AK 61	66.0/7.7	Et (1.11), Ac (0.30), H_2 (0.54)	ND
Clostridium strain AK 1	47.8/7.6	Et (1.50), Ac (0.38), H_2 (0.55)	x, ga, m, f, l, se, pe
Clostridium strain AK 14	55.3/7.8	Et (0.20), Ac (0.35), Bu (0.55), H_2 (1.90)	x, ga, m, f, s, st
Clostridium strain AK 19	40.7/4.8	Et (1.10), Ac (0.60), H_2 (0.51)	ND
Clostridium strain AK 28	45.9/8.0	Et (0.60), Ac (0.50), H_2 (0.30)	ND
Clostridium strain AK 30	50.4/7.9	Et (1.00), Ac (0.80), H_2 (1.40)	ND
Clostridium strain AK 35	52.5/7.5	Et (1.10), Ac (0.50), H_2 (0.92)	ND
Clostridium strain AK 55	53.4/7.0	Et (0.82), Ac (0.75), H_2 (1.21)	ND
Caloramator strain AK 34	49.0/5.8	Et (0.80), Ac (0.40), H_2 (0.67)	ND
Caloramator strain AK 38	50.3/7.7	Et (0.95), Ac (0.65), H_2 (0.96)	ND
Caloramator strain AK 47	60.9/6.7	Et (0.20), Ac (1.20), H_2 (1.11)	ND
Caloramator strain AK 49	60.7/7.7	Et (0.89), Ac (0.76), H_2 (1.23)	ND
Caldicellulosiruptor strain AK 80	69.5/8.0	Et (0.05), Ac (1.34), H_2 (2.45)	ND
Caldicellulosiruptor strain AK 81	71.5/8.2	Et (0.11), Ac (1.21), H_2 (2.02)	ND
Caldicellulosiruptor strain AK 82	71.6/8.2	Et (0.08), Ac (1.12), H_2 (2.10)	ND

Supplementary Table 2. End product formation from branched-chain amino acids (20 mM) in the presence of thiosulfate (20 mM) within the genus *Thermoanaerobacter*

	Leucine						Isoleucine					
Substrate	Amino acid degradation (mM)	3-methyl-1-butyrate (mM)	3-methyl-1-butanol (mM)	H_2 (mmol/L)	H_2S (mmol/L)	Carbon balances (%)	Amino acid degradation (mM)	2-methyl-1-butyrate (mM)	2-methy-l-butanol (mM)	H_2 (mmol/L)	H_2S (mmol/L)	Carbon balances (%)
T. wiegelii (DSM 10319)	18.0±1.1	12.0±0.5	2.5±0.7	6.3±0.9	7.8±0.3	80.5	19.0±1.0	13.8±1.3	3.0±0.5	5.3±1.2	12.6±0.7	88.4
T. brockii (DSM 1457)*	16.0±1.3	13.6±01.1	1.5±0.1	0.5±0.0	9.8±01.1	94.4	17.4±0.4	14.4±1.3	1.2±0.1	0.2±0.0	12.6±1.7	95.1
T. thermohydrosulfuricus (DSM 567)	15.4±0.9	10.7±0.7	1.8±0.0	0.6±0.1	3.5±1.0	81.2	20.0±0.0	15.5±1.7	1.5±0.3	8.0±0.3	11.4±0.7	85.0
T. ethanolicus (DSM 2246)	7.8±0.4	5.1±0.7	0.6±0.2	2.4±0.1	2.0±0.2	73.1	9.0±0.2	6.8±0.9	0.6±0.0	2.1±0.1	2.7±0.5	82.2
AK15 (*T. uzonensis*)	18.2±1.1	13.9±0.5	2.1±0.2	1.4±0.1	11.2±1.4	88.4	19.8±0.0	14.5±0.1	1.9±0.0	0.8±0.2	12.0±1.2	82.8
AK46 (*T. uzonensis*)	14.3±0.8	10.0±0.3	1.3±0.2	3.5±0.1	2.7±0.6	79.0	15.2±0.8	12.0±0.5	1.8±0.3	12.0±0.5	1.8±0.3	90.7
AK62 (*T. ethanolicus*)	9.2±0.0	7.0±0.1	0.5±0.0	1.6±0.3	7.8±0.5	81.5	13.5±1.0	9.3±0.7	2.0±0.0	2.4±0.1	8.1±0.4	83.7
AK68 (*T. thermohydrosulfuricus*)	18.0±0.8	13.0±0.8	2.5±0.6	0.3±0.0	12.5±0.9	86.1	17.9±1.2	12.8±0.8	4.0±0.6	0.6±0.1	11.4±0.6	93.8
AK85 (*T. uzonensis*)	15.1±1.3	11.0±0.7	1.5±0.2	5.5±0.5	6.6±0.2	82.3	17.0±0.8	13.3±1.2	2.1±0.1	4.4±0.4	8.2±0.5	90.6
AK90 (*T. thermohydrosulfuricus*)	19.5±1.8	14.6±1.2	3.0±0.3	0.2±0.0	9.3±0.4	90.2	20.0±0.0	13.6±2.1	4.1±0.5	0.6±0.2	8.8±1.1	88.5
AK98 (*T. ethanolicus*)	5.2±0.2	3.4±0.1	0.6±0.0	1.5±0.3	1.3±0.2	76.9	9.3±0.3	6.6±0.5	0.9±0.1	1.3±0.3	3.1±0.3	80.6
AK106 (*T. ethanolicus*)	8.8±0.4	6.5±0.4	1.0±0.1	1.1±0.2	3.3±0.2	85.2	12.6±0.8	9.2±0.5	1.6±0.2	2.1±0.6	4.1±0.5	85.7
AK107 (*T. ethanolicus*)	14.1±0.7	11.0±0.3	1.3±0.1	0.4±0.0	8.5±1.1	87.2	13.7±0.1	9.5±0.8	1.8±0.2	0.2±0.0	7.4±0.6	82.4
AK110 (*T. pseudoethanolicus*)	5.1±0.3	3.5±0.4	0.6±0.1	2.7±0.1	1.5±0.1	80.4	7.2±0.3	5.9±0.4	1.0±0.1	4.4±0.0	3.1±1.1	95.8
AK152 (*T. thermohydrosulfuricus*)	12.7±0.2	8.8±0.6	2.3±0.2	2.9±0.4	7.8±1.1	87.4	18.3±0.5	12.7±1.1	3.2±0.3	1.4±0.2	8.2±0.5	86.8

*Data from Scully & Orlygsson, 2014

Supplementary Table 2 (Continued) - End product formation from branched-chain amino acids (20 mM) in the presence of thiosulfate (20 mM) within the genus *Thermoanaerobacter*

	Valine					
Substrate	Amino acid Degradation (mM)	2-methyl-1-propionate (mM)	2-methy-l-propanol (mM)	H_2 (mmol/L)	H_2S (mmol/L)	Carbon balances (%)
T. wiegelii (DSM 10319)	18.8±0.6	14.0±0.8	1.8±0.3	7.8±	9.5±0.7	84.0
T. brockii (DSM 1457)*	15.5±0.8	11.0±1.5	1.3±0.1	0.2±0.0	5.0±0.5	80.6
T. thermohydrosulfuricus (DSM 567)	20.0±0.0	15.0±0.9	1.5±0.5	15±1.5	14.4±0.6	82.5
T. ethanolicus (DSM 2246)	8.6±0.4	6.2±1.0	0.3±0.0	1.7±0.3	1.8±0.5	75.6
AK15 (*T. uzonensis*)	17.5±1.3	14.0±0.7	0.9±0.1	1.8±0.2	12.1±1.5	85.1
AK46 (*T. uzonensis*)	15.2±0.7	11.5±1,2	1.4±0.0	3.8±0.2	11.6±0.8	84.9
AK62 (*T. ethanolicus*)	9.3±0.6	7.0±0.7	0.4±0.1	1.7±0.1	8.0±0.3	84.9
AK68 (*T. thermohydrosulfuricus*)	17.4±1.2	12.2±0.5	2.3±0.1	0.2±0.0	13.1±1.0	83.3
AK85 (*T. uzonensis*)	15.3±0.9	12.5±0.8	1.2±0.1	3.0±0.3	4.0±0.3	89.5
AK90 (*T. thermohydrosulfuricus*)	20.0±0.0	16.4±1.5	1.3±0.3	0.6±0.1	10.3±0.9	88.5
AK98 (*T. ethanolicus*)	8.5±0.3	6.6±0.2	0.4±0.1	0.5±0.2	2.1±0.4	82.3
AK106 (*T. ethanolicus*)	9.3±0.6	7.5±0.5	0.8±0.1	1.2±0.3	3.7±0.4	89.2
AK107 (*T. ethanolicus*)	7.6±0.7	5.9±0.3	0.4±0.0	1.0±0.1	4.4±0.2	82.9
AK110 (*T. pseudoethanolicus*)	9.0±0.5	6.1±0.4	1.2±0.1	4.6±0.4	1.6±0.6	81.1
AK152 (*T. thermohydrosulfuricus*)	17.0±0.9	13.7±0.7	1.6±0.2	1.3±0.1	6.9±0.3	90.0

*Data from Scully & Orlygsson, 2014

Table 3. End product formation from branched-chain amino acids (20 mM) in the presence of thiosulfate (20 mM) within the genus *Caldanaerobacter*

Substrate	Leucine						Isoleucine					
	Amino acid degradation (mM)	3-methyl-1-butyrate (mM)	3-methyl-1-butanol (mM)	H_2 (mmol/L)	H_2S (mmol/L)	Carbon balances (%)	Amino acid Degradation (mM)	2-methyl-1-butyrate (mM)	2-methyl-1-butanol (mM)	H_2 (mmol/L)	H_2S (mmol/L)	Carbon balances (%)
C. yonseinesis (DSM 13777)*	19.7±0.3	16.5±2.1	3.5±1.0	3.3±1.8	11.9±1.6	101.5	20.0±0.0	17.4±0.8	4.0±0.4	11.5±0.2	17.0±0.8	107.0
C. subterraneus subsp. *subterraneus* (DSM 13054)	20.0±0.0	14.1±0.9	5.7±0.7	0.2±0.0	13.0±1.2	99.0	19.1±0.6	14.0±1.0	3.4±0.4	0.6±0.1	12.6±0.9	91.1
AK70 (subsp. *subterraneus*)	5.2±0.2	3.9±0.2	0.8±0.0	1.2±0.1	2.6±0.4	90.3	12.6±0.6	9.0±1.2	1.2±0.1	3.7±0.5	4.7±0.4	80.9
AK72 (subsp. *subterraneus*)	9.0±0.6	5.8±0.5	2.0±0.1	6.8±0.4	5.0±0.5	86.7	12.0±0.3	9.6±0.5	2.1±0.2	10.6±0.6	5.4±0.4	97.5
AK76 (subsp. *subterraneus*)	2.6±0.3	1.6±0.2	0.4±0.0	1.3±0.1	5.3±0.6	76.9	5.4±0.2	3.4±0.2	1.0±0.1	3.8±0.4	2.8±0.6	81.4
AK77 (subsp. *subterraneus*)	7.3±0.4	4.8±0.3	1.3±0.1	4.1±0.3	2.4±0.4	83.5	8.8±0.6	5.8±0.4	1.6±0.3	6.3±0.4	4.5±0.3	84.1
AK101 (subsp. *subterraneus*)	10.0±0.9	7.1±0.5	1.3±0.2	0.5±0.0	4.1±0.3	84.0	13.3±0.7	10.0±1.0	2.2±0.2	0.2±0.0	5.4±0.6	91.7
AK102 (subsp. *subterraneus*)	9.0±0.8	5.7±0.3	2.0±0.1	5.8±0.5	5.8±0.2	85.5	14.3±1.3	10.1±0.6	2.7±0.3	8.4±0.5	7.3±1.0	89.5
AK112 (subsp. *yonseiensis*)	18.0±1.0	11.7±1.2	4.0±0.6	9.6±2.9	10.2±0.6	87.2	17.9±0.5	12.5±0.9	3.5±0.3	15.4±2.0	7.5±0.4	89.3
AK113 (subsp. *yonseiensis*)	20.0±0.0	12.0±1.2	5.3±0.4	6.5±0.5	12.1±1.1	86.5	14.9±0.7	10.0±0.7	2.6±0.3	10.0±2.1	10.3±0.9	84.5
AK131 (subsp. *subterraneus*)	20.0±0.0	11.1±0.7	6.5±0.1	3.7±1.4	13.1±0.9	88.0	14.2±0.9	8.5±0.4	3.9±0.0	6.1±0.1	9.9±0.7	87.3

*Data from Scully & Orlygsson, 2014

Table 3 (continued). End product formation from branched-chain amino acids (20 mM) in the presence of thiosulfate (20 mM) within the genus *Caldanaerobacter*

Substrate	Valine					
	Amino acid degradation (mM)	2-methyl-1-propionate (mM)	2-methyl-1-propanol (mM)	H_2 (mmol/L)	H_2S (mmol/L)	Carbon balances (%)
C. yonseinesis (DSM 13777)*	20.0±0.0	17.4±1.0	2.1±0.3	5.5±0.1	16.7±0.7	97.5
C. subterraneus subsp. subterraneus (DSM 13054)	20.0±0.0	15.6±1.2	3.5±1.2	0.9±0.3	13.3±1.1	95.5
AK70 (subsp. *subterraneus*)	14.3±0.8	9.9±0.4	1.6±0.1	1.4±0.3	6.0±0.9	80.4
AK72 (subsp. *subterraneus*)	13.9±1.0	9.3±1.0	1.5±0.1	10.8±1.1	6.1±0.5	77.7
AK76 (subsp. *subterraneus*)	7.9±0.6	5.7±0.3	0.9±0.1	4.2±0.2	5.5±0.4	83.5
AK77 (subsp. *subterraneus*)	8.3±0.5	6.0±0.5	1.0±0.1	4.0±1.0	7.4±0.3	84.3
AK101 (subsp. *subterraneus*)	15.3±0.9	11.0±1.1	1.8±0.1	0.8±0.3	6.9±0.5	83.7
AK102 (subsp. *subterraneus*)	11.2±0.8	8.9±1.2	1.0±0.1	4.1±0.3	8.0±0.7	88.4
AK112 (subsp. *yonseiensis*)	17.4±2.2	12.9±4.1	1.4±0.4	13.5±2.4	10.0±0.4	82.8
AK113 (subsp. *yonseiensis*)	15.5±0.9	10.9±1.1	1.2±0.2	9.8±0.5	10.1±0.6	78.1
AK131 (subsp. *subterraneus*)	15.2±0.2	11.5±0.6	1.2±0.0	6.4±1.4	11.2±0.8	83.6

*Data from Scully & Orlygsson, 2014

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Research Article

Amino Acid Metabolism of *Thermoanaerobacter* Strain AK90: The Role of Electron-Scavenging Systems in End Product Formation

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The catabolism of the 20 amino acids by *Thermoanaerobacter* strain AK90 (KR007667) was investigated under three different conditions: as single amino acids without an electron-scavenging system, in the presence of thiosulfate, and in coculture with a hydrogenotrophic methanogen. The strain degraded only serine without an alternative electron acceptor but degraded 11 amino acids (alanine, cysteine, isoleucine, leucine, lysine, methionine, phenylalanine, serine, threonine, tyrosine, and valine) under both of the electron-scavenging systems investigated. Acetate was the dominant end product from alanine, cysteine, lysine, serine, and threonine under electron-scavenging conditions. The branched-chain amino acids, isoleucine, leucine, and valine, were degraded to their corresponding fatty acids under methanogenic conditions and to a mixture of their corresponding fatty acids and alcohols in the presence of thiosulfate. The partial pressure of hydrogen seems to be of importance for the branched-chain alcohol formation. This was suggested by low but detectable hydrogen concentrations at the end of cultivation on the branched-chain amino acid in the presence of thiosulfate but not when cocultured with the methanogen. A more detailed examination of the role of thiosulfate as an electron acceptor was performed with *Thermoanaerobacter ethanolicus* (DSM 2246) and *Thermoanaerobacter brockii* (DSM 1457).

1. Introduction

Thermoanaerobacter and *Caldanaerobacter* species have been intensively investigated in the context of biofuel production due to their broad substrate spectrum, especially among the sugars present in lignocellulosic biomass, and due to their high ethanol and hydrogen yields [1–4]. However, the metabolism of both proteins and amino acids by thermophilic bacteria has received much less attention.

Branched-chain amino acids (BCAAs) are known to be degraded to their corresponding branched-chain fatty acids (BCFAs) under anaerobic conditions [5, 6]. The majority of catabolic studies of BCAAs have focused on aerobic bacteria such as species of *Staphylococcus* and *Enterococcus* [7–9], aerotolerant anaerobes including *Lactobacillus sakei* [10], or yeasts that use the so-called Ehrlich pathway [11, 12]. These studies have often focused on the formation of compounds that contribute to the flavor profile of foods and beverages (branched-chain and aromatic aldehydes, alcohols, and acids) [13, 14]. Amino acid metabolism has been investigated in some detail for *Thermoanaerobacter brockii* which degrades the BCAAs (isoleucine, leucine, and valine) by using an oxidative deamination and decarboxylation mechanism, but only in the presence of a hydrogen-scavenging system [6]. An external electron acceptor is required due to the unfavorable thermodynamics; $\Delta G^{\circ\prime}$ for the degradation of these three BCAAs is between +4.2 and +9.7 kJ/mol [6, 15]. However, the addition of thiosulfate or coculturing amino acid degrading thermoanaerobes with hydrogen-scavenging methanogens allows for the degradation of these amino acids to their corresponding fatty acids [6]. Thus, leucine is degraded to 3-methylbutyrate, isoleucine to 2-methylbutyrate, and valine to 2-methylpropionate. Serine, however, is degraded to acetate, ethanol, hydrogen, and carbon dioxide by *T. brockii* without an external electron acceptor. The addition of an external electron acceptor, however, shifts the fermentation products of this amino acid to acetate and greatly reduces ethanol formation [6]. Recent investigation

in our research group showed that most species within the genera of *Thermoanaerobacter* and *Caldanaerobacter* degrade the BCAAs not only to their corresponding fatty acids but also to a mixture of BCFAs and branched-chain alcohols (BCOHs) under thiosulfate reducing conditions [16, 17].

The present study focuses on the amino acid catabolism of *Thermoanaerobacter* AK90, which was isolated from a hot spring in Iceland. Special emphasis was given to amino acid degradation by the strain in the presence of thiosulfate or in coculture with a hydrogenotrophic methanogen, *Methanothermobacter* M39. For comparison, the degradation of BCAAs by *Thermoanaerobacter ethanolicus* (DSM 2246) and *Thermoanaerobacter brockii* (DSM 1457) was investigated under the same growth conditions as well as using different concentrations of thiosulfate to reveal its effect on end product distribution between the corresponding BCFA and BCOH.

2. Materials and Methods

2.1. Bacterial Strains. *Thermoanaerobacter* AK90 (KR007667) was isolated from a hot spring in Grensdalur (Southwest Iceland) using Timothy grass (*Phleum pratense*) hydrolysate as a carbon source according to methods already described [18]. *Thermoanaerobacter ethanolicus* (DSM 2246) and *Thermoanaerobacter brockii* (DSM 1457) were purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ). The hydrogenotrophic methanogen, *Methanothermobacter* strain M39, used in coculture experiments was isolated as described earlier [19].

2.2. Phylogenic Characterization. Phylogenetic characterization of strain AK90 has been described earlier [17]. The *Methanothermobacter* strain M39 was analyzed for 16S rDNA by DSMZ. Genomic DNA extraction was carried out using MasterPure Gram Positive DNA Purification Kits from Epicentre Biotechnologies, Germany, according to the manufacturer's instructions. PCR mediated amplification of the 16S rDNA and purification of the PCR product was carried out as previously described [20]. Purified PCR products were sequenced using the BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems) as described in the manufacturer's protocol. Sequence reactions were electrophoresed using the 3500 xL Genetic Analyzer from Applied Biosystems. The resulting sequence data was put into the alignment editor ae2 [21], aligned manually, and compared with representative 16S rRNA sequences of organisms belonging to Archaea [21]. For comparison, 16S rRNA sequences were obtained from the EMBL database or RPD [21].

2.3. Culture Conditions. The medium (per liter), hereafter referred to as BM medium, consisted of NH_4Cl 0.3 g, NaCl 0.3 g, $CaCl_2$ 0.11 g, $MgCl_2 \times 6H_2O$ 0.1 g, yeast extract 2.0 g, resazurin 1 mg, trace element solution 1 mL, vitamin solution 1 mL, and $NaHCO_3$ 0.8 g. Phosphate buffers were also used where 1 M stock solutions of NaH_2PO_4 and Na_2HPO_4 were made and added to the media to give a buffer capacity of 50 mM. The vitamin solution was prepared according to DSM141. The trace element solution consists of (g/L) $FeCl_2 \times 4H_2O$, 2.0, EDTA, 0.5, $CuCl_2$, 0.03, H_3BO_4, $ZnCl_2$, $MnCl_2 \times 4H_2O$, $(NH_4)_6Mo_7O_{24} \times 4H_2O$, $AlCl_3$, $CoCl_2 \times 6H_2O$, $NiCl_2$, all 0.05 mg, and 1 mL of concentrated HCl. The medium was prepared by adding the phosphate buffer, yeast extract, and resazurin to distilled water, which was then boiled for 5–10 min and cooled while flushing with nitrogen. The mixture was then transferred to cultivation bottles and autoclaved for 60 minutes. All other components of the medium were added separately through filter-sterilized solutions. The gas phase in all fermentation experiments consisted of 5.0 nitrogen (<5 ppm O_2). All experiments were performed at 65°C and pH 7.0 without agitation. When *Methanothermobacter* M39 was used in a coculture with other strains, it was pregrown on hydrogen and carbon dioxide (80/20 v/v) for one week. Prior to inoculating strain AK90 into the methanogenic cultures, the bottles were flushed with nitrogen. The inoculum volume of strain AK90 was 2% (v/v) from the exponential growth phase of stock cultures grown on glucose (20 mM) in all cases. Substrate solutions were added to culture media after autoclaving (121°C for 60 minutes) through a syringe filter (Whatman PES, 0.45 μm). All experiments were done in duplicate.

2.4. Degradation of Amino Acids. The ability of strain AK90 to utilize amino acids was tested using the BM medium supplemented with different amino acids (20 mM) in the presence or absence of thiosulfate (40 mM), or in a coculture with *Methanothermobacter* M39. The samples were grown for five days, at which time liquid (1 mL) and gas (0.2 mL) samples were withdrawn and the end products analyzed. Fermentation of single amino acids and amino acids in the presence of thiosulfate was performed in 24.5 mL serum bottles with a liquid-gas phase ratio of 1 : 1. Experiments with amino acid degradation in the presence of the methanogen were performed in 117.5 mL serum bottles with a liquid-gas ratio of 0.2. Similar procedure was used for investigating the growth of *T. brockii* and *T. ethanolicus* on BCAAs (20 mM) in the presence of the methanogen, but when thiosulfate was used, initial concentrations of thiosulfate varied between 5 and 80 mM.

2.5. Analytical Methods. Hydrogen and methane were analyzed using a PerkinElmer Auto System XL gas chromatograph equipped with a thermoconductivity (TCD) detector. Nitrogen was used as a carrier gas at a rate of 3 mL/min, with another 17 mL/min as make-up gas. The column used was Supelco 1010 Carboxen GC Plot Capillary Column. The oven temperature was 80°C, and the injector and detector temperatures were kept at 200°C. Alcohols and volatile fatty acids were measured by gas chromatography using a PerkinElmer Clarus 580 gas chromatograph equipped with a flame ionization detector (FID) using standards purchased from Sigma Aldrich. The column used was 30 m DB-FFAP capillary column (Agilent Industries Inc., Palo Alto, CA, USA). Amino acids were analyzed using the ninhydrin

method by mixing 100 μL of sample and 100 μL of 1% (w/v) ninhydrin reagent (60% v/v 2-propanol and 40 mM acetate buffer, pH 5.5) in a microtiter plate and incubating at 100°C for 20 minutes. After cooling, 200 μL of 50% (v/v) 2-propanol was added and the absorbance read at 580 nm on a Bioscreen C (Oy Growth Curves AB, Finland). Hydrogen sulfide was analyzed according to the method described by Cord-Ruwisch [22]. Thiosulfate was analyzed according to Westley [23] modified for use in microplates. Sulfur was detected by microscopic examination.

3. Results

3.1. Strains AK90 and M39. Strain AK90 was isolated from a hot spring in Iceland [17]. According to partial 16S rRNA sequence data, the strain belongs to the genus *Thermoanaerobacter* with the most closely related strains being *T. thermohydrosulfuricus* (100.0% similarity), *T. ethanolicus* (99.7%), and *T. pseudoethanolicus* (99.5%). Strain M39 was also isolated from a hot spring in Iceland [19]. According to partial 16S rRNA (about 800 nucleotides), the strain belongs to the genus *Methanothermobacter* and is most closely related to *M. marburgensis* Marburg^T (99.7% similarity).

3.2. Degradation of Amino Acids by Strain AK90 without an External Electron Acceptor. The following amino acids were not degraded under any culture conditions by strain AK90: arginine, asparagine, aspartic acid, glycine, glutamate, glutamine, histidine, proline, and tryptophan. Of the 20 amino acids tested as single substrates, only serine was degraded (Table 1). Serine (16.0 mM) was degraded to ethanol (5.7 mM), acetate (15.7 mM), hydrogen (9.7 mmol/L), and carbon dioxide (calculated; 21.4 mmol/L). When the quantity of end products in the control bottles (yeast extract) was subtracted from these values, a carbon balance of 87.5% was obtained. Other amino acids were only degraded to a small extent with end product formation similar to or slightly higher than that observed in the control bottles.

3.3. Degradation of Amino Acids in the Presence of Methanothermobacter M39. During degradation of amino acids in the presence of *Methanothermobacter* M39, strain AK90 was capable of degrading a much wider spectrum of amino acids as compared to cultures without any electron-scavenging system (Table 1). The strain converted more than 90% of cysteine, isoleucine, methionine, phenylalanine, serine, threonine, and tyrosine. Additionally, between 11.5 and 16.7 mM (57.5 to 83.5%) of alanine, leucine, lysine, and valine were degraded. End products from alanine, cysteine, lysine, serine, and threonine were primarily acetate, although small amounts of butyrate (2.3 mM) were produced from lysine.

The BCAAs were degraded to their corresponding BCFAs (leucine to 3-methylbutyrate, isoleucine to 2-methylbutyrate, and valine to 2-methylpropionate). Methane concentrations from BCAA fermentation were similar, ranging from 8.9 to 11.7 mmol L^{-1} (Table 1). The stoichiometry for the BCAAs under methanogenic conditions was as follows (control subtracted):

1.00 leucine $\rightarrow$ 0.71 3-methylbutyrate + 0.38 CH_4

1.00 isoleucine $\rightarrow$ 0.77 2-methylbutyrate + 0.47 CH_4

1.00 valine $\rightarrow$ 0.72 2-methylpropionate + 0.60 CH_4

Methane production by a hydrogenotrophic methanogen uses 4 moles of hydrogen to produce 1 mole of methane. The oxidative deamination and decarboxylation from one mole of a single BCAA should thus yield 0.5 moles of methane since, for each mole of degraded BCAA, 2 moles of hydrogen are produced [6]. This is reasonably consistent with the data obtained.

The aromatic amino acids phenylalanine and tyrosine, as well as methionine, were almost completely degraded under methanogenic conditions. Several unidentified peaks were observed late in the gas chromatograph run (between 10 and 14 min) from these amino acids and methane concentrations were similar as produced from the BCAAs. Ethanol was only a minor product from all amino acids as is shown in Table 1 and usually in similar concentrations as was observed in control bottles.

3.4. Degradation of Amino Acids in the Presence of Thiosulfate. The addition of thiosulfate in the present investigation resulted in similar degradation spectra as observed for methanogenic conditions. Six amino acids were completely degraded (alanine, cysteine, isoleucine, leucine, serine, and valine). Degradation of methionine, phenylalanine, and tyrosine was between 11.7 and 16.4 mM (58.7 to 82.0%), but lysine and threonine were only partially degraded (Table 1). As for methanogenic cultures on alanine, cysteine, and serine, acetate was the major end product under thiosulfate conditions. Similarly, unidentified peaks were observed from methionine, phenylalanine, and tyrosine. In all experimental bottles, the thiosulfate concentrations were negligible (<0.2 mM) at the end of cultivation and hydrogen sulfide and sulfur were end products from thiosulfate reduction, but only hydrogen sulfide was quantified. The concentrations of hydrogen sulfide from the BCAAs were between 12.4 and 13.5 mM.

During the degradation of the BCAAs under these conditions, not only the corresponding BCFAs but also the BCOHs were produced. Thus, leucine was degraded to a mixture of 3-methylbutyrate and 3-methylbutanol, isoleucine to 2-methylbutyrate and 2-methylbutanol, and valine to 2-methylpropionate and 2-methylpropanol. The stoichiometry for the BCAAs degradation was as follows (controls subtracted):

1.00 leucine $\rightarrow$ 0.49 3-methylbutyrate + 0.18 3-methylbutanol + 0.47 H_2S

1.00 isoleucine $\rightarrow$ 0.78 + 2-methylbutyrate + 0.22 2-methylbutanol + 0.60 H_2S

1.00 valine $\rightarrow$ 0.95 2-methylpropionate + 0.09 2-methylpropanol + 0.60 H_2S

In all cases, the branched-chain fatty acid concentration was greater than that of the corresponding alcohol.

Table 1: End product formation from amino acids by *Thermoanaerobacter* strain AK90. Initial amino acid concentration was 20 mM in all cases. Experiments were done with and without electron-scavenging systems, either with 40 mM of thiosulfate ($S_2O_3^{2-}$) or in a coculture with a hydrogenotrophic methanogen (M39). Data represent the average of two replicate experiments ± standard deviation. Experiments without added carbon source only contained 2 g/L of yeast extract (YE). Branched-chain fatty acids (BCFAs) are 3-methylbutyrate from leucine, 2-methylbutyrate from isoleucine, and 2-methylpropionate from valine. Branched-chain alcohols (BCOHs) are 3-methylbutanol from leucine, 2-methylbutanol from isoleucine, and 2-methylpropanol from valine.

Substrate/conditions	Amino acids (end of fermentation)	End products (mmol/L)							Carbon balance (%)
		Ethanol	Acetate	BCFA	BCOH	H_2	H_2S	CH_4	
YE	ND[1]	2.1 ± 0.1	5.2 ± 0.1	0.4 ± 0.1[2]	<0.1[3]	7.1 ± 0.2	ND	ND	ND
YE + S_2O_3	ND	2.5 ± 0.2	8.9 ± 0.3	0.9 ± 0.1[2]	0.1 ± 0.0[3]	<0.1	0.3 ± 0.1	ND	ND
YE + M39	ND	1.0 ± 0.1	8.7 ± 0.2	0.9 ± 0.2[2]	<0.1[3]	<0.1	ND	2.2 ± 0.1	ND
Ala	19.2 ± 1.5	1.2 ± 0.1	3.4 ± 0.3	<1.0	<0.1	10.9 ± 0.2	ND	ND	ND
Ala + S_2O_3	0.0 ± 0.0	5.5 ± 0.6	25.4 ± 0.8	<1.0	<0.1	1.3 ± 0.2	11.5 ± 0.8	ND	97.5[4]
Ala + M39	6.1 ± 1.6	1.4 ± 0.1	21.4 ± 2.8	<1.0	<0.1	<0.1	ND	8.0 ± 0.1	94.2[4]
Cys	19.3 ± 0.0	1.2 ± 0.1	3.4 ± 0.1	<1.0	<0.1	6.3 ± 0.5	ND	ND	ND
Cys + S_2O_3	0.2 ± 0.1	3.0 ± 1.1	24.2 ± 1.6	<1.0	<0.1	1.6 ± 0.1	12.9 ± 0.4	ND	79.8[4]
Cys + M39	0.0 ± 0.0	1.5 ± 0.1	25.6 ± 1.7	<1.0	<0.1	<0.1	ND	8.6 ± 0.4	87.0[4]
Ile	16.2 ± 0.4	1.4 ± 0.3	3.0 ± 0.5	3.8 ± 0.2	<0.1	11.1 ± 0.9	ND	ND	ND
Ile + S_2O_3	0.0 ± 0.0	2.9 ± 1.2	8.1 ± 1.1	15.9 ± 0.7	4.4 ± 0.3	0.2 ± 0.0	12.5 ± 0.6	ND	100.0
Ile + M39	0.0 ± 0.0	1.4 ± 0.3	8.4 ± 1.4	15.6 ± 1.6	0.2 ± 0.0	0.0 ± 0.0	ND	11.7 ± 1.3	77.5
Leu	18.5 ± 0.4	4.9 ± 0.5	4.2 ± 0.3	2.0 ± 0.1	<0.1	8.9 ± 0.3	ND	ND	ND
Leu + S_2O_3	0.0 ± 0.0	2.9 ± 1.0	8.1 ± 0.4	9.7 ± 2.1	3.5 ± 0.3	0.6 ± 0.3	13.5 ± 0.7	ND	64.5
Leu + M39	4.0 ± 0.4	1.3 ± 0.2	4.3 ± 0.7	11.7 ± 0.4	0.4 ± 0.1	<0.1	ND	9.9 ± 0.3	73.1
Lys	20.0 ± 0.0	1.1 ± 0.1	2.6 ± 0.1	<1.0	<0.1	5.8 ± 0.1	ND	ND	ND
Lys + S_2O_3	15.9 ± 2.2	3.7 ± 0.4	9.9 ± 0.5	<1.0	<0.1	0.1 ± 0.0	2.2 ± 0.4	ND	ND[5]
Lys + M39	8.5 ± 1.2	1.6 ± 0.2	19.5 ± 6.2	<1.0	<0.1	<0.1	ND	5.5 ± 0.3	ND[5]
Met	19.5 ± 0.5	3.0 ± 0.9	4.0 ± 0.2	<1.0	<0.1	8.2 ± 0.4	ND	ND	ND
Met + S_2O_3	3.6 ± 0.5	3.0 ± 0.3	6.1 ± 0.3	<1.0	<0.1	0.7 ± 0.1	12.4 ± 0.9	ND	ND
Met + M39	0.4 ± 0.1	1.8 ± 0.2	9.7 ± 0.7	<1.0	<0.1	<0.1	ND	12.2 ± 0.6	ND
Phe	20.4 ± 0.6	1.1 ± 0.1	2.6 ± 0.1	<1.0	<0.1	8.0 ± 0.1	ND	ND	ND
Phe + S_2O_3	8.3 ± 1.0	2.8 ± 0.3	6.3 ± 0.4	<1.0	<0.1	1.4 ± 0.1	7.4 ± 0.6	ND	ND
Phe + M39	0.1 ± 0.0	1.5 ± 0.2	10.4 ± 1.1	<1.0	<0.1	<0.1	ND	11.7 ± 0.4	ND
Ser	4.0 ± 0.5	5.7 ± 0.5	15.7 ± 0.6	<1.0	<0.1	9.7 ± 0.5	ND	ND	87.5[4]
Ser + S_2O_3	0.0 ± 0.0	3.3 ± 1.1	26.8 ± 1.4	<1.0	<0.1	0.1 ± 0.1	12.5 ± 1.2	ND	93.5[4]
Ser + M39	0.0 ± 0.0	1.5 ± 0.3	27.5 ± 2.0	<1.0	<0.1	<0.1	ND	8.8 ± 0.2	96.5[4]
Thr	19.0 ± 1.3	1.1 ± 0.1	2.6 ± 0.1	<1.0	<0.1	8.0 ± 0.8	ND	ND	ND
Thr + S_2O_3	14.5 ± 2.5	3.0 ± 0.4	25.4 ± 2.1	<1.0	<0.1	0.1 ± 0.0	11.5 ± 1.0	ND	ND[6]
Thr + M39	1.5 ± 0.2	0.8 ± 0.1	31.2 ± 1.8	<1.0	<0.1	<0.1	ND	6.9 ± 0.5	ND[6]
Tyr	19.3 ± 0.5	1.1 ± 0.1	2.6 ± 0.1	<1.0	<0.1	6.3 ± 0.5	ND	ND	ND
Tyr + S_2O_3	6.8 ± 1.3	3.4 ± 0.1	7.7 ± 0.3	<1.0	<0.1	0.9 ± 0.2	9.5 ± 0.3	ND	ND
Tyr + M39	1.2 ± 0.2	1.4 ± 0.1	10.0 ± 1.1	<1.0	<0.1	<0.1	ND	7.1 ± 2.0	ND
Val	17.4 ± 1.0	1.1 ± 0.1	2.6 ± 0.1	3.3 ± 0.1	<0.1	9.5 ± 0.1	ND	ND	ND
Val + S_2O_3	0.0 ± 0.0	3.2 ± 1.1	7.6 ± 0.6	19.2 ± 1.0	1.8 ± 0.5	0.6 ± 0.2	12.4 ± 0.5	ND	103.5
Val + M39	3.3 ± 1.7	1.4 ± 0.0	6.4 ± 1.6	12.3 ± 2.4	<0.1	<0.1	ND	8.9 ± 2.0	71.8

[1]ND: not determined.
[2]Total of 3-methylbutyrate, 2-methylbutyrate, and 2-methylpropionate.
[3]Total of 3-methylbutanol, 2-methylbutanol, and 2-methylpropanol.
[4]Assuming that CO_2 is produced in equimolar ratio with the production of acetate and ethanol.
[5]Butyrate was produced (2.2 mM) but not shown in the table for simplicity reasons.
[6]Degradation pathway unknown and thus not calculated (see results and discussion).

Table 2: Amino acid degradation and end product formation from branched-chain amino acids by *Thermoanaerobacter brockii* and *Thermoanaerobacter ethanolicus* in a coculture with *Methanothermobacter* strain M39. Initial amino acid concentration was 20 mM in all cases. Data represent the average of two replicate experiments ± standard deviation. The branched-chain fatty acids from leucine, isoleucine, and valine were 3-methylbutyrate, 2-methylbutyrate, and 2-methylpropionate, respectively. #Data from [16].

Strain and substrates	Amino acid (end of cultivation)	Branched-chain fatty acid	Methane
	Concentration (mmol/L)		
T. brockii: leucine#	1.5 ± 0.2	15.9 ± 2.3	9.3 ± 0.8
T. brockii: isoleucine#	1.7 ± 0.3	15.4 ± 1.3	8.3 ± 0.8
T. brockii: valine#	2.4 ± 0.2	14.8 ± 0.9	7.6 ± 1.0
T. ethanolicus: leucine	3.5 ± 0.3	15.0 ± 1.3	7.4 ± 1.1
T. ethanolicus: isoleucine	2.7 ± 0.4	14.3 ± 0.8	7.8 ± 0.9
T. ethanolicus: valine	12.4 ± 0.8	7.6 ± 0.6	3.4 ± 1.3

3.5. Degradation of Branched-Chain Amino Acids by Thermoanaerobacter ethanolicus and Thermoanaerobacter brockii. The type species of the genus *Thermoanaerobacter* is *T. ethanolicus*^{T} (DSM 2246) and was investigated in this study as well as *T. brockii* (DSM 1457) for the ability to produce BCOHs (and BCFAs) from BCAAs. These species were cultivated at 20 mM concentrations of the BCAAs both in the presence and absence of electron-scavenging systems as was done for strain AK90, but the initial concentration of thiosulfate varied between 5 and 80 mM. When cultivated without any electron-scavenging system, the BCAAs were only degraded to a minor extent, producing low amounts of BCFAs (<2.0 mM) (Figures 2 and 3). An earlier study performed in our laboratory on *T. brockii* showed that, under methanogenic conditions, the BCAAs (20 mM) were almost completely degraded and the production of BCFAs varied between 14.8 and 15.9 mM, and similar amounts of methane were produced as compared to strain AK90 (between 7.6 and 9.3 mmol/L) (Table 2) [18]. A similar pattern was also observed for *T. ethanolicus* when cocultivated with the methanogen on leucine and isoleucine; almost complete degradation of the BCAAs and the amounts of the BCFAs were 15.0 and 14.3 mM for leucine and isoleucine, respectively; methane was observed in similar concentrations (7.4 to 7.8 mmol/L) as before (Table 2). Valine, however, was only partially degraded under these conditions by *T. ethanolicus*, resulting in lower concentrations of BCFAs and methane yields (Figure 3 and Table 2).

Similarly, cultivation with thiosulfate as an electron scavenger by these two species resulted in the production of BCFAs from BCAAs (20 mM), as well as the formation of their corresponding BCOHs (Figures 2 and 3). However, the amount of alcohol formation was lower as compared to strain AK90. By increasing the concentration of thiosulfate from 5 to 80 mM, an increase in the BCFA formation as well as in amino acid degradation was observed for *T. brockii* (Figures 2(a)–2(c)). At lower concentrations of thiosulfate, the electron sink is not in excess, leading to hydrogen accumulation and inhibition of further amino acid degradation. The highest amounts of BCOHs were produced under these conditions. By increasing the initial thiosulfate concentration, hydrogen was kept at lower concentrations, allowing for almost complete degradation of the amino acids. The concentration of hydrogen sulfide is not correlated to increased initial thiosulfate concentrations except for the step from 5 to 10 mM thiosulfate concentrations. At higher thiosulfate concentrations, H_2S actually decreased in the experimental bottles; microscopic observations as well as the formation of a strong yellow color, especially at high initial thiosulfate loadings, showed the presence of elemental sulfur.

T. ethanolicus produced much lower amounts of BCFAs and BCOHs compared to strains AK90 and *T. brockii*, in the presence of thiosulfate with only between 3.0 and 10.0 mM of the amino acids being degraded (Figures 3(a)–3(c)). Hydrogen concentrations at high initial thiosulfate concentrations were also higher as compared to *T. brockii*. Hydrogen sulfide and BCOHs concentrations showed similar spectrum as with *T. brockii*, increasing from 5 to 10 mM initial thiosulfate concentrations, but decreasing at higher concentrations. These values, however, were always found to be in lower concentrations as compared with *T. brockii*.

4. Discussion

The amino acid metabolism of thermophilic bacteria has been investigated previously [5, 6, 24, 25], though to a lesser extent as compared to the metabolism of carbohydrates. Many of these investigations focus on the thermodynamics of amino acid degradation. BCAAs can only be degraded when the electrons are scavenged either by the addition of thiosulfate or by coculturing with a methanogen [6] or with sulfate reducing bacteria [26]. Strain AK90 could only degrade one amino acid (serine) when cultivated without any electron-scavenging system; the end product under these culture conditions was predominantly acetate and 80% of the amino acid was degraded (Table 1). When serine was degraded in the presence of thiosulfate or in a coculture with *Methanothermobacter* M39, complete degradation occurred and increased acetate concentrations were observed. This is similar to a study in which *T. brockii* was grown on serine where a shift from ethanol to acetate was reported under hydrogen-scavenging conditions [6]. For most of the other amino acids, only a small fraction is degraded with the accumulation of hydrogen leading to thermodynamic hindrance for further degradation.

Under methanogenic conditions, much wider spectra of amino acids were degraded (Table 1). This is in agreement with previous studies by others where proteins are degraded to a greater extent under methanogenic conditions [15, 27]. This was most clearly shown during the degradation of

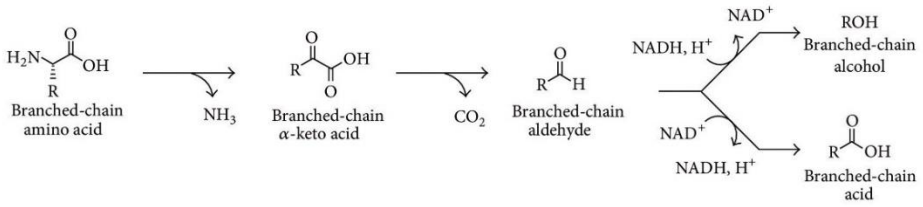

FIGURE 1: The Ehrlich pathway (from [17]). Catabolism of branched-chain amino acids (leucine, isoleucine, and valine) leading to the production of branched-chain acids and alcohols.

the BCAAs where more than 80% of them were degraded to their corresponding BCFAs. As stated previously, *Thermoanaerobacter* strain AK90 threonine was not degraded as a single substrate but under methanogenic conditions it was almost completely converted to acetate (Table 1). This implies that its degradation pathway is more complex and it has been shown in other studies that threonine is degraded to propionate or to a mixture of propionate and butyrate [5, 28]. The amount of methane produced in experimental bottles supplemented with the aromatic amino acids and methionine was between 6.3 and 8.2 mmol/L which was lower as compared to methane from the BCAAs (between 8.9 and 11.1 mmol/L), but well above the control value (yeast extract only). This, together with the fact that these four amino acids were almost completely degraded under these conditions, indicates an oxidative mechanism for their initial degradation pathways. This was also indicated by a number of unidentified products observed during the gas chromatography run from these amino acids. Thus, methionine is likely to be degraded to 3-methylthiopropionate, phenylalanine to 2-phenylethanoate, and tyrosine to 2-(4-hydroxyphenyl)ethanoate. It is known that these end products have been produced by anaerobic bacteria [29].

Thiosulfate reduction to sulfide and sulfur is a common characteristic among the genera *Thermoanaerobacter*, *Caldanaerobacter*, and *Thermoanaerobacterium* [6, 24, 30]. Fardeau and coworkers demonstrated a shift in end product formation by *Thermoanaerobacter finnii* (now *T. brockii* subsp. *finnii*) on glucose in the presence and absence of thiosulfate [24]. Both ethanol and lactate decreased during thiosulfate reduction to hydrogen sulfide, while acetate and biomass increased. The influence of using hydrogen-scavenging systems has also been investigated during the amino acid degradation by *Thermoanaerobacter brockii* [6]. Both thiosulfate and the presence of a hydrogen-scavenging methanogen were crucial for the oxidative deamination of the BCAAs by this strain.

During the degradation of the BCAAs in the presence of thiosulfate, strain AK90 produced not only the corresponding fatty acids, but also their corresponding alcohols (Table 1). This has only recently been shown by some species within the genera of *Thermoanaerobacter* and *Caldanaerobacter* [16, 17] and is known to occur in lactic acid bacteria [10] and yeasts [12] through the Ehrlich pathway (Figure 1). The reason for a mixture of a fatty acid and alcohol produced from the BCAAs can most likely be directly linked to the partial pressure of hydrogen and regulation of the $NADH/NAD^+$ and the corresponding hydrogenases are involved. Although the concentrations of hydrogen are very low under both electron-scavenging systems (methanogenic/thiosulfate), it was detectable under thiosulfate reduction conditions which may explain the formation of the reduced alcohol.

Interestingly, threonine was only partially (5.5 mM) degraded under methanogenic conditions by strain AK90 (Table 1). This is in contrast to the relatively large amounts of acetate produced. The reason might be that the strain is using a pathway postulated by Barker [31]: formation of acetate from threonine can be accomplished by direct cleavage of threonine to acetaldehyde and glycine by threonine aldolase reaction, followed by oxidation of acetaldehyde to acetate and the conversion of glycine to acetate. Since glycine is not utilized as a single substrate under any culture conditions by strain AK90, it could be accumulating in the culture broth (assuming this pathway is active), thus explaining the seemingly partial degradation for threonine. From the H_2S and S formation observed during growth on phenylalanine, tyrosine, and methionine in the presence of thiosulfate and the fact that the same unidentified peaks were also observed as under methanogenic conditions, it can be deduced that similar end products were produced.

The degradation of the BCAAs in the presence of thiosulfate by *T. brockii* and *T. ethanolicus* led to the production of both BCOHs and BCFAs, similar to strain AK90, though to a lesser extent (Figures 2 and 3). By increasing the initial concentrations of thiosulfate from 5 to 10 mM in *T. brockii* cultures on the three BCAAs, an increase of hydrogen sulfide was observed. However, by further increasing the thiosulfate concentrations to 20, 40, and 80 mM, the hydrogen sulfide concentrations did not increase in the experimental bottles; the hydrogen sulfide concentrations were, in fact, lower at higher thiosulfate loadings. The most reasonable explanation is either thiosulfate or H_2S is converted to sulfur. Microscopic analysis revealed the presence of sulfur granules in the cultures and a strong yellow color formation was observed.

T. ethanolicus degraded only between 3.0 and 10.0 mM of the BCAA in the presence of thiosulfate. Additionally, hydrogen concentrations were higher at the end of incubation as compared with *T. brockii* and strain AK90. Thus, *T. ethanolicus* seems to be less effective in reducing thiosulfate, but hydrogen was found to be above 1 mmol L^{-1} even at very high thiosulfate concentrations. This is most likely the reason for incomplete BCAA degradation by this strain.

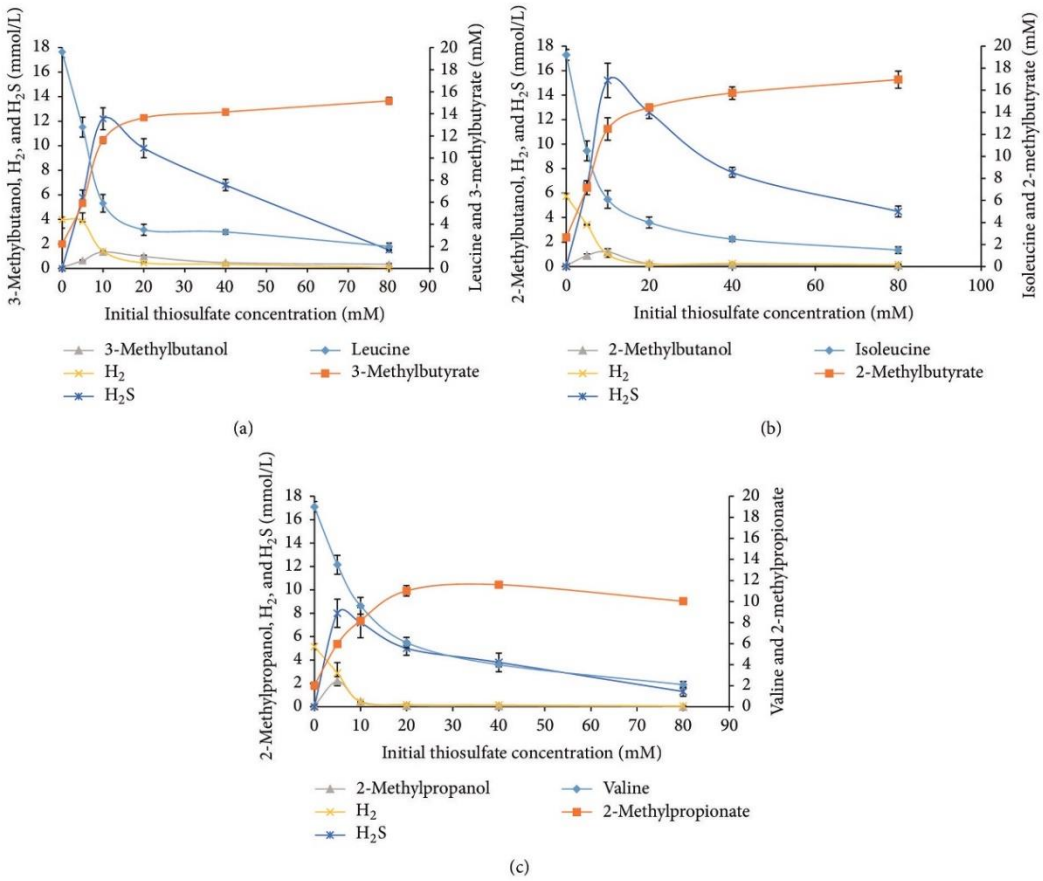

FIGURE 2: Amino acid degradation and end product formation at five different initial thiosulfate (5, 10, 20, 50, and 80 mM) concentrations by *Thermoanaerobacter brockii*. (a) Leucine degradation, (b) isoleucine degradation, and (c) valine degradation. Bars represent standard deviation from two replicates.

The original characterization paper on *T. ethanolicus* indicated that the strain was incapable of utilizing yeast extract only, but its presence was crucial for sugar degradation [1]. Faudon and coworkers [25], however, showed that both *T. brockii* and *T. ethanolicus* were capable of peptide and amino acid degradation and that the presence of thiosulfate was indeed of importance resulting in more efficient degradation. However, *T. brockii* was much more efficient than *T. ethanolicus*, which is in line with our results.

Comparison of the three strains, AK90, *T. brockii*, and *T. ethanolicus*, shows that, under the same growth conditions (20 mM BCAA, 20 mM thiosulfate), strain AK90 produces most of the BCOH (from 1.8 to 4.4 mM) and *T. brockii* the least (between 0.1 and 1.0 mM) (Table 1; Figures 2 and 3). Strains AK90 and *T. brockii* produced between 9.7 and 19.2 mM of the BCFA from the BCAA, but *T. ethanolicus* only between 5.1 and 6.8 mM, which is reflected in lower BCAA degradation. Under methanogenic conditions, strains AK90 and *T. brockii* almost completely degraded the BCAA, which was also true for *T. ethanolicus* on both leucine and isoleucine, but not on valine (only 7.6 mM degraded) (Tables 1 and 2). This apparent ability to degrade valine by *T. ethanolicus* may be caused by differences in enzyme specificity.

The production of BCFAs and BCOHs from amino acids has been well established in yeasts such as *Saccharomyces cerevisiae* via the Ehrlich pathway [12, 32]. Recent investigations on the degradation of BCAAs to BCOHs by *Thermoanaerobacter brockii* and *Caldanaerobacter subterraneus* subsp. *yonseiensis* were recently reported [16]. This work led to a screening of various species within these two genera as well as within *Clostridium*, *Caldicellulosiruptor*, *Caloramator*, and *Thermoanaerobacterium* [17]. From these studies, only *Thermoanaerobacter* and *Caldanaerobacter* species showed the capacity to produce BCOHs from BCAAs. In both of these studies, it was clear that a factor of importance for BCOH formation was mainly the partial pressure of hydrogen [16, 17]. The present investigation shows that it is very likely that strain AK90 is also producing a mixture of aromatic fatty

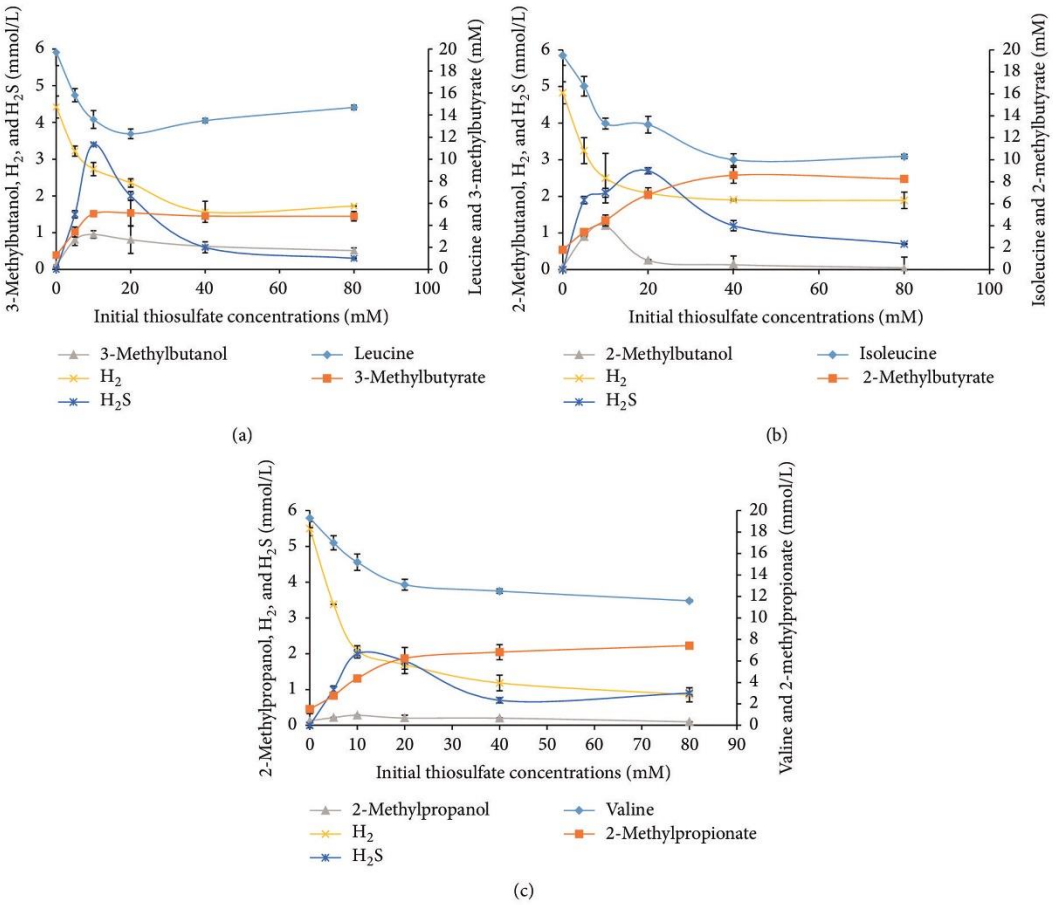

FIGURE 3: Amino acid degradation and end product formation at five different initial thiosulfate (5, 10, 20, 50, and 80 mM) concentrations by *Thermoanaerobacter ethanolicus*. (a) Leucine degradation, (b) isoleucine degradation, and (c) valine degradation. Bars represent standard deviation from two replicates.

acids and aromatic alcohols during hydrogen-scavenging conditions.

The degradation of amino acids presents a renewable route to potentially important feedstock chemicals. The interest in BCAA degradation has often been directed towards the formation of flavor compounds (branched- and aromatic chain aldehydes, alcohols, and acids) in food and beverage products [12]. Additionally, (*S*)-2-methylbutanol is a potential biofuel [33] and some of the BCOHs may serve as building blocks [34]. Recently, some studies have focused on the production of branched-chain alcohols from protein-rich waste using genetically engineered *Escherichia coli* and *Bacillus subtilis* with the main focus being that BCOHs are promising biofuel candidates [35, 36].

5. Conclusion

Thermoanaerobacter strain AK90 degraded only serine when used as a single substrate but degraded nine amino acids under electron-scavenging conditions (thiosulfate and a coculture with a hydrogenotrophic methanogen). Branched-chain amino acids were degraded to their corresponding branched-chain fatty acids under methanogenic conditions and to a mixture of branched-chain fatty acids and alcohols under thiosulfate reducing conditions. This phenomenon was also exhibited by *Thermoanaerobacter brockii* (DSM 1457) and *Thermoanaerobacter ethanolicus* (DSM 2246), though to a lesser extent. The formation of these end products seems to be highly dependent upon partial pressure of hydrogen.

Conflict of Interests

The authors declare that there is no conflict of interests.

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IV

Branched-chain amino acid catabolism of *Thermoanaerobacter* strain AK85 and the influence of culture conditions on branched-chain alcohol formation

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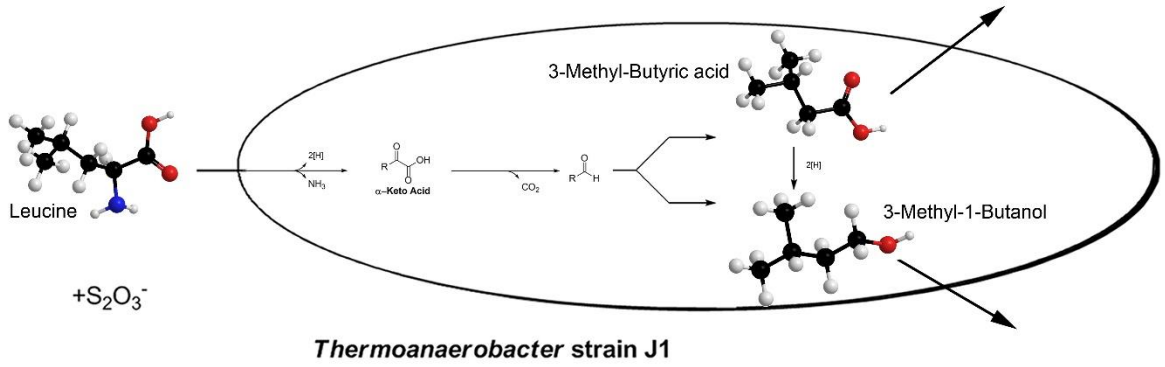


ABSTRACT

The bioprocessing of amino acids to branched-chain fatty acids and alcohols are described using *Thermoanaerobacter* strain AK85. The amino acid utilization profile was performed without an electron scavenger, with thiosulfate, and in a co-culture with a methanogen. There was an emphasis on the production of branched-chain alcohols and fatty acids from the branched-chain amino acids, particularly the influence of culture conditions which was investigated using isoleucine, which revealed that the concentration of thiosulfate was of great importance for the branched-chain alcohols/fatty acid ratio produced. Kinetic studies show that branched-chain amino acids fermentation is relatively slow with the concentrations of the alcohol increasing over time. To understand the flow of electrons and to investigate if the branched-chain fatty acid was being converted to branched-chain alcohol, enzyme assays and fermentation studies using ^{13}C-labeled leucine and 3-methyl-1-butyrate were performed which indeed suggest carboxylic acid reduction as a source of branched-chain alcohols with thiosulfate.

Highlights

- *Thermoanaerobacter* strain AK85 degrades val, ile, leu, ser, and thr
- An electron scavenger is needed for BCAA and thr degradation
- Val, ile, and leu are converted to branched-chain fatty acids alcohols
- Thiosulfate concentration is a major factor for the BCOH/BCFA
- Branched-chain amino acid metabolism is slow

1. Introduction

The protein fraction represents a major underutilized component of lignocellulosic and algal biomass with potential applications for production of biofuels and fine chemicals. The vast majority of the work on the utilization of biomass has focused on the conversion of the carbohydrate fraction to low-value biofuels such as bioethanol, biohydrogen, and biomethane. Thermophilic *Clostridia*, particularly those within the genus of *Thermoanaerobacter*, are attractive bioprocessing agents due to their wide catabolic capabilities, often degrading hexoses, pentoses, disaccharides, starch, and xylan (Jessen and Orlygsson, 2012; Slobodkin et al., 1999; Tomás et al., 2013; Wiegel and Ljungdahl, 1981; Xue et al., 2001). While the majority of studies on the catabolic capabilities of thermophilic *Clostridia* have predominately focused on their carbohydrate catabolism, particularly highly ethanol or hydrogen-producing strains, this has left the catabolism of protein and amino acids largely unexplored.

Historically, protein and amino acid utilization patterns have been important as a part of understanding the metabolism of microorganisms in the rumen of domestic animals or the infectious nature of clostridial pathogens such as *Clostridium botulinum* or food spoilage organism including *Clostridium perfringens* and *Clostridium sporogenes* (McInerney, 1988). Specific studies on the degradation of the branched-chain amino acids (BCAA) within class *Clostridia* (Elsden & Hilton, 1978) and *Thermoanaerobacter brockii* (Fardeau et al., 1997) have previously shown that BCAA are typically converted to a one carbon shorter branched-chain fatty acid (BCFA). Systematic studies of the proteolytic and amino acid utilizing capabilities of many genera in characterization papers of novel species often ignore amino acid catabolism altogether. The anaerobic degradation of amino acids can be oxidative, reductive or without oxidation/reduction (Orlygsson, Anderson, & Svensson, 1995). For instance, in the Stickland reaction, one amino acid is oxidized (e.g. alanine) and the reducing equivalents used to reduce another amino acid (such as glycine) (Andreesen, 1994). The catabolism of reduced amino acids (e.g. alanine, glutamate, branched-chain amino acids) are thermodynamically unfavorable, often with $\Delta G^{o\prime}$ values between +5 to +10 kJ/mol (Orlygsson, Houwen, & Svensson, 1995). Thus, these amino acids will not be degraded unless the reducing equivalents, produced in both the oxidative deamination and decarboxylation steps, are scavenged. This has indeed been shown for several thermophilic bacteria where leucine, isoleucine, and valine can be degraded to their corresponding one carbon shorter fatty acid in the presence of thiosulfate or in a co-culture with a hydrogenotrophic methanogen (Faudon et al., 1995; Orlygsson et al., 1995a; Scully et al., 2015; Scully and Orlygsson, 2015, 2014; Scully and Orlygsson, 2014).

Thermoanaerobacter strain AK85 (previously named as strain J1), an isolate from a geothermal area in Iceland, is closely related to *Thermoanaerobacter uzonensis*. This strain is highly ethanologenic and, unlike other *Thermoanaerobacter* species, tolerates relatively high initial substrate concentrations (Jessen and Orlygsson, 2012) making it a potentially promising bioprocessing organism. Previously, it was revealed that this strain degraded a mixture of BCAAs to a mixture of the corresponding BCFAs and branched-chain alcohols (BCOHs) although its ability to utilize other amino acids and the conditions that are most favorable for BCOH formation was not reported (Scully, Iloranta, Myllymaki, & Orlygsson, 2015).

The present study focuses on the amino acid catabolism of *Thermoanaerobacter* strain AK85 by using two electron-scavenging systems with an emphasis on the production of

BCOHs from the BCAAs. Here we present the influence of various culture parameters on the degradation of isoleucine to a mixture of its corresponding fatty acid and alcohol. Enzymatic assays of the various alcohol dehydrogenases were performed and in order to ascertain if the BCFA was being converted to the corresponding alcohol, NMR studies using ^{13}C-labled leucine and 3-methyl-1-butyrate were performed.

2. Experimental

2.1 General methods

All materials were obtained from Sigma Aldrich unless otherwise noted. ^{13}C2-labeled leucine was obtained from Cambridge Isotope Laboratories (Tewksbury, MA) while ^{13}C1-labled 3-methyl-1-butyric acid was obtained from Sigma-Aldrich. Nitrogen gas was of 5.0 quality (< 5 ppm O_2) obtained from AGA gas. Nucleotide cofactors were obtained from Megazyme with the exception of NADPH which was obtained from Sigma.

2.2 Bacterial strains and Culture conditions

Thermoanaerobacter strain AK85 (Jessen and Orlygsson, 2012) and *Methanothermobacter* strain M39 (Brynjarsdottir et al. 2012) were reactivated from our culture collection. Strain AK85 was cultivated in basal mineral medium (BM) prepared as previously described by (Chades et al., 2018). All fermentation experiments were performed in serum bottles as independent triplicates at 65°C and pH 7.0 without agitation at a liquid-gas (L-G) phase ratio of 1:1 unless noted otherwise. The inoculum volume was 2% (v/v) from the exponential growth phase of stock cultures grown on glucose (20 mM). Substrates, vitamins, and trace element solutions were added to culture media after autoclaving (121°C for 60 minutes) through a syringe filter (Whatman PES, 0.45 µm).

In experiments involving co-culture with *Methanothermobacter* M39, experimental bottles were pre-cultivated on hydrogen and carbon dioxide (80/20 v/v) for one week in 117.5 mL with 20 mL liquid medium (L-G phase 0.2). Prior to inoculating strain AK85 into the methanogenic cultures, the bottles were flushed with nitrogen and aseptically titrated to pH 7.0.

2.3 Amino acids utilization spectrum

The ability of strain AK85 to utilize the 20 proteogenic amino acids (20 mM) in the presence or absence of thiosulfate (40 mM) or in a co-culture with *Methanothermobacter* M39 was accessed in batch culture. After a 5 day incubation period, end products were analyzed. Fermentation of amino acids as a single substrate and in the presence of thiosulfate were done in 24.5 mL serum bottles with a L-G phase ratio of 1:1. Experiments with degradation of amino acids in the presence of the methanogen were performed in 117.5 mL serum bottles with a L-G ratio of 0.2.

2.4 Influence of initial pH

To investigate the influence of initial pH on isoleucine fermentation, BM medium with initial pH values between pH 4.0 and 9.0 in 0.5 unit increments, obtained by titration with HCl or NaOH, were prepared in Hungate tubes (16x150 mm) with a L-G ratio of 1:1. 20 mM isoleucine was used as the carbon source with the addition of thiosulfate (20 mM); the tubes were incubated in a water bath for 14 days.

2.5 Influence of cultivation temperature

The impact of cultivation temperature was investigated between 50-85°C in 5°C increments in BM containing Ile (20 mM) and thiosulfate (20 mM). Fermentations were performed in Hungate tubes as described above.

2.6 L-G phase experiment

The strain was cultivated in BM containing 20 mM of isoleucine with the addition of 20 mM sodium thiosulfate in 117.5 mL serum bottles. Five different L-G phases were used; 0.05, 0.34, 0.98, 2.08 and 5.40. Cultivations were carried out for 14 days.

2.7 Influence of initial thiosulfate concentration

The effect of thiosulfate was investigated on isoleucine (20 mM) with thiosulfate concentrations between 10 and 60 mM in 10 mM increments as otherwise described above.

2.8 L-G phase and thiosulfate experiments

To investigate the impact of L-G phase ratio and thiosulfate concentration, on the fermentation profile of isoleucine (20 mM) by strain AK85, serum bottles with liquid-gas phase ratios of 0.05, 0.98, and 5.4 were prepared in 117.5 mL serum bottles containing either 0, 10, 20, or 40 mM of exogenously added thiosulfate.

2.9 Kinetic Experiments

Kinetic experiments on the fermentation of valine, leucine, and isoleucine (20 mM) in the presence of thiosulfate (20 mM) was were performed in 125 mL serum bottles with a L-G of 1:1 at 65°C. Hydrogen sulfide was analyzed at the end of the incubation period of 7 days. Periodically, 1 mL samples were taken for the analysis of soluble analyses and 0.2 mL of headspace gas was removed for analysis.

2.10 ^{13}C NMR experiments

Strain AK85 was cultivated in the 20 mM ^{13}C2-labled leucine +/- 40 mM thiosulfate (L-G 0.98) for 7 days and analyzed. ^{13}C NMR spectra were obtained on a Bruker AV400 at 298K after spiking with D_2O to obtain a signal lock (0.3 mL addition of D_2O to 1 mL of aqueous sample). In one experimental set up strain AK85 was cultivated on leucine (20 mM) and thiosulfate (20 mM) in the presence of 20 mM ^{13}C1-labled 3-methyl-butyrate.

2.11 Analytical methods

Hydrogen and methane were analyzed by Perkin Elmer Auto System XL gas chromatograph equipped with a thermo-conductivity (TCD) detector as previously described (Orlygsson & Baldursson, 2007). Alcohols and volatile fatty acids were measured by gas chromatography using a Perkin-Elmer Clarus 580 gas chromatograph equipped with flame ionization detector (FID) as previously described (Orlygsson & Baldursson, 2007). Amino acids were analyzed as previously described in (Scully & Orlygsson, 2015). Hydrogen sulfide was analyzed according to the method described by Cord-Ruwisch (Cline, 1969). Thiosulfate was analyzed according to Westley (Westley, 1987) modified for use in microplates. Optical density was determined using a Shimadzu UV-1800 at a wavelength of 600 nm (l=1 cm).

2.12 Enzymatic assays

All solutions were prepared in using rigorously degassed (sonication + vacuum) and syringe filtered into nitrogen flushed serum bottles. Cells were otherwise manipulated in an anaerobic glove box under nitrogen atmosphere. Protein was quantified according to the method of (Bradford, 1976) using bovine serum albumin as a standard.

Cells grown in 1 L serum bottles containing 500 mL of medium and were harvested after 24 hours and after 72 hours grown on 20 mM Ile + 20 mM thiosulfate. Cell-free extracts were prepared by harvesting cells by centrifugation (4700 rpm, 0-4°C) after the addition of potassium dithionite at a final concentration of 5 mg/L. Cells were suspended in 5 mL of 50 mM Tris buffer (pH 8.5) and were disrupted by adding an equal volume of 0.1 mm silica beads and vortexed in 4 cycles of 30 s followed by cooling in an ice bath for 1 min 60 s between cycles. Cell debris was removed centrifugation (5 min, 13000 x g) and the resulting cell extract stored under inert atmosphere in a nitrogen-flushed serum bottles at 4°C until use.

Alcohol dehydrogenase (ADH) and Aldehyde Oxidase (AOR) activity was determined oxidatively using a nitroblue tetrazolium (NBT) and phenazinemethosulfate (PMS) to the formation blue-purple formazan according to the method described by (Fibla & Gonzhlez-Duarte, 1993) with minor modifications. Briefly, 50 µL of diluted enzyme solution, 135 µL of reagent solution (330 µM NAD^+ or $NADP^+$, 330 µM NBT, 0.13% w/v gelatin) in 50 mM Tris buffer, pH 8.0) containing 5.5 mM of the relevant alcohol, and 15 µL of 10X PMS solution (80 µM) into microplates. Samples were anaerobically incubated at 65°C in a Bioscreen C (GrowthCurves, Ltd, Finland) and read every 2 minutes at 580 nm. A standard curve was generated using NADH or NADPH. ADH activity was calculated according to the formula below where *v* the sample volume in mL and *t* is the time in minutes.

$$ADH\ activity\ \left(\frac{mU}{mL}\right) = \frac{nmol\ NADH}{v \cdot t} = nmol\ NADHx2$$

3 Results and Discussion

3.1 Amino acid utilization spectrum

The ability of *Thermoanaerobacter* strain AK85 to utilize the 20 proteogenic amino acids was examined as a sole carbon source with no exogenous electron acceptor, as well as with the addition of 40 mM of thiosulfate, and in a co-culture with a hydrogenotrophic methanogen. It should be noted that some amino acids cannot be degraded unless the electron produced in the oxidation steps of the degradation pathway are scavenged. This has been observed by several studies on thermophilic bacteria where for instance the BCAA are not degraded because of the thermodynamic nature of their oxidative deamination step (Fardeau et al., 1997; Orlygsson, et al., 1995). Strain AK85 was found to utilize only serine partially when not external electron acceptor was provided (end products were acetate, 13.0 mM and ethanol, 4.6 mM; Table 1).

Table 1 - Utilization of amino acids by *Thermoanaerobacter* strain AK85 with or without electron scavenging systems (either thiosulfate; $S_2O_3^{2-}$) or in the presence of a methanogen (M).

	No	$S_2O_3^{2-}$	M
Serine	+	+	+
Threonine	-	+	+
Isoleucine	-	+	+
Leucine	-	+	+
Valine	-	+	+

In the presence of thiosulfate, serine was completely degraded with major end product being acetate. Threonine was partially degraded to acetate by strain AK85 under electron scavenging conditions. The four carbon threonine can be degraded by several different pathways to various end products (Barker, 1981; McInerney, 1988) while the main end products in our study was acetate. Additionally, all three BCAA in the prescence of thiosulfate were degraded to a mixture of their corresponding BCFA and BCOH. Recent investigations in our laboratory have shown that many thermophiles can degraded the BCAA to a mixture of their corresponding acids and alcohols when thiosulfate is used as an electron acceptor (Scully et al., 2015; Scully and Orlygsson, 2014; Scully and Orlygsson, 2015) as was observed with strain AK85 in present investigation. The stoichiometry for the BCAAs degradation was as follows (controls subtracted):

1.00 Leucine → 0.83 3-Methyl-1-butyrate + 0.17 3-Methyl-1-butanol + 0.32 H_2S

1.00 Isoleucine → 0.87 + 2-Methyl-1-butyrate + 0.13 2-Methyl-1-butanol + 0.24 H_2S

1.00 Valine → 0.97 2-Methyl-1-propionate + 0.03 2-Methyl-1-propanol + 0.07 H_2S

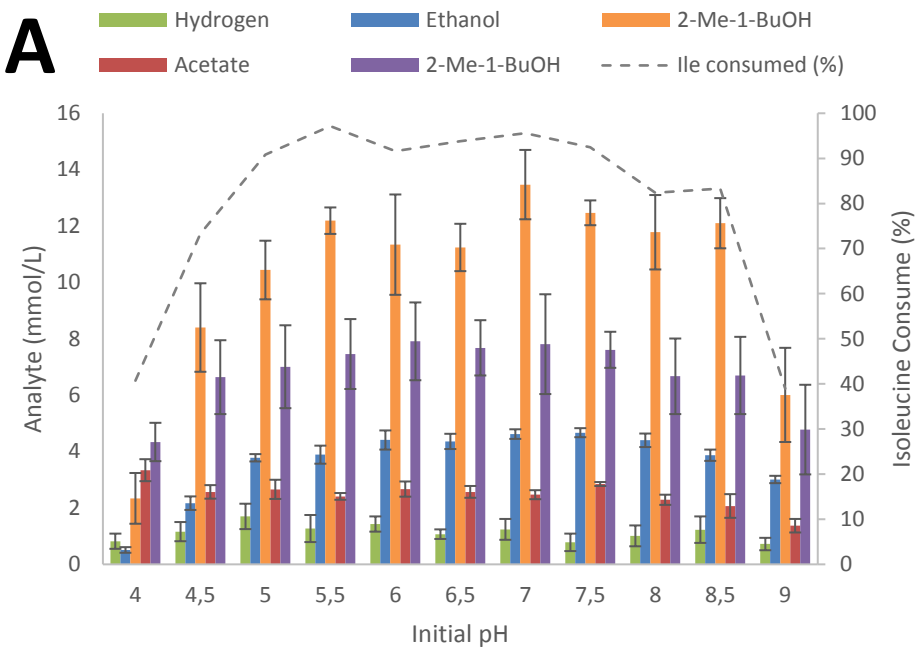
In all cases, the concentration of the acid was greater than of the alcohol. In the presence of a hydrogentrophic methanogen the same amino acids were degraded as with thiosulfate addition (Table 1). However, less of the BCAA were degraded as compared with thiosulfate addition and also no alcohol was formed. From the data obtained it is not clear why different electron systems (thiosulfate, hydrogenotrophic methanogen) result in a different end products when cultivated on the BCAA. Therefore various experiments on the effect of several environmental factors on the degradation of BCAA were performed.

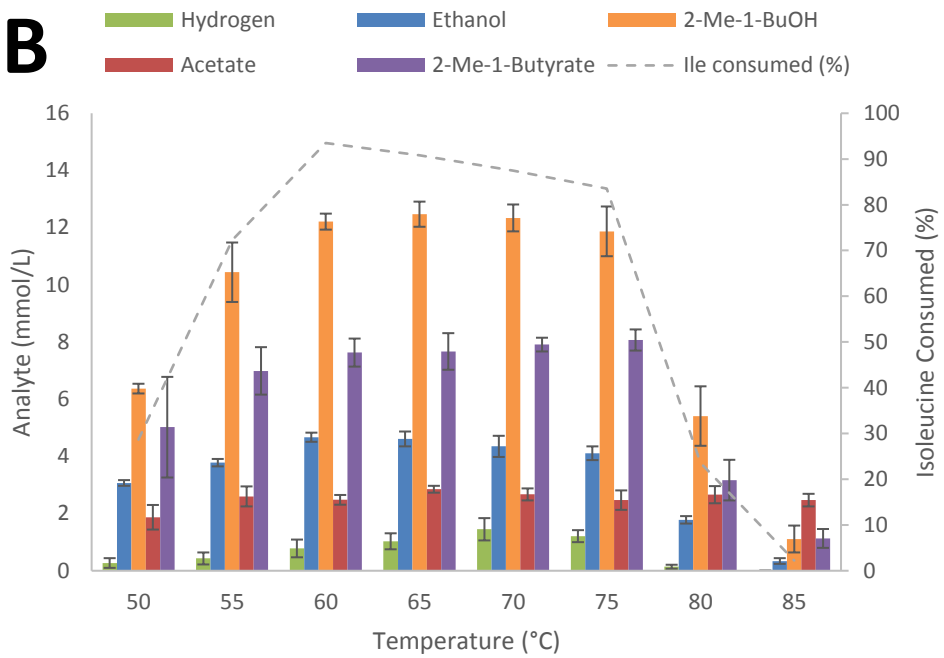
3.2 Effect of Culture Conditions on Isoleucine

To examine the influence of culture conditions on the catabolism of BCAAs and the resultant ratio of BCOH to BCFA, strain AK85 was cultivated on isoleucine as a model compound. Fermentation experiments involving isoleucine (20 mM) with thiosulfate (20 mM) at various pH values, temperatures, and different L-G phase volumes. Additionally, experiment involving the importance of initial thiosulfate was performed. In all cases, acetate and ethanol formation originates from the yeast extract and were confirmed in controls.

In these experiments, analytes were analyzed after 14 days of incubation. Strain AK85 shows a broad pH range for growth, producing between 10.44 and 13.47 mM of 2-methyl-1-butanol between initial pH values of 5.0 to 8.5, but between 6.7 to 7.0 mM of 2-methyl-1-butyrate (Figure 1A). Less end product formation occurred below pH 4.5 or at pH 9.0. The ratio of the alcohols versus the fatty acids show a good correlation for both ethanol/acetate and 2-methyl-1-butanol/2-methyl-1-butyrate at all pH values tested. This ratio was higher at pH between 5.0 to 8.5 (between 1.5 and 1.9) as compared to lower pH values. Hydrogen production was low at all pH values tested, ranging from 0.8 to 1.7 mmol/L. Hydrogen sulfide ranged from 2.02 to 4.21 mmol/L and in all cases thiosulfate was below 0.5 mM except for pH value of 4.0 where it was 5.34 mM. The carbon balances ranged from 92.7 to 115.0%.

The strain almost completely degraded isoleucine at temperatures between 60 to 70°C resulting in a production of 2-methyl-1-butyrate and 2-methyl-1-butanol (Fig. 1B). As before the concentration of the alcohols were most often higher than of the fatty acids. Partial degradation of isoleucine occurred at 50 to 55° as well as 80°C, resulting in lower amounts of end products with no degradation of isoleucine at all occurred at 85°C. The ratio of the 2-methyl-1-butanol over the acid was quite stable between 60 and 75 °C or between 1.49 and 1.70 but less at higher and lower temperatures. The ratio of end product formation from the yeast extract, ethanol and acetate were also produced with similar ratio as for the end product formation from isoleucine. Hydrogen production was low at all temperatures, ranging from cero to 1.45 mmol/L at 70°C. Hydrogen sulfide ranged from 0.17 to 3.71 mM but thiosulfate was in all cases lower than 0.5 mM. In all cases, the carbon balances were higher than 100% (between 105.7 to 128.3%).


A
Hydrogen
Ethanol
2-Me-1-BuOH
Acetate
2-Me-1-BuOH
Ile consumed (%)
Analyte (mmol/L)
Isoleucine Consume (%)
Initial pH
4
4,5
5
5,5
6
6,5
7
7,5
8
8,5
9


B
Hydrogen
Ethanol
2-Me-1-BuOH
Acetate
2-Me-1-Butyrate
Ile consumed (%)
Analyte (mmol/L)
Isoleucine Consumed (%)
Temperature (°C)
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55
60
65
70
75
80
85

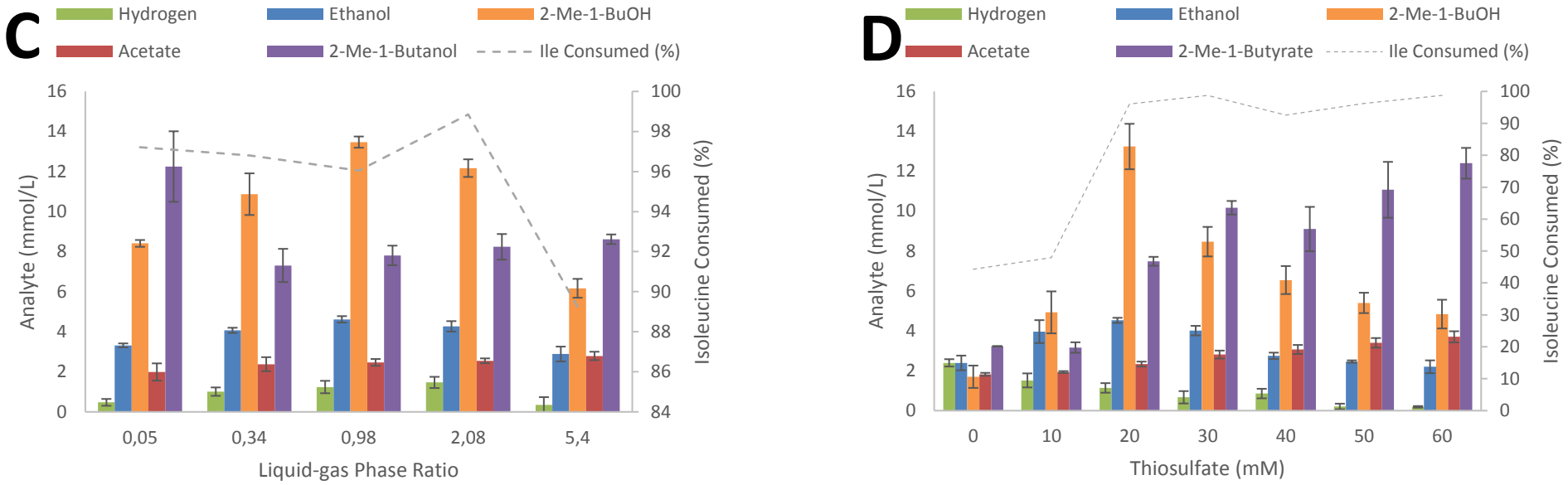

Figure 1 - Influence of initial pH (A), temperature (B), initial thiosulfate concentration (C), and L-G ratio (D) on end product profile of isoleucine (20 mM) fermentation with the addition of thiosulfate (20 mM). The insert highlights the ratio of the alcoholic end product and the corresponding carboxylic acid.

Results in earlier experiments of BCOH formation indicate that concentrations of hydrogen during fermentation may be of importance as has been observed for the formation of other substrates. In presence of thiosulfate, hydrogen concentrations are most often few mmol/L but during growth of strain AK85 in a co-culture with the hydrogenotrophic methanogen the concentration of hydrogen at end of experiments were always much lower and often lower than is the detection limit of the gas chromatograph. Thus, by increasing the partial pressure of hydrogen by cultivating strain AK85 at higher L-G phase ratios, it should be theoretically possible increase the amount of the alcohol over the fatty acid.

Cultivation of strain AK85 at five different L-G phases (between 0.05 to 5.4) showed that most of the amino acid was degraded in all conditions resulting in production of BCOH and BCFA (Fig. 1C). The concentration of hydrogen at end of cultivation was very low in all cases. Thus, it is likely that it is not only the efficiency of thiosulfate that serves as an electron acceptor but that other molecules are serving as electron acceptors (see later discussion). The concentration of H_2S and thiosulfate were also very low under all L-G phase conditions. The carbon balances were good, ranging from 84.7% to 110.4%. The ratio of the alcohols was higher than of the acid at the between L-G phase of 0.34 to 2.08 (ranging from 1.46-1.48.

To investigate the impact of thiosulfate concentration, AK85 was cultivated between 0 and 60 mM of added thiosulfate. Degradation of isoleucine without any electron accepter resulted in small quantities of both 2-methyl-1-butyrate and 2-methyl-1-butanol with only 10% of isoleucine was degraded (Figure 1D). The addition of 10 mM of thiosulfate increased the amount of the amino acid degraded (9.6 mM; 48%) and resulted in higher amounts of the 2-methyl-1-butyrate and 2-methyl-1-butanol, or 3.16 and 4.93 mM, respectively. By increasing the thiosulfate to 20 mM and above, isoleucine was almost completely degraded. However, at thiosulfate concentrations above 20 mM, the ratio of the alcohols to acid shifted in favor for BCFA formation; at 20 mM thiosulfate addition the ratio of the acid over the alcohol was 0.57 but changed to 2.56 at 60 mM initial thiosulfate concentration with 2-methyl-1-butyrate being the dominant end product above 30 mM initial thiosulfate concentration. Not surprisingly, the concentration of hydrogen gradually lowers with increased thiosulfate concentrations. Concentration of H_2S varied from 2.33 to 4.86 mM and thiosulfate was always below 0.5 mM.

The 2-methyl-1-butanol titers initially reported in 3.1 are lower than those reported. There are several potential explanations the different ratios of the BCOH over the BCFA. In the experiment presented in Table 1, the cultivation time was five days and the amount of thiosulfate added was 40 mM. In the experiments discussed in section 3.3 below, the serum bottles were incubated for 14 days and the concentration of thiosulfate added was lower (20 mM). It is clear from the data presented in Figure 1D where initial thiosulfate was stepwise increased that the amount of the BCFA increases proportionally, as compared with the production of the BCOH. However, it seems that the high concentrations of BCFA produced during the first five days of cultivation are converted to their corresponding BCOH when cultivated for a longer period. This is supported by the subsequent kinetic experiments and ^{13}C-labled studies in 3.4 and 3.6.

3.3 Effect of partial pressure of hydrogen and initial thiosulfate concentration on end product formation

To investigate the influence of both thiosulfate concentration and partial pressure of hydrogen, strain AK85 was cultivated on 20 mM of isoleucine in the presence of 0 to 40 mM of thiosulfate and at L-G ratios of 0.05, 0.98, and 5.40. As before, thiosulfate was almost completely consumed although the amount of hydrogen sulfide accounted for at the end of the experiment was less than 2.4 mM in all case.

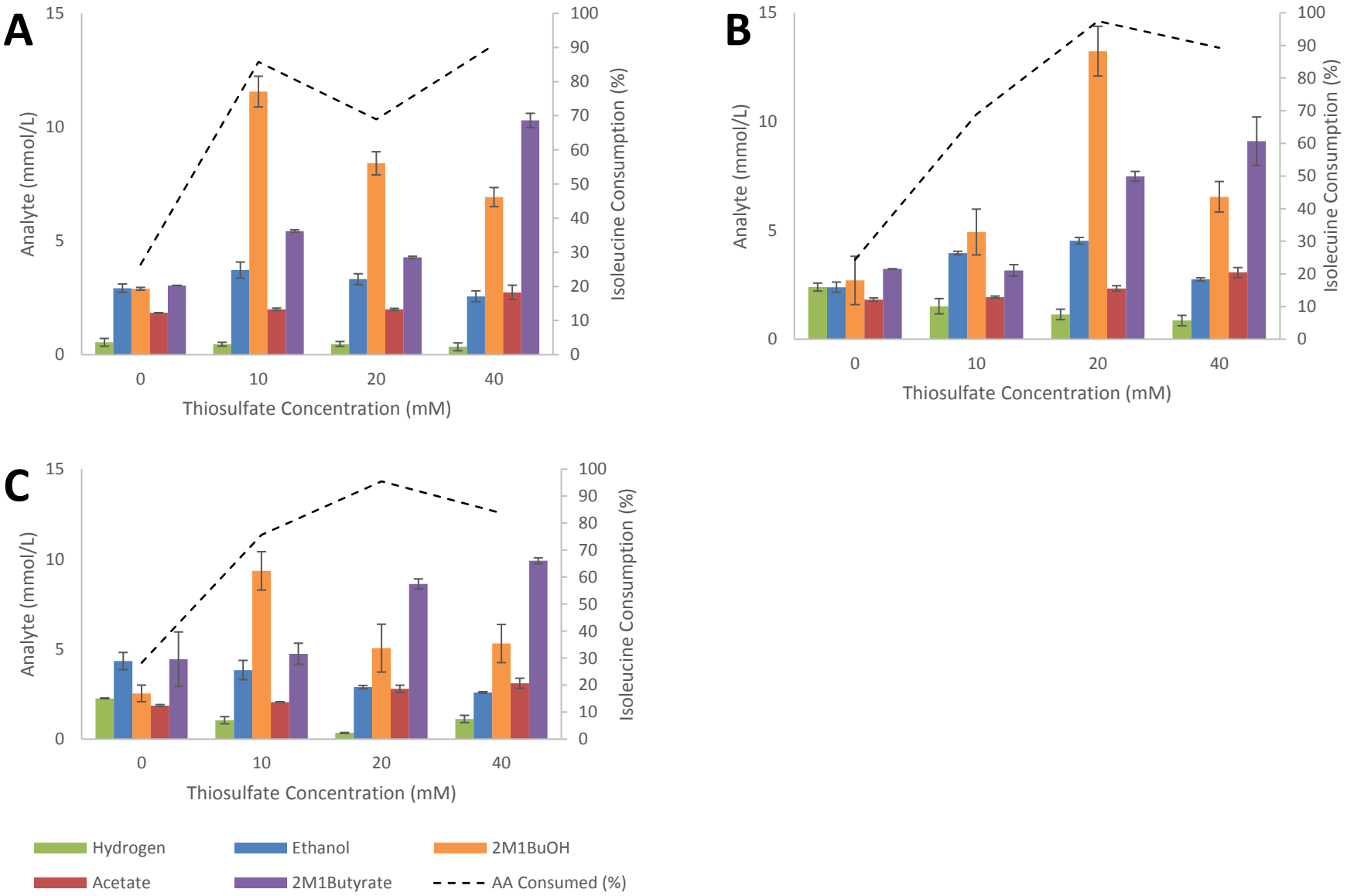

Figure 2 – Influence of liquid-gas phase ratio and initial thiosulfate concentration on the fermentation of isoleucine (20 mM) by *Thermoanaerobacter* strain AK85; A- low (L-G = 0.05), B – medium (L-G 0.98), C – high (L-G 5.4).

During isoleucine degradation and end-product formation at low L-G phase without thiosulfate only 26.4% of the amino acid was degraded resulting in a similar amounts of the BCOH and the BCFA, or 2.9 and 2.1 mM, respectively (Figure 2A). Addition of thiosulfate resulted in between 69 to 91% degradation of the amino acid. By increasing the thiosulfate from 10 mM to 40 mM the ratio of the alcohol over the acid changed from 2.12 to 0.67. The concentrations of ethanol, acetate, and hydrogen were relatively stable at all thiosulfate concentrations used.

In intermediate L-G phase ratios end-product formation without thiosulfate resulted similarly in only partial degradation of the amino acid and end product formation (BCOH; 2.5 mM, BCFA; 2.4 mM) (Figure 2B). In the presence of thiosulfate, between 68.9 to 97.5% of the amino acid was degraded. Again, a shift in the ratio of the alcohol over the acid was profound, especially when increasing the thiosulfate from 20 to 40 mM where the ratio of 2-methyl-1-butanol to 2-methyl-1-butyrate changed from 1.76 to 0.72. Concentration of ethanol and acetate were between 2.39 to 4.53, and 1.82 to 3.07, respectively. Hydrogen decreased from 2.4 mmol/L (no thiosulfate added) to 0.86 mmol/L when the thiosulfate concentration was 40 mM.

At the highest L-G phase ratio used similar spectra was shown concerning the amount of the amino acid degraded (only 28.3 % without thiosulfate versus 75 to 95% degradation in the presence of thiosulfate) (Figure 2C). Similarly, the ratio of the alcohol over the acid decreased from 1.96 (at 10 mM thiosulfate) to 0.53 (at 20 mM thiosulfate). Ethanol decreased from 4.34 mM (no thiosulfate) to 2.59, acetate increased from 1.87 to 3.10 and hydrogen was lowest at the two highest thiosulfate concentrations applied.

The results obtained and shown in Figures 2A-C strengthen the data obtained from the various physiological experiments performed (Figures 1A-D); at all L-G phase ratios used the concentration of the acid is higher than of the alcohol at the highest thiosulfate concentration used. At 20 mM initial thiosulfate concentration, the acid was higher only at the high L-G phase concentration. Hydrogen concentrations were considerable lower at the low L-G phase used (between 0.4 and 2.4 mmol/L) as compared with medium and high L-G phases. The main reason for lower concentrations of the BCOH initially is most likely due to the difference in the cultivation time and the concentration of thiosulfate used. Longer fermentation times may therefore be required for the system to reach equilibrium and the maximum BCOH titer assuming that it is the favored reduced product.

3.4 Kinetic experiments

Fermentation kinetics by *Thermoanaerobacter* strain AK85 on the three branched-chain amino acids was investigated with the fermentation of isoleucine being described in Figure 3.

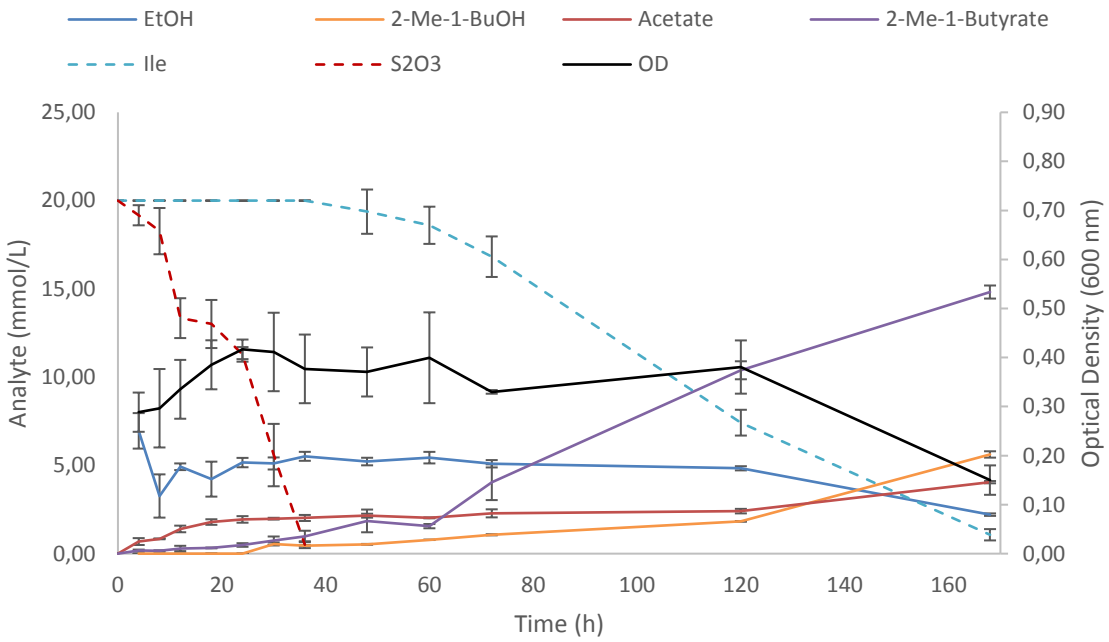

Figure 3 - Fermentation kinetics of *Thermoanaerobacter* strain AK85 grown on 20 mM Isoleucine (C) in the presence of thiosulfate (20 mM).

During growth on isoleucine in the presence of 20 mM of thiosulfate, strain AK85 reaches max OD of 0.42 after 24 h (Figure 3). Production of ethanol and acetate (from the yeast extract) also accumulates during the first 24 h while the formation of 2-methyl-1-butyrate and 2-methyl-1-butanol coincides with a decrease in isoleucine concentration after this initial growth period. Interestingly, the ratio of the BCFA over the BCOH is different as compared with valine which may be due to the substrate preferences of the enzymes involved. The concentration of 2-methyl-1-butyrate is higher than of the alcohol but increases until the end of monitoring after 7 days. The lower 2-methyl-1-butanol titer than those observed in earlier experiments with isoleucine is likely due to shorter incubation time as the exchange of reducing potential between analytes may not yet have reached equilibrium as suggested by the higher titers observed in Figures 2A-D when 20 mM of thiosulfate was used except incubated for 14 days. It is also noteworthy that ethanol reaches a maximum of 5.5 mM at 36 h but decrease to 2.2 mM at the end of the experiment whereas acetate increases from 2.0 mM to 5.6 mM while the concentration of 2-methyl-1-butanol starts to slowly increase after 48 hours. This suggests that reducing equivalence is being transferred from ethanol to another electron acceptor, such as 3-methyl-1-butryate to yield the corresponding alcohol. It is worth noting that low molecular weight alcohols are

routinely used in organic synthesis as a source of reducing potential with *Thermoanaerobacter* secondary alcohol dehydrogenases (Faber, 2011). Growth on valine and leucine show a similar pattern as for isoleucine.

One possibility for the formation of 2-methyl-1-butanol is the conversion of the fatty acid which acts as a sink for the reducing potential liberated from the amino acid. This was further investigated using ^{13}C-labled substrates in subsequent experiments.

3.5 Enzyme activities of isoleucine-grown cells

The activities of alcohol dehydrogenase (ADH) and aldehyde ferredoxin oxidoreductase (AOR) were determined by using cells that were harvested after 24 and 72 hours growth using isoleucine (20 mM) as a substrate with 20 mM thiosulfate butanol as shown in Figure 4. The specific activity of various primary and secondary alcohols were lower after 72 hours as compared to 24 hours with similar activities for 2-methyl-1-propanol, 2-methyl-1-butanol, and 3-methyl-1-. Interestingly, NAD-linked oxidation of C2 and C3 alcohols was not detectable after 72 hours while the activities of NADP-linked oxidations was marginally lower as compared with activity after 24h. After 24 hours, the specific activities of acetaldehyde oxidation were similar using either NAD or NADP while the NAD-dependent oxidation of acetaldehyde was higher after 68 hours. The activities of 2- and 3-methyl-1-butyrate oxidation were generally lower after 68 hours but were still detectable. Given the relative low activities of secondary alcohol oxidation, it is likely that the primary alcohol dehydrogenase is most active. It is known that ADH activity for *Thermoanaerobacter* strains have multiple alcohol dehydrogenases with different specificities and cofactor preferences as exemplified by *T. pseudoethanolicus* with three different ADHs which include a primary and secondary-specific ADHs (Zhou et al., 2017). It has long been known that the NAD(P)-dependent ADHs of *T. brockii* can reduce small ketones, often with high enantiospecificity, to their corresponding alcohols and it has also been demonstrated that *T. ethanolicus* posses a primary ADH that can oxidize branched-chain primary alcohols (Bryant, Wiegel, & Ljungdahl, 1992; Lamed & Zeikus, 1981). Earlier work demonstrated that *T. ethanolicus* produces different ADHs at different phases of growth (Bryant et al., 1988) which can be controlled by manipulating the growth temperature with higher temperatures favoring the primary ADH et al., 1992).

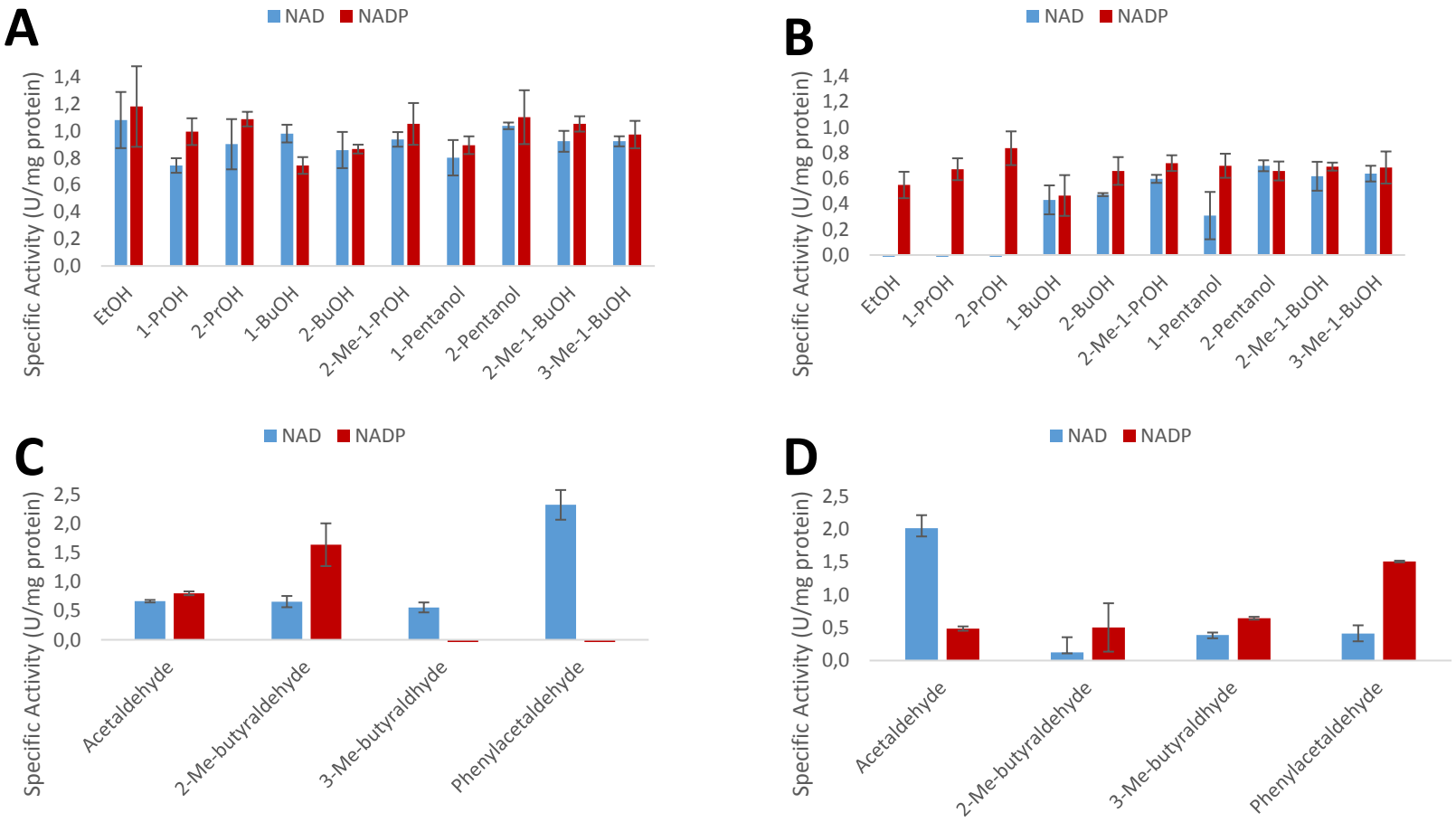

Figure 4 – Specific activities of alcohol and aldehyde oxidation in cell-free extracts of *Thermoanaerobacter* strain AK85 grown on isoleucine (20 mM) in the presence of 20 mM of thiosulfate after 24 hours (A and C) or 72 hours (B and D).

3.5 Degradation of ^{13}C2-labeled leucine and its corresponding BCOH

To demonstrate that the source of the BCOH was indeed from the degradation of its corresponding BCAA such as leucine, the strain was cultivated with ^{13}C2-labled leucine with and without the addition of 20 mM thiosulfate as show in in Figure 5. ^{13}C2-labled leucine was used instead of ^{13}C2-labled isoleucine due to the cost of the substrate.

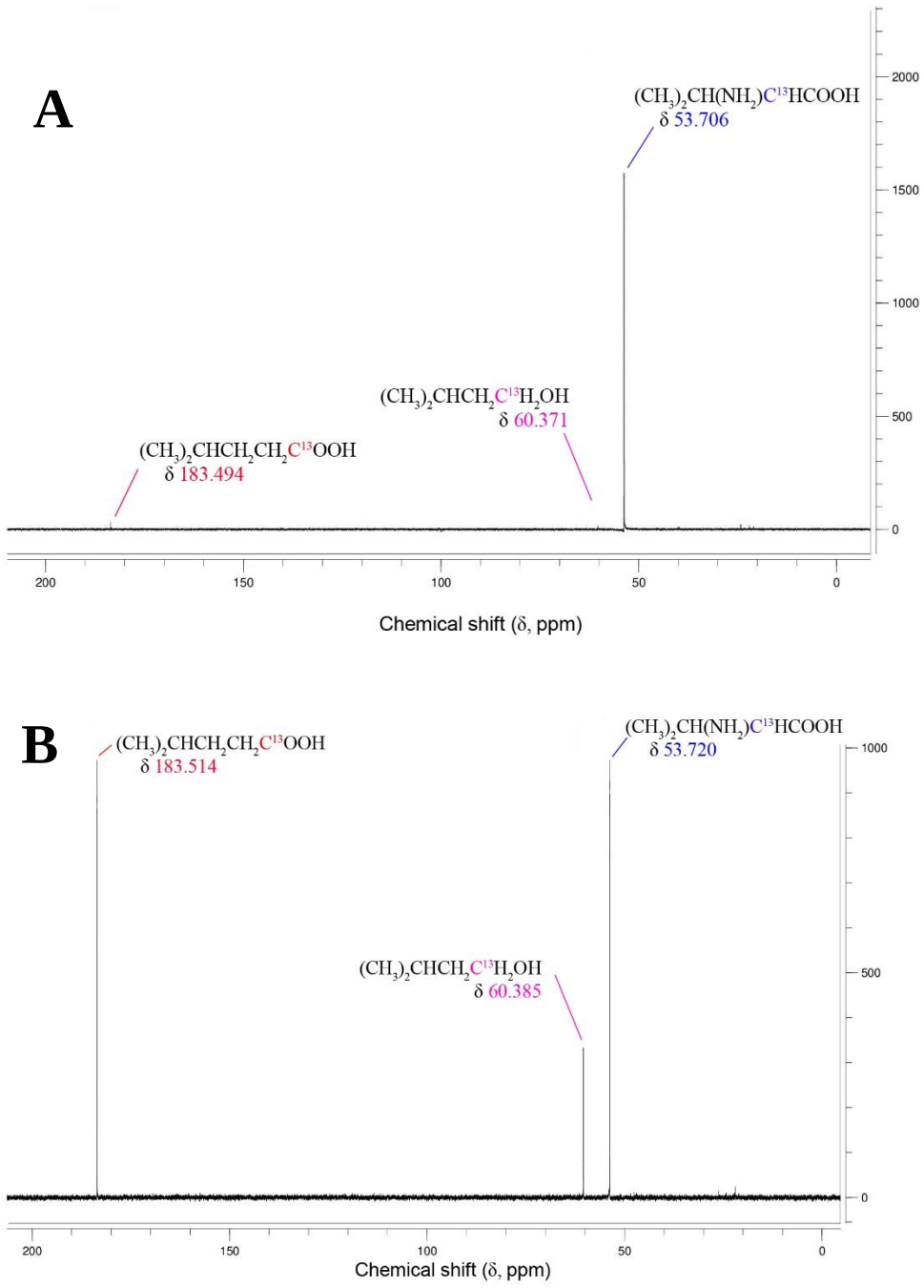

Figure 5 - Fermentation of ^{13}C2-labeled L-leucine by *Thermoanaerobacter* strain AK85 without thiosulfate (A) and with added thiosulfate at a final concentration of 20 mM (B) after 60 hours of incubation.

Analysis of end product formation from fermentation of ^{13}C2-labeled leucine without thiosulfate supplementation did not result in growth (results not shown) nor formation of any significant amounts of the corresponding BCOH and BCFA.

Samples taken from ^{13}C2-labled leucine in the presence of thiosulfate resulted in the formation of two peaks, one appearing at 60.4 and the other at 185.5 ppm which can be attributed to 3-methyl-1-butanol and 3-methyl-1-butyrate, respectively. The peak with a chemical shift of 53.7 ppm can be attributed to the C2 of leucine.

Bearing in mind the different ratios of the acid versus the alcohol observed in the various physiological experiments discussed above, it was also decided to add ^{13}C1-labeled 2-methyl-1-butyrate to a culture of strain AK85 together with leucine and thiosulfate. Peaks appearing at 60.3 ppm can be attributed to the C1 of 3-methyl-1-butanol suggesting that leucine, can serve as a source of reducing potential for the reduction of a BCFA into the corresponding alcohol. Thus, it seems that the BCOH comes both from leucine via fermentation and from the BCFA via a reductive biotransformation thus serving as an alternative electron sink.

Conversion of the BCAA are considered to occur in at least three steps. Isoleucine is for example first oxidatively deaminated producing its corresponding α-keto acid (3-methyl-2-oxopentanoate), ammonia and hydrogen. The α-keto acid is then most likely oxidatively degraded to its corresponding aldehyde and further to a mixture of the BCFA (oxidation) or BCOH (reduction) together with carbon dioxide and hydrogen. Since the strain can utilize the BCFA as an electron sink (Figure 3C) it is also possible that the strain is producing the fatty acid from the aldehyde through a CoA intermediate to gain ATP and after its formation it competes with thiosulfate for the electrons producing either H_2S (from the thiosulfate) or BCOH (from the BCFA). The use of carboxylic acids as an electron sink has been previously demonstrated for *Clostridium* species such as *Clostridium acetireducens* (Orlygsson, et al., 1996) and acetogens such as "*Clostridium ragsdalei*" (Isom, Nanny, & Tanner, 2015). Recently, *Thermoanaerobacter* species have been demonstrated to reduce carboxylic acids to their corresponding alcohols using glucose as a source of reducing potential (Hitschler et al., 2018) and specifically investigated for *T. pseudoethanolicus* which also converts C2 to C6 fatty acids, including 2- and 3-methyl-1-butyrate, to the corresponding alcohol in the presence of glucose as a source of reducing potential (Scully, Brown, Ross, & Orlygsson, 2019).

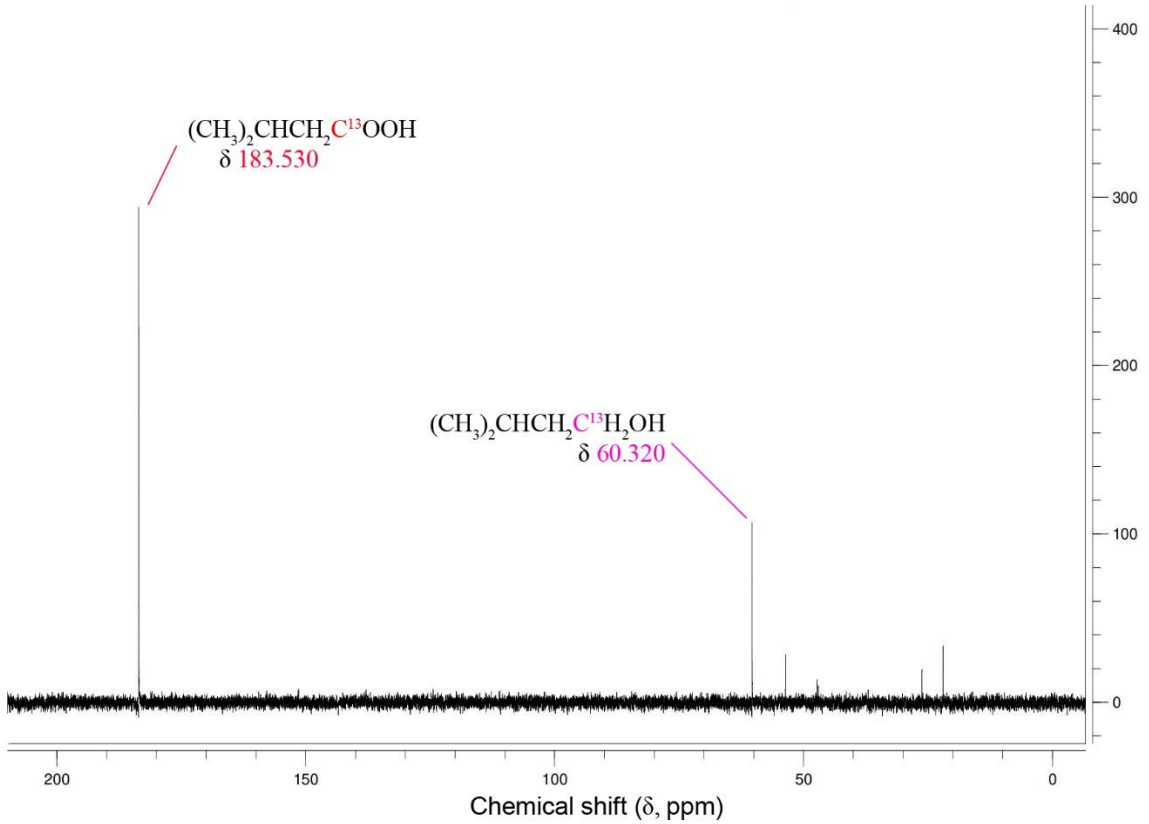

Figure 6 - Fermentation of various substrates in the presence of ^{13}C1 3-methyl-1-butyrate (20 mM) with 20 mM L-leucine + 20 mM thiosulfate

This study has implications for the valorization of at least part of the protein fraction of biomass and suggests a route for the conversion of carboxylic acids to alcohols. Future work will focus on the reduction of carboxylic acids as well as the proteolytic capabilities of *Thermoanaerobacter* strain AK85 and the conversion of protein-rich biomass, such as macro algae and lupine, to useful higher-order alcohols.

4. Conclusions

Strain AK85 degraded only serine as a single substrate. By adding an external electron acceptor like thiosulfate or co-cultivating the strain with a hydrogenotrophic methanogen, a broader substrate spectra was observed and the strain converts BCAA to a mixture of BCFA and BCOH. It is not clear from the data provided if the strain converts the BCAA to a mixture of their corresponding BCFA and BCOH or whether it only produces the acid by fermentation and then converts it to its corresponding alcohol. Initial concentration of thiosulfate is of great importance and directs the flow more to the fatty acid rather than the alcohol. However, by using isotopically labeled BCFA it is clear that it can be converted to its corresponding alcohol and this is most likely dependent on the oxidation states of the electron carriers the strain uses; $NAD(P)^+$/NADPH, Ferredoxin (ox/red) and the ratio of the BCFA/BCOH.

E-supplementary data of this work can be found in online version of the paper.

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Plese note that the data tables described in **Paper IV** and presented were published separately as a dataset in *Data in Brief* published under the Creative Commons Liscense (Scully, S.M., Orlygsson, J. (2019).Dataset Describing the Amino Acid Catabolism of *Thermoanaerobacter* strain AK85: the Influence of Culture Conditions on End Product Formation. Data in Brief, 24, DOI: 10.1016/j.dib.2019.103938).

Supplementary Table 1. Amino acid degradation and end product formation from amino acids by *Thermoanaerobacter* strain AK85. Initial amino acid concentration was 20 mM in all cases. Experiments were done with and without electron scavenging systems and with either different concentration of thiosulfate ($S_2O_3^{2-}$) or in a co-culture with *Methanothermobacter* strain M39. Data represent the average of two replicate experiments ± standard deviation. Controls are experimental bottles with only yeast extract (2.0 g/L).

	Analyte (mmol/L)											
Substrate	Ethanol	2/3-methyl-1-butanol	2-methyl-1-propanol	Acetate	2-methyl-1-propionate	2/3-methyl-1-butyrate	H_2	H_2S	CH_4	Amino acid degraded (mM)	pH final	Carbon balance*	OD
Control	2.71 ± 0.15	<0.05	<0.05	2.76 ± 0.06	0.22±0.04	0.44±0.10	3.24±0.05	ND	ND	ND	7.0±0.0	ND	0.26±0.02
Control + S_2O_3 (40 mM)	1.64 ± 0.28	<0.05	0.91 ± 0.12	6.23 ± 0.27	0.87±0.07	0.87±0.16	<0.05	0.11±0.02	ND	ND	6.9±0.0	ND	0.23±0.03
Control + M39	1.71±0.11	<0.05	<0.05	3.77 ± 0.11	0.44±0.96	0.80±0.11	<0.05	ND	1.41	ND	8.0±0.1	ND	0.44±0.03
Serine	4.49±0.53	<0.05	<0.05	12.77±0.550	0.11±0.02	0.34±0.04	4.82±0.05	ND	ND	12.20±0.75	6.9±0.1	83.3±0.8	0.24±0.03
Serine + S_2O_3 (40 mM)	3.76±0.37	<0.05	0.72±0.16	22.51±0.44	0.59±0.11	0.70 ± 0.12	1.00±0.36	2.13±0.11	ND	20.00±0.00	6.8±0.1	81.4±2.1	0.26±0.03
Serine + M39	1.36±0.12	<0.05	<0.05	20.92±1.12	0.76±0.14	1.11±0.12	<0.05	ND	5.55±0.23	20.00±0.00	7.5±0.1	85.8±2.4	0.45±0.02
Threonine	1.67±0.28	<0.05	<0.05	3.15±0.56	0.10±0.02	0.40±0.06	4.03±0.36	ND	ND	2.22±0.03	6.7±0.1	ND	0.21±0.02
Threonine + S_2O_3 (40 mM)	3.23±0.20	<0.05	0.77±0.14	15.76±0.22±	0.64±0.11	0.69±0.08	0.14± 0.03	2.65±0.14	ND	12.23±0.21	6.8±0.0	ND	0.29±0.01
Threonine+ M39	1.14±0.11	<0.05	<0.05	23.22±0.70	0.54±0.12	0.84±0.11	<0.05	ND	3.32±0.20	16.53±0.22	7.9±0.1	ND	0.45±0.02
Isoleucine	2.93±1.07	1.1±0.11	<0.05	1.92±0.22	0.22±0.04	2.20±0.21	5.04±0.27	ND	ND	1.80±0.12	6.8±0.1	97.8±2.3	0.21±0.03
Isoleucine + S_2O_3 (40 mM)	2.41±0.42	2.00±0.17	0.70±0.12	3.58±0.43	1.00±0.03	13.05±0.66	0.38±0.01	3.12±0.17	ND	16.50±0.86	6.9±0.0	85.9±1.8	0.29±0.00
Isoleucine + M39	1.24±0.07	<0.05	<0.05	3.02±0.15	0.43±0.08	9.07±0.18	<0.05	ND	3.27±0.23	8.66±0.34	7.9±0.1	95.4±2.0	0.28±0.03
Leucine	0.13±0.14	1.43±0.21	<0.05	1.94±0.03	0.20±0.05	2.21±0.34	4.05±0.19	ND	ND	1.33±0.11	6.7±0.0	98.3±1.1	0.23±0.03
Leucine + S_2O_3 (40 mM)	2.23±0.23	2.22±0.29	0.34±0.03	3.58±0.14	0.67±0.22	10.60±0.4	0.57±0.14	3.23±0.21	ND	12.22±1.21	6.9±0.0	97.8±1.5	0.27±0.05
Leucine + M39	2.69±0.25	<0.05	<0.05	2.80±0.12	0.40±0.09	8.31±0.12	<0.05	ND	4.54±0.32	8.23±0.87	7.6±0.2	91.2±2.1	0.34±0.03
Valine	1.98±0.25	<0.05	1.22±0.21	4.19±0.12	1.51±0.45	0.34±0.02	6.38±0.40	ND	ND	2.54±0.21	6.4±0.2	100.0±0.0	0.23±0.05
Valine + S_2O_3 (40 mM)	2.09±0.16	<0.05	0.43±0.18	4.02±0.20	12.50±0.87	0.63±0.09	0.30±0.15	3.44±0.23	ND	14.43±0.98	6.9±0.1	83.6±0.8	0.23±0.04
Valine + M39	3.49±0.11	<0.05	0.10±0.02	1.87±0.12	7.96±0.45	0.77±0.10	<0.05	ND	4.39±0.22	8.43±1.10	7.8±0.1	89.0±1.2	0.24±0.05

* Carbon balances are calculated by subtracting values of end products from controls. In case of threonine, carbon balances were not calculated since degradation pathways are unknown. In case of serine and the BCAA the carbon balances were calculated assuming that 1 CO_2 is produced in the degradation pathways.

Supplementary Table 2 - Influence of pH on the fermentation of isoleucine (20 mM) and thiosulfate (20 mM) by *Thermoanaerobacter* strain AK85 after 14 days of cultivation. Values represent the average of triplicate measures ± standard deviation.

	Analyte (mmol/L)											
Initial pH	Ethanol	2-methyl-1-butanol	Acetate	2-methyl-1-butyrate	H_2	H_2S	S_2O_3	Ile	Ile degraded (%)	Carbon balance (%)	OD	pH
4.0	0.51±0.10	2.34±0.90	3.34±0.39	4.34±0.68	0.82±0.27	3.04±0.21	5.34±0.37	11.85±0.83	40.8	92.7	0.05±0.00	4.3±0.2
4.5	2.17±0.24	8.40±1.57	2.57±0.24	6.64±1.31	1.16±0.34	2.68±0.33	<0.50	5.34±1.01	73.3	101.9	0.13±0.01	4.9±0.1
5.0	3.78±0.13	10.44±1.04	2.66±0.34	7.01±1.47	1.70±0.45	2.48±0.39	<0.50	1.83±0.43	90.9	96.4	0.17±0.03	5.2±0.0
5.5	3.89±0.32	12.19±0.47	2.41±0.12	7.46±1.24	1.27±0.48	2.02±0.45	<0.50	0.57±0.32	97.2	101.1	0.17±0.02	5.8±0.1
6.0	4.41±0.34	11.34±1.78	2.67±0.27	7.91±1.38	1.43±0.27	2.37±0.41	<0.50	1.68±0.71	91.6	104.7	0.18±0.01	6.6±0.2
6.5	4.36±0.27	11.24±0.84	2.57±0.21	7.68±0.98	1.07±0.17	2.47±0.20	<0.50	1.24±0.50	93.8	100.8	0.19±0.02	6.8±0.0
7.0	4.62±0.17	13.47±1.23	2.47±0.16	7.81±1.77	1.24±0.37	2.33±0.31	<0.50	0.89±0.36	95.6	110.9	0.19±0.01	7.3±0.1
7.5	4.67±0.16	12.47±0.44	2.85±0.07	7.61±0.64	0.78±0.31	2.46±0.13	<0.50	1.51±0.33	92.5	108.0	0.21±0.02	7.6±0.1
8.0	4.40±0.24	11.78±1.32	2.29±0.18	6.67±1.34	1.01±0.37	2.64±0.37	<0.50	3.53±0.85	82.4	109.9	0.13±0.05	8.2±0.1
8.5	3.87±0.20	12.10±0.89	2.07±0.42	6.70±1.37	1.23±0.47	3.71±0.25	<0.50	3.34±0.91	83.3	110.7	0.12±0.03	8.8±0.0
9.0	3.01±0.13	6.01±1.67	1.37±0.24	4.78±1.59	0.72±0.22	4.21±0.36	<0.50	12.21±2.87	39.0	115.0	0.09±0.01	9.3±0.1

Supplementary Table 3 - Influence of temperature on the fermentation of isoleucine (20 mM) and thiosulfate (20 mM) by *Thermoanaerobacter* strain AK85 after 14 days of cultivation. Values represent the average of triplicate measures ± standard deviation.

	Analyte (mmol/L)											
Temp (°C)	Ethanol	2-methyl-1-butanol	Acetate	2-methyl-1-butyrate	H_2	H_2S	S_2O_3	Ile	Ile degraded (%)	Carbon balance (%)	OD	pH
50	3.07±0.10	6.37±0.17	1.87±0.43	5.02±1.76	0.27±0.17	0.87±0.33	<0.50	14.27±2.57	28.7	128.3	0.08±0.01	7.2±0.1
55	3.78±0.13	10.44±1.04	2.60±0.35	6.99±0.83	0.43±0.21	1.78±0.27	<0.50	5.55±0.54	72.3	114.9	0.09±0.02	7.3±0.2
60	4.67±0.16	12.21±0.28	2.48±0.17	7.63±0.49	0.78±0.31	2.57±0.53	<0.50	1.30±0.41	93.5	105.7	0.18±0.03	7.4±0.0
65	4.61±0.26	12.47±0.44	2.85±0.12	7.67±0.64	1.03±0.28	2.46±0.13	<0.50	1.84±0.27	90.8	109.9	0.20±0.02	7.3±0.1
70	4.35±0.37	12.34±0.47	2.67±0.21	7.91±0.24	1.45±0.39	3.71±0.22	<0.50	2.51±0.47	87.5	113.8	0.20±0.02	7.4±0.0
75	4.11±0.24	11.87±0.87	2.47±0.34	8.07±0.37	1.21±0.21	1.56±0.46	<0.50	3.29±0.34	83.6	116.2	0.16±0.01	7.3±0.2
80	1.78±0.13	5.41±1.04	2.66±0.30	3.17±0.71	0.14±0.07	1.86±0.17	<0.50	15.28±1.24	23.6	119.3	0.10±0.02	7.4±0.1
85	0.34±0.10	1.11±0.47	2.47±0.22	1.13±0.33	0.00±0.00	0.17±0.10	<0.50	19.55±0.57	2.3	109.0	0.05±0.01	7.2±0.2

Supplemental Table 4 - Influence of liquid-gas phase ratio concentration on the fermentation of isoleucine (20 mM) and thiosulfate (20 mM) by *Thermoanaerobacter* strain AK85. Values represent the average of triplicate measures ± standard deviation.

	Analyte (mmol/L)											
L-G	Ethanol	2-methyl-1-butanol	Acetate	2-methyl-1-butyrate	H_2	H_2S	S_2O_3	Ile	Ile degraded (%)	Carbon balance (%)	OD	pH
0.05	3.32±0.15	8.41±0.51	1.99±0.04	12.25±0.14	0.47±0.11	1.20±0.02	<0.50	0.56±0.17	97.2	106.1	0.22±0.02	7.3±0.2
0.34	4.07±0.40	10.87±0.66	2.38±0.21	7.31±0.56	1.01±0.35	1.88±0.47	<0.50	0.64±0.27	96.8	94.1	0.24±0.03	7.1±0.1
0.98	4.62±0.17	13.47±1.23	2.47±0.16	7.81±1.77	1.24±0.37	2.33±0.31	<0.50	0.79±0.14	96.1	110.4	0.19±0.01	7.4±0.1
2.08	4.27±0.44	12.17±0.87	2.55±0.27	8.24±0.48	1.47±0.28	2.78±0.27	<0.50	0.23±0.05	98.9	103.2	0.19±0.01	7.2±0.1
5.4	2.89±0.09	6.17±1.33	2.79±0.20	8.62±0.28	0.34±0.02	3.32±0.75	<0.50	2.15±0.43	89.3	84.7	0.17±0.01	7.3±0.0

Supplemental Table 5 - Influence of thiosulfate concentration on the fermentation of isoleucine (20 mM) by *Thermoanaerobacter* strain AK85 after 14 days of cultivation. Values represent the average of triplicate measures ± standard deviation.

	Analyte (mmol/L)											
[S_2O_3] (mM)	Ethanol	2-methyl-1-butanol	Acetate	2-methyl-1-butyrate	H_2	H_2S	S_2O_3	Ile	Ile degraded (%)	Carbon balance (%)	OD	pH
0	2.39±0.37	1.70±0.56	1.82±0.08	3.23±0.01	2.40±0.18	2.33±0.31	<0.50	11.14±2.74	44.3	80.3	0.19±0.02	7.2±0.1
10	3.96±0.57	4.93±1.05	1.93±0.05	3.16±0.26	1.52±0.35	2.35±2.16	<0.50	10.41±0.68	48.0	92.5	0.20±0.01	7.3±0.1
20	4.53±0.13	13.24±1.14	2.34±0.12	7.49±0.22	1.14±0.24	2.33±0.31	<0.50	0.78±0.24	96.1	107.5	0.20±0.09	7.1±0.0
30	4.01±0.24	8.47±0.74	2.81±0.20	10.17±0.34	0.67±0.31	2.56±0.47	<0.50	0.25±0.07	98.8	94.5	0.22±0.05	7.4±0.2
40	2.75±0.16	6.55±0.70	3.07±0.23	9.10±1.11	0.86±0.24	2.69±0.87	<0.50	1.49±0.18	92.6	85.7	0.21±0.02	7.3±0.1
50	2.46±0.06	5.40±0.51	3.40±0.24	11.07±1.40	0.21±0.14	4.01±0.44	<0.50	0.76±0.05	96.2	86.2	0.25±0.04	7.6±0.3
60	2.20±0.32	4.84±0.72	3.70±0.28	12.40±0.77	0.19±0.04	4.86±0.49	<0.50	0.25±0.11	98.7	87.5	0.26±0.07	7.5±0.2

Supplemental Table 6 - Influence of liquid-gas phase ratio and initial thiosulfate concentration on the fermentation of isoleucine (20 mM) by *Thermoanaerobacter* strain AK85. Values represent the average of triplicate measures ± standard deviation.

Substrate	Thiosulfate (mM)	L-G	Analyte (mmol/L)								% AA degraded	Carbon balance (%)	OD	pH
			Ethanol	2-methyl-1-butanol	Acetate	2-methyl-1-butyrate	H_2	H_2S	S_2O_3	Ile				
Control (YE)	0	0.05	2.71 ±0.23	<0.50	2.13±0.20	<0.50	0.54±0.17	<0.50	ND	ND	ND	ND	0.17±0.01	6.9±0.1
	10	0.05	3.53±0.34	<0.50	3.00±0.17	<0.50	0.46±0.08	<0.50	<0.50	ND	ND	ND	0.21±0.01	7.1±0.2
	20	0.05	3.66±0.26	<0.50	2.18±0.27	<0.50	0.47±0.11	<0.50	<0.50	ND	ND	ND	0.22±0.02	7.2±0.1
	40	0.05	2.49±0.10	<0.50	2.72±0.14	<0.50	0.35±0.17	<0.50	<0.50	ND	ND	ND	0.39±0.04	6.8±0.1
	0	0.98	2.13 ±0.37	<0.50	1.63±0.04	<0.50	2.40±0.18	<0.50	ND	ND	ND	ND	0.19±0.02	7.0±0.1
	10	0.98	3.62±0.12	<0.50	2.18±0.23	<0.50	1.52±0.35	<0.50	<0.50	ND	ND	ND	0.20±0.01	6.8±0.0
	20	0.98	3.76±0.28	<0.50	3.26±0.19	<0.50	1.14±0.24	<0.50	<0.50	ND	ND	ND	0.19±0.01	6.7±0.1
	40	0.98	3.10±0.34	<0.50	3.37±0.27	<0.50	0.86±0.24	<0.50	<0.50	ND	ND	ND	0.21±0.04	6.7±0.2
	0	5.4	3.97 ±0.28	<0.50	1.93±0.14	<0.50	2.27±0.00	<0.50	ND	ND	ND	ND	0.16±0.03	6.9±0.1
	10	5.4	4.03±0.24	<0.50	2.45±0.26	<0.50	1.05±0.20	<0.50	<0.50	ND	ND	ND	0.15±0.02	6.8±0.1
	20	5.4	3.15±0.25	<0.50	2.61±0.26	<0.50	0.34±0.02	<0.50	<0.50	ND	ND	ND	0.17±0.02	6.8±0.3
	40	5.4	2.9±0.16	<0.50	3.45±0.17	<0.50	1.12±0.20	<0.50	<0.50	ND	ND	ND	0.14±0.0	6.6±0.2
Isoleucine														
	0	0.05	2.93 ±0.23	2.90±0.06	1.84±0.01	3.04±1.01	0.54±0.17	<0.50	<0.50	14.24±1.32	26.4	103.3	0.17±0.01	7.2±0.1
	10	0.05	3.72±0.08	11.56±0.67	2.00±0.05	5.44±0.12	0.46±0.08	0.61±0.34	<0.50	2.85±0.43	85.8	99.3	0.21±0.01	7.6±0.2
	20	0.05	3.32±0.15	8.41±0.51	1.99±0.04	4.28±0.14	0.47±0.11	2.33±0.31	<0.50	6.21±0.63	69.00	94.5	0.22±0.02	7.5±0.2
	40	0.05	2.71±0.07	6.93±0.42	2.74±0.31	10.29±1.60	0.35±0.17	2.69±0.87	<0.50	1.85±0.43	90.8	95.3	0.39±0.04	7.6±0.1
	0	0.98	2.39 ±0.37	2.70±1.11	1.82±0.08	3.23±0.01	2.40±0.18	<0.50	<0.50	13.35±2.17	24.4	105.3	0.19±0.02	7.0±0.2
	10	0.98	3.96±0.57	4.93±1.05	1.93±0.05	3.16±0.26	1.52±0.35	2.25±0.14	<0.50	12.22±1.01	68.9	101.5	0.20±0.01	7.4±0.2
	20	0.98	4.53±0.13	13.24±1.14	2.34±0.12	7.49±0.22	1.14±0.24	2.54±1.10	<0.50	0.51±0.22	97.5	106.2	0.19±0.01	7.6±0.1
	40	0.98	2.75±0.16	6.55±0.70	3.07±0.23	9.10±1.11	0.86±0.24	1.36±0.59	<0.50	2.14±0.09	89.3	88.9	0.21±0.04	7.4±0.0
	0	5.4	4.34 ±0.48	2.54±0.46	1.87±0.05	4.44±1.51	2.27±0.00	<0.50	<0.50	14.35±0.47	28.3	106.7	0.16±0.03	7.2±0.2
	10	5.4	3.84±0.54	9.35±1.07	2.06±0.01	4.74±0.58	1.05±0.20	3.06±0.83	<0.50	4.87±0.38	75.7	94.8	0.15±0.02	7.7±0.2
	20	5.4	2.89±0.09	5.05±1.33	2.79±0.20	8.62±0.28	0.34±0.02	1.36±0.04	<0.50	0.91±0.23	95.5	72.9	0.17±0.02	7.7±0.1
	40	5.4	2.59±0.04	5.31±1.06	3.10±0.28	9.91±0.16	1.12±0.20	2.98±0.87	<0.50	3.27±0.44	83.7	92.4	0.14±0.0	7.8±0.2

Table 7 - Fermentation kinetics of valine (20 mM) in the presence of thiosulfate (20 mM) by *Thermoanaerobacter* strain AK85. Values represent the average of triplicate measures ± standard deviation.

Time (h)	Analyte (mmol/L)							% AA degraded	Carbon balance (%)	OD
	Ethanol	2-methyl-1-propanol	Acetate	2-methyl-1-propionate	H_2	S_2O_3	Val			
4	3.37± 1.35	0.00± 0.00	2.36± 1.59	0.06±0.00	0.32±0.02	20.00±0.00	20.00±0.00	0.0	100.8	0.35±0.09
8	3.54± 0.28	0.00± 0.00	1.10± 0.03	0.15±0.19	0.45±0.13	18.53±1.01	20.00±0.00	0.0	101.5	0.36±0.04
12	5.87± 1.62	0.23± 0.01	1.15± 0.10	0.07±0.01	1.07±0.28	16.49±0.77	20.00±0.00	0.0	102.5	0.41±0.02
18	6.76± 1.84	0.26± 0.01	1.69± 0.13	0.24±0.04	1.14±0.22	13.61±0.75	20.00±0.00	0.0	103.8	0.45±0.04
24	7.43± 0.24	0.29± 0.03	1.97± 0.17	0.47±0.06	1.59±0.40	5.15±0.78	20.00±0.00	0.0	102.6	0.51±0.12
30	7.19± 0.27	0.31± 0.02	1.91±0.06	0.80±0.16	1.44±0.18	0.23±0.04	19.41±1.14	3.0	99.7	0.34±0.06
36	6.90± 0.58	0.33± 0.03	1.97±0.08	1.03±0.17	2.48±0.37	0.00± 0.00	18.58±1.17	7.1	101.2	0.33±0.07
48	6.58± 0.30	0.37± 0.07	2.04±0.16	1.84±0.40	2.23±0.63	0.00± 0.00	18.03±1.21	9.8	96.6	0.32±0.08
60	7.39± 0.72	0.54± 0.09	2.05±0.06	2.53±0.32	1.94±0.31	0.00± 0.00	16.25±1.04	18.8	100.5	0.34±0.05
72	7.42± 0.06	0.54± 0.11	2.08±0.14	3.78±0.68	2.31±0.49	0.00± 0.00	15.78±0.51	21.1	96.9	0.31±0.10
120	3.00± 0.10	1.47± 0.11	2.37±0.19	9.46±1.03	1.83±0.42	0.00± 0.00	8.44±1.41	57.8	101.0	0.22±0.03
168	1.54± 0.12	4.34± 0.24	3.78±0.15	14.37±0.82	1.07±0.37	0.00± 0.00	1.49±0.32	92.6	100.8	0.16±0.04

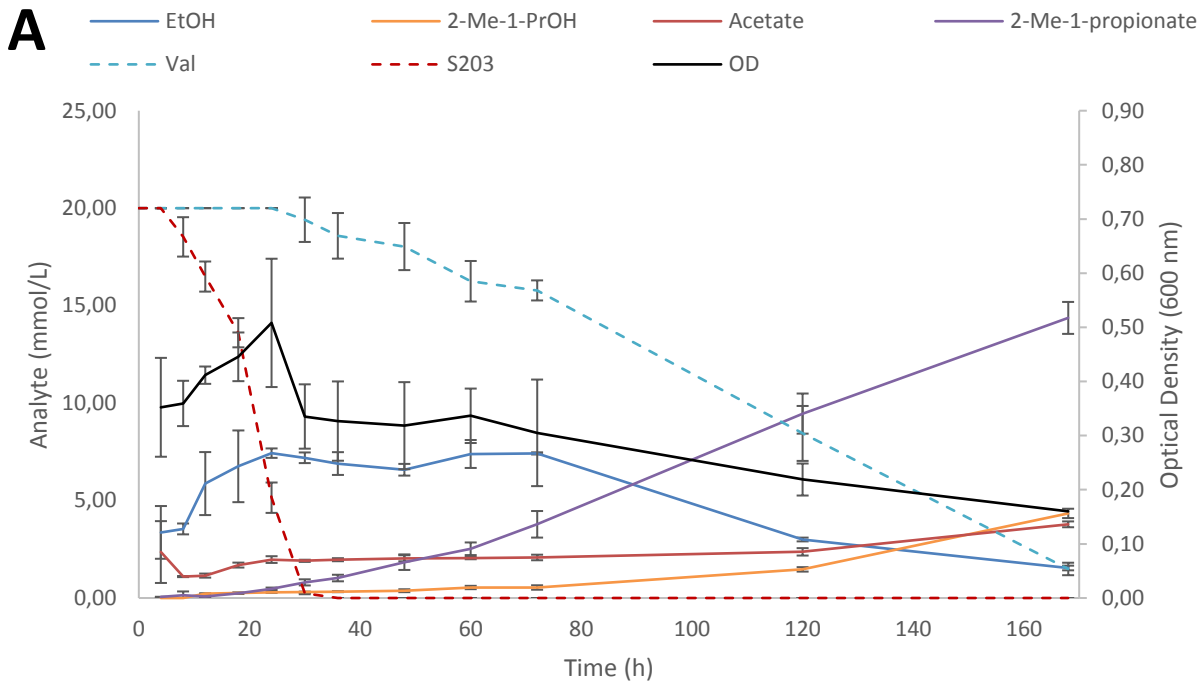
Table 8 - Fermentation kinetics of isoleucine (20 mM) in the presence of thiosulfate (20 mM) by *Thermoanaerobacter* strain AK85. Values represent the average of triplicate measures ± standard deviation.

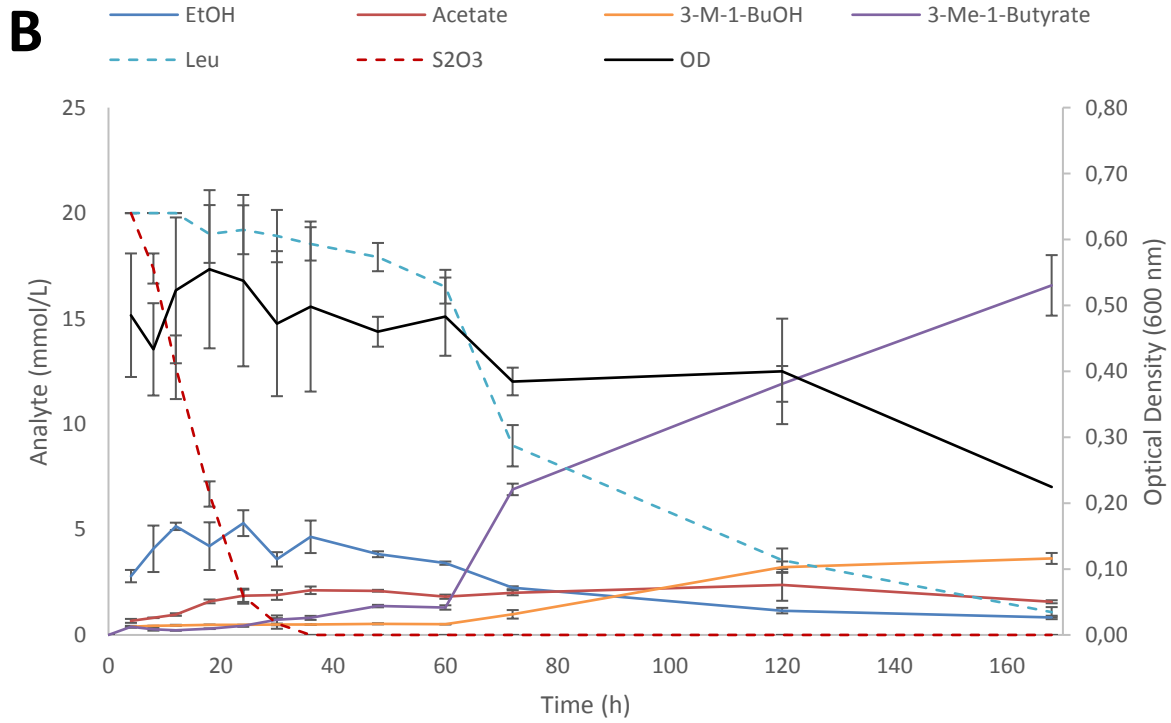
Time (h)	Analyte (mmol/L)							% AA degraded	Carbon balance (%)	OD
	Ethanol	2-methyl-1-butanol	Acetate	2-methyl-1-butyrate	H_2	S_2O_3	Ile			
4	6.96± 1.01	0.00± 0.00	0.69±0.19	0.19±0.04	0.17±0.05	19.17±0.57	20.00±0.00	0.0	100.9	0.29± 0.04
8	3.27± 1.22	0.00± 0.00	0.84±0.02	0.17±0.02	0.63±0.19	18.27±1.31	20.00±0.00	0.0	100.9	0.30±0.08
12	4.92± 0.19	0.00± 0.00	1.40±0.19	0.29±0.15	1.43±0.21	13.34±1.13	20.00±0.00	0.0	101.5	0.34± 0.06
18	4.22± 0.99	0.00± 0.00	1.79±0.16	0.32±0.02	1.72±0.43	13.01±1.36	20.00±0.00	0.0	101.6	0.39± 0.05
24	5.16± 0.27	0.00± 0.00	1.94±0.19	0.49±0.10	1.63±0.38	11.29±0.41	20.00±0.00	0.0	102.5	0.42± 0.02
30	5.11± 0.34	0.55± 0.09	1.98±0.05	0.73±0.24	1.84±0.55	5.58±1.77	20.00±0.00	0.0	106.4	0.41± 0.08
36	5.51± 0.25	0.46± 0.01	2.02±0.17	0.97±0.32	2.07±0.20	0.51±0.19	20.00±0.00	0.0	107.2	0.38± 0.07
48	5.22± 0.21	0.53± 0.02	2.15±0.11	1.85±0.64	2.43±0.42	0.00± 0.00	19.37±1.25	3.1	108.8	0.37± 0.05
60	5.44± 0.33	0.78± 0.01	2.03±0.03	1.56±0.12	1.20±0.38	0.00± 0.00	18.60±1.05	7.0	104.7	0.40± 0.09
72	5.09± 0.21	1.07± 0.03	2.28±0.23	4.05±1.01	1.40±0.23	0.00± 0.00	16.82±1.15	15.9	109.7	0.33± 0.00
120	4.84± 0.12	1.83± 0.02	2.41±0.13	10.39±0.51	1.03±0.13	0.00± 0.00	7.42±0.73	62.9	98.2	0.38± 0.05
168	2.21± 0.07	5.62± 0.18	4.05±0.07	14.82±0.37	0.79±0.05	0.00± 0.00	1.07±0.32	94.7	107.6	0.15± 0.03

Table 9 - Fermentation kinetics of leucine (20 mM) in the presence of thiosulfate (20 mM) by *Thermoanaerobacter* strain AK85. Values represent the average of triplicate measures ± standard deviation.

Time (h)	Analyte (mmol/L)									
	Ethanol	3-methyl-1-butanol	Acetate	3-methyl-1-butyrate	H_2	S_2O_3	Leu	% AA degraded	Carbon balance (%)	OD
4	6.96±1.01	0.00± 0.00	0.69±0.19	0.19±0.04	0.34±0.12	19.17±0.57	20.00±0.00	0.0	100.9	0.49±0.09
8	3.27±1.22	0.00± 0.00	0.84±0.02	0.17±0.02	0.78±0.33	18.27±1.31	20.00±0.00	0.0	100.9	0.43±0.07
12	4.92±0.19	0.00± 0.00	1.40±0.19	0.29±0.15	1.07±0.24	13.34±1.13	20.00±0.00	0.0	101.5	0.52±0.11
18	4.22±0.99	0.00± 0.00	1.79±0.16	0.32±0.02	1.40±0.47	13.01±1.36	20.00±0.00	0.0	101.6	0.55±0.12
24	5.16±0.27	0.00± 0.00	1.94±0.19	0.49±0.10	1.66±0.23	11.29±0.41	20.00±0.00	0.0	102.5	0.54±0.13
30	5.11±0.34	0.55±0.09	1.98±0.05	0.73±0.24	1.83±0.56	5.58±1.77	20.00±0.00	0.0	106.4	0.47±0.11
36	5.51±0.25	0.46±0.01	2.02±0.17	0.97±0.32	2.25±0.38	0.51±0.19	20.00±0.00	0.0	107.2	0.50±0.13
48	5.22±0.21	0.53±0.02	2.15±0.11	1.85±0.64	2.41±0.47	0.00± 0.00	19.37±1.25	3.1	108.8	0.46±0.02
60	5.44±0.33	0.78±0.01	2.03±0.03	1.56±0.12	2.87±0.69	0.00± 0.00	18.60±1.05	7.0	104.7	0.48±0.06
72	5.09±0.21	1.07±0.03	2.28±0.23	4.05±1.01	1.63±0.43	0.00± 0.00	16.82±1.15	15.9	109.7	0.38±0.02
120	4.84±0.12	1.83±0.02	2.41±0.13	10.39±0.51	1.28±0.18	0.00± 0.00	7.42±0.73	62.9	98.2	0.40±0.08
168	2.21±0.07	5.62±0.18	4.05±0.07	14.82±0.37	1.32±0.21	0.00± 0.00	1.07±0.32	94.7	107.6	0.22±0.08

Supplementary Figure 1 – Fermentation kinetics of *Thermoanaerobacter* strain AK85 grown on of 20 mM Valine (A), 20 mM Leucine (B) in the presence of thiosulfate (20 mM).





V

Branched-chain amino acid catabolism of *Thermoanaerobacter pseudoethanolicus* reveals potential route to branched-chain alcohol formation

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ABSTRACT

The bioprocessing of branched-chain amino acids (BCAA) to branched-chain fatty acids (BCFA) and branched-chain alcohols (BCOH) are described using *Thermoanaerobacter pseudoethanolicus* (DSM 2355). The BCAA were not degraded to any extent without electrons scavenging system, but were degraded to a mixture of their BCFA and BCOH when thiosulfate was added to the culture. The amount of the acid was always considerable higher than the alcohol. Various environmental parameters on BCAA metabolism using isoleucine as a model compound were investigated (pH, liquid-gas phase ratio, and initial thiosulfate concentrations) and the major outcome showed that by increasing the partial pressure of hydrogen through using higher liquid-gas phase ratios formation with 2-methyl-1-butanol achieving a maximal titer of 3.4 mM at a 1:1 liquid-gas phase ratio. Alternately, increasing the thiosulfate concentration shifting end product formation in favor of the more oxidized produce (BCFA) and decreased the amount of the BCOH. Kinetic monitoring of BCAA degradation revealed that the formation of BCOH occurs slowly after the onset of BCFA formation. ^{13}C-labelled studies of leucine confirmed the production of a mixture of 3-methyl-1-butyrate and 3-methyl-1-butanol from leucine in the presence of thiosulfate and also showed that leucine could be degraded without addition of thiosulfate if a volatile fatty acid was present. Finally, enzymatic assays showed that alcohol dehydrogenase and aldehyde oxidoreductase activities for C2-C6 substrates are present on leucine grown cells supplemented with thiosulfate supporting their roles in BCOH formation. Thus, the role of carboxylic acid reduction is likely of importance in the production of BCOH formation during the degradation of BCAA such as leucine.

Introduction

The capability of *Clostridium* and closely related species to degrade proteins and amino acids has been well-documented, particularly among the pathogenic *Clostridia*, (Andreesen et al., 1989; Mitchell, 2001; Reid & Stutz, 2005). Studies of thermophilic *Clostridia* have been limited to *Caloramator* strains such as *Caloramator fervidus* and *Caloramator proteoclasticus* (Speelmans, de Vrij, & Konings, 1989; Tarlera et al., 1997) and to a lesser extent, *Thermoanaerobacter* species (Fardeau et al., 1997; Faudon et al., 1995; Scully & Orlygsson, 2014, 2015a; Scully, Iloranta, Myllymaki, & Orlygsson, 2015). Understanding the amino acid and protein metabolism, as well as the need for associated electron acceptors such as thiosulfate, is important for understanding the role of specific organisms in nutrient cycling in an environment as well developing knowledge of how such substrates are catabolized during bioprocessing.

Under anaerobic conditions, the catabolism of many amino acids is not thermodynamically favorable unless an electron scavenging system is used. For instance, the $\Delta G°'$ values for the degradation of branched-chain amino acids (BCAAs) to their corresponding branched-chain fatty acids (BCFAs) ranges from + 4.2 to + 9.7 kJ/mol. Degradation of BCAAs occurs in several steps, the first being oxidative deamination of the amino acid to its corresponding keto acid (and $NH_4^+ + H_2$). The keto acid is further oxidatively decarboxylated to its corresponding aldehyde (and $CO_2 + H_2$) which is finally converted to its corresponding fatty acid. The initial step (deamination) for the three BCAA is the bottleneck from thermodynamic point of view, $\Delta G°'$ values ranging from + 51.5 to 57.0 (Orlygsson, Houwen, & Svensson, 1995). Thus, oxidative deamination of the BCAA cannot occur unless the reducing equivalents produced are scavenged. This can occur when the amino acid degrading bacteria is co-cultivated in the presence of hydrogen scavenging organism, e.g. hydrogenotrophic methanogen or sulfate reducing bacteria or by adding external electron acceptor (e.g. thiosulfate) to the culture, in case the amino acid degrading bacteria can use it (Gottschalk, 1986; McInerney, 1988).

Early work on the ability of *Thermoanaerobacter* species demonstrated that the BCAAs are degraded only in the presence of external electron acceptor (e.g. thiosulfate) or under methanogenic conditions (in a co-culture with a hydrogenotrophic methanogen) to yield the corresponding, one carbon shorter BCFA (Fardeau et al., 1997; Faudon et al., 1995). Recent studies on the amino acid catabolism of *Thermoanaerobacter* and *Caldanaerobacter* species demonstrates that the degradation of BCAA yields a mixture of both BCFA and branched-chain alcohol (BCOH) when thiosulfate is used as an electron scavenging system (Scully, et al., 2015; Scully & Orlygsson, 2014, 2015). Thus, valine is degraded to a mixture of 2-methyl-1-propionate and 2-methyl-1-propanol, isoleucine to 2-methyl-1-butyrate and 2-methyl-1-butanol, and leucine to 3-methyl-1-butyrate and 3-methyl-1-butanol. This has recently been shown by *Thermoanaerobacter* strain AK85 producing a mixture of BCFAs and BCOHs from their corresponding BCAAs when thiosulfate was used as an electron scavenger (Scully and Orlygsson, 2019; **Paper IV**). Interestingly, the marine anaerobe *Spirochaeta isovalerica* also produces detectable quantities of BCOHs from BCAAs (Harwood & Canale-Parola, 1981, 1983).

Beyond the ability of *Thermoanaerobacter* species to produce ethanol from carbohydrates, the genus has other aspects that are of biotechnological potential such as a number of thermostable enzyme systems. For instance, the amylases, pectinases, and xylanases of *Thermoanaerobacter* species have been scrutinized (Kozianowski et al., 1997; Lee, Saha, & Zeikus, 1990; Xue et al., 2001) as well as their highly-selective secondary alcohol dehydrogenase (SADH) from *T. pseudoethanolicus* has been widely explored (Bsharat et al., 2017; Hitschler et al., 2018; Musa et al., 2018; Musa & Phillips, 2011). Recent work by our group has also demonstrated that *T. pseudoethanolicus* can reduce carboxylic acids to their corresponding alcohols (Scully, Brown, Ross, & Orlygsson, 2019; Scully and Orlygsson, 2019) while work by (Hitschler et al., 2018) found that *Thermoanaerobacter* strains containing an aldehyde:ferredoxin oxidoreductase gene which is likely involved in carboxylic acid reduction and may explain the highly ethanologenic nature of some *Thermoanaerobacter* species. This phenomenon seems to be at least partially responsible for the formation of BCOHs during the catabolism of BCAAs by *Thermoanaerobacter* strain AK85 (**Paper IV**).

The present study focuses on the BCAA catabolism of the well-studied *T. pseudoethanolicus* with an emphasis on the impact of culture conditions on BCFA and BCOH formation. Of particular interest is the role of carboxylic acid reduction in the formation of BCOH formation during BCAA metabolism.

2 MATERIALS AND METHODS

2.1 Bacterial strains and Culture conditions

Thermoanaerobacter pseudoethanolicus (DSM 2355) was acquired from DSMZ and cultivated in basal mineral (BM) medium which was prepared as previously described (Chades et al., 2018). All experiments were performed at 65°C and pH 7.0 without agitation and at liquid-gas phase ratio of 1:1 unless noted otherwise. The inoculum volume was 2% (v/v) from the exponential growth phase of stock cultures grown on glucose (20 mM). Substrates solutions were added to culture media after autoclaving (121°C for 60 minutes) through a syringe filter (Whatman PES, 0.45 µm). Cultivation experiments were performed in triplicate.

2.2 Influence of Culture conditions on BCAA Fermentation

The ability of *T. pseudoethanolicus* to utilize the BCAA (20 mM) in the absence or presence of thiosulfate (40 mM) was performed. The samples were grown for 7 days, at which time, liquid (1 mL) and gas (0.2 mL) samples were withdrawn and the end products analyzed.

2.2.1 Liquid-gas phase experiment

The strain was cultivated in BM containing 20 mM of isoleucine with the addition of 20 mM sodium thiosulfate in 117.5 mL serum bottles. Five different liquid-gas (L-G) phases were used; 0.05, 0.34, 0.98, 2.08, and 5.40. Cultivations were carried out for 14 days.

2.2.2 Influence of Initial pH

To investigate the influence of initial pH on isoleucine fermentation, BM medium with initial pH values between pH 4.0 and 9.0 in 0.5 unit increments, obtained by titration with HCl or NaOH, were prepared in Hungate tubes (16x150 mm) with a L-G ratio of 1:1. 20 mM isoleucine was used as the carbon source with the addition of thiosulfate (20 mM); the tubes were incubated for 14 days.

2.2.3 Effect of initial thiosulfate concentration

The impact of cultivation of isoleucine (20 mM) in varying concentrations of sodium thiosulfate was examined at concentrations ranging from 0 mM to 60 mM of thiosulfate. Experiments were performed at pH 7.0 as otherwise described in the previous section for 14 days.

2.3 Kinetic Experiments

Kinetic experiments on the fermentation of valine, leucine, and isoleucine (20 mM) in the presence of thiosulfate (20 mM) was were performed in 125 mL serum bottles with a L-G of 1:1 at 65°C. Periodically, 1 mL samples were taken for the analysis of soluble analyses and 0.2 mL of headspace gas was removed for analysis of hydrogen.

2.4 ^{13}C NMR Experiments

T. pseudoethanolicus was cultivated in the 20 mM ^{13}C2 leucine with and without the addition of 40 mM thiosulfate for 5 days as previously described (**Paper IV**). Additionally, the bacterium was cultivated with ^{13}C1 3-methyl-1-butyrate with and without the addition of an electron donor (leucine or glucose, 20 mM) and also with leucine, ^{13}C1 3-methyl-1-butyrate and thiosulfate. ^{13}C NMR spectra were obtained using a Bruker ^{13}C NMR spectra were obtained on a Bruker AV400 at 298K after spiking with D_2O to obtain a signal lock as previously described (**Paper IV**).

2.5 Analytical methods

Hydrogen was analyzed by GC-TCD as described by (Orlygsson & Baldursson, 2007). Alcohols and volatile fatty acids were measured by gas chromatography using a Perkin-Elmer Clarus 580 gas chromatograph equipped with flame ionization detector (FID) as previously described (Orlygsson & Baldursson, 2007). Hydrogen sulfide was analyzed according to the method described (Cline, 1969). Thiosulfate was analyzed according to Westley (Westley, 1987) modified for use in microplates. Sulfur was detected by microscopic examination. Optical density was determined using a Shimadzu UV-1800 with a 1 cm pathlength at a wavelength of 600 nm. Protein was quantified according to the method of Bradford (1976) with modifications as previously described (**Paper IV**).

2.6 Enzyme assays

Cells for enzymatic assays were prepared by cultivating *T. pseudoethanolicus* under two conditions: and isoleucine (20 mM) + thiosulfate (20 mM), and leucine (20 mM) supplemented with 3-methyl-1-butyrate and thiosulfate. Cells for enzyme assays were harvested from the exponential growth phase by centrifugation (4700 rpm 0-4°C) after the addition of potassium dithionite (5 mg/L) and stored at -80°C. Cells were twice washed with 50 mM Tris-HCl containing 5 mM $CaCl_2$ and 5 mg/L potassium dithionite. Cells extracts were prepared using glass beads and vortexing as previously described (Paper IV) and stored in a nitrogen-flushed serum bottle at 4°C prior to use. Procedures describing assays for oxidative alcohol dehydrogenase (ADH) and aldehyde oxidation (AOR) were performed using the NBT-based methods in microplates as described in **Paper IV**.

3 RESULTS

3.1 Fermentation of Branched-chain Amino Acids

The ability of *T. pseudoethanolicus* to utilize the BCAAs was examined either by using no electron acceptor or by the addition of 40 mM of thiosulfate. End product formation from the three BCAAs with and without the addition of thiosulfate as illustrated in Figure 1.

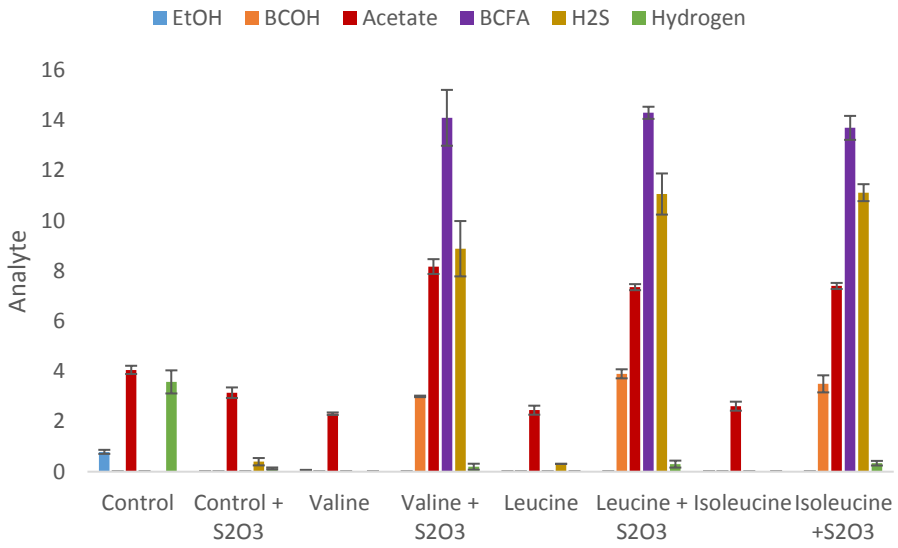

Figure 1. Fermentation of the BCAA by *T. pseudoethanolicus* with and without thiosulfate (40 mM). Values represent the average of triplicate fermentations with standard deviation represented as error bars.

The strain degraded only small quantity of the BCAAs provided when cultivated without thiosulfate resulting only traces corresponding BCFAs and corresponding BCOHs (Figure 1). During degradation of the BCAAs in the presence of thiosulfate, not only were the corresponding BCFAs produced, but also the BCOHs. Degradation of valine (18.3 mM degraded) resulted in production of a mixture of 2-methyl-1-propionate (14.1 mM) and 2-methyl-1-propanol (3.0 mM). Leucine degradation (19.0 mM) resulted in production of 3-methyl-1-butyrate (14.3 mM) and 3-methyl-1-butanol (3.9 mM), and isoleucine degradation (18.5 mM degraded) rendered 2-methyl-1-butyrate (13.7 mM) and 2-methyl-1-butanol (3.5 mM). The stoichiometry for the BCAAs degradation was as follows (controls subtracted):

1.00 Valine → 0.77 2-Methyl-1-propionate + 0.16 2-methyl-1-propanol + 0.60 H_2S

1.00 Leucine → 0.75 3-Methyl-1-butyrate + 0.20 3-methyl-1-butanol + 0.58 H_2S

1.00 Isoleucine → 0.74 + 2-Methyl-1butyrate + 0.219 2-methyl-1-butanol + 0.48 H_2S

In all cases the concentration of the acid was greater than of the alcohol.

3.2 Effect of Culture Conditions

To further examine the effect of culture conditions on the utilization of BCAAs, the strain was cultivated on 20 mM of isoleucine with 20 mM of thiosulfate unless otherwise noted.

3.2.1 Effect of pH

Only a small fraction of isoleucine was degraded at pH values below 5.5 (Figure 2). Almost complete degradation of isoleucine was degraded between pH 5.5 to 7.5 but at pH 5.0 and 8.0 – 9.0 between 50 to 80% of the amino acid was degaded. Between pH 5.5 and 8.0 the main end product was 2-methyl-1-butyrate (15.34 to 16.71 mM) but to a much lesser extent 2-methyl-1-butanol (3.45 to 4.36 mM). The ratio of 2-methyl-1-butanol to 2-methyl-1-butyrate is realtively stable from pH 6 to 8.5 with a value around 0.25.

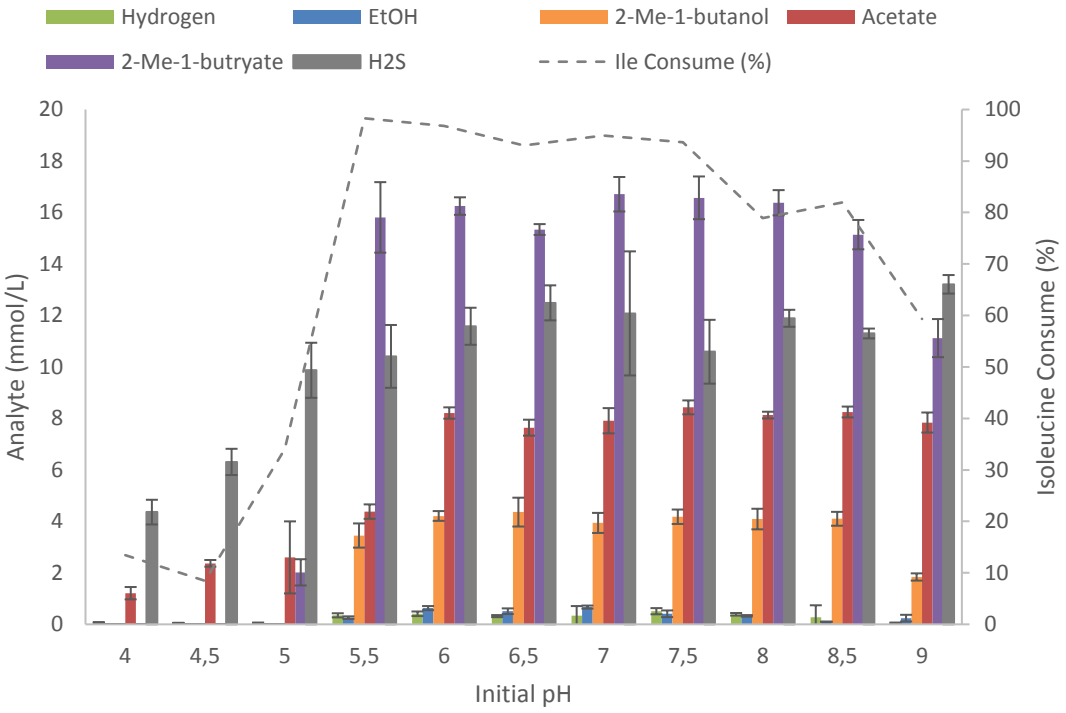

Figure 2. Influence of initial pH on end product profile of isoleucine (20 mM) fermentation with the addition of thiosulfate (20 mM). Values represent the average of triplicate fermentations with standard deviation represented as error bars.

3.2.2 Effect of L-G ratio

To determine the impact of hydrogen accumulation on isoleucine degradation using thiosulfate as an electron scavenger, *T. pseudoethanolicus* was cultivated at five different L-G phases (between 0.05 to 5.4) as displayed in Figure 3.

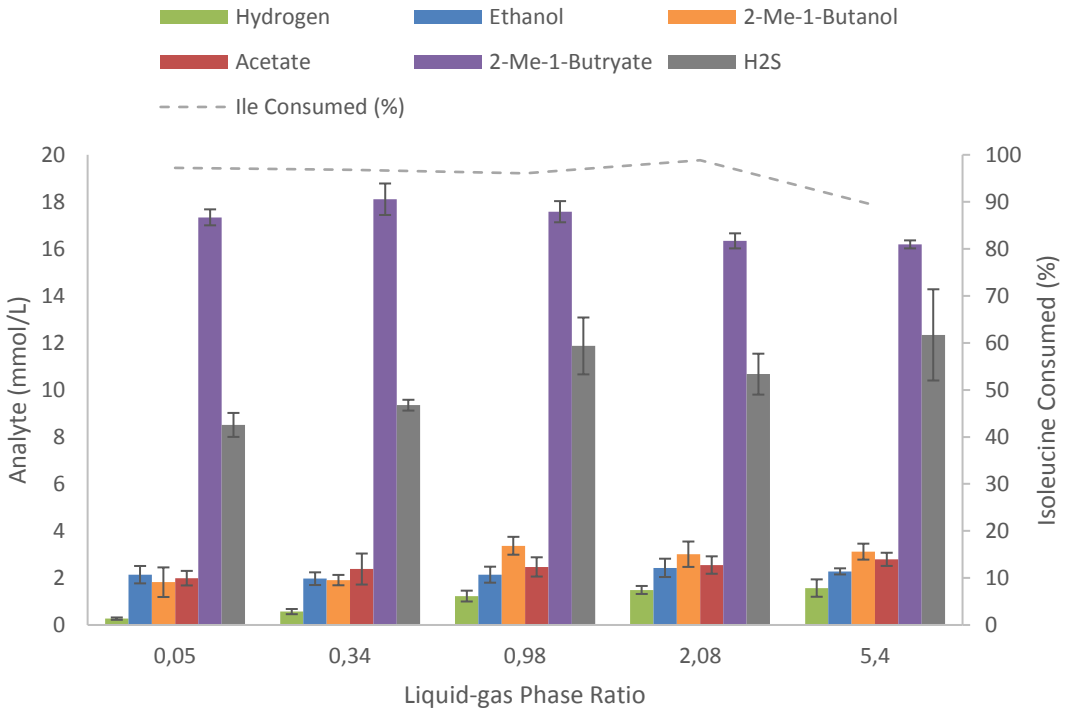

Figure 3. Influence of liquid-gas phase ratio on the fermentation of isoleucine (20 mM) by *Thermoanaerobacter pseudoethanolicus*. Values represent the average of triplicates ± standard deviation.

At all L-G ratios examined, isoleucine was almost completely consumed resulting in the formation of 2-methyl-1-butyrate as the dominant end product and lower quantities of 2-methyl-1-butanol (Figure 3). Ethanol and acetate are most likely not products from the isoleucine but from the yeast extract. The concentration of the BCFA varied between 16.19 to 18.11 mM and was higher at low L-G phases while the concentration of the BCOH varied between 1.82 to 3.37 mM (higher at high L-G phases). At the two lower L-G phases the ratio between the BCFA and BCOH was 9.5 but at the three higher L-G phases the ratio was between 5.18 to 5.42. The concentration of hydrogen at end of cultivation was very low in all cases (lowest at L-G phase of 0.05; 0.57 mmol/L and highest at L-G phase of 5.4; 1.57). The concentration of H_2S was lowest at the lowest L-G phase (8.51 mmol/L) and increased with increasing L-G phase ratios with end concentration at the highest L-G phase of 12.34 mmol/L). End concentrations of thiosulfate was always below 0.5 mM). The carbon balances ranged from 97.9 to 108.7%.

3.2.3 Effect of initial thiosulfate concentration

In order to investigate the impact of initial thiosulfate concentration on isoleucine catabolism, *T. pseudoethanolicus* was cultivated on isoleucine (20 mM) with the addition of 0 and 60 mM of thiosulfate as summarized in Figure 4.

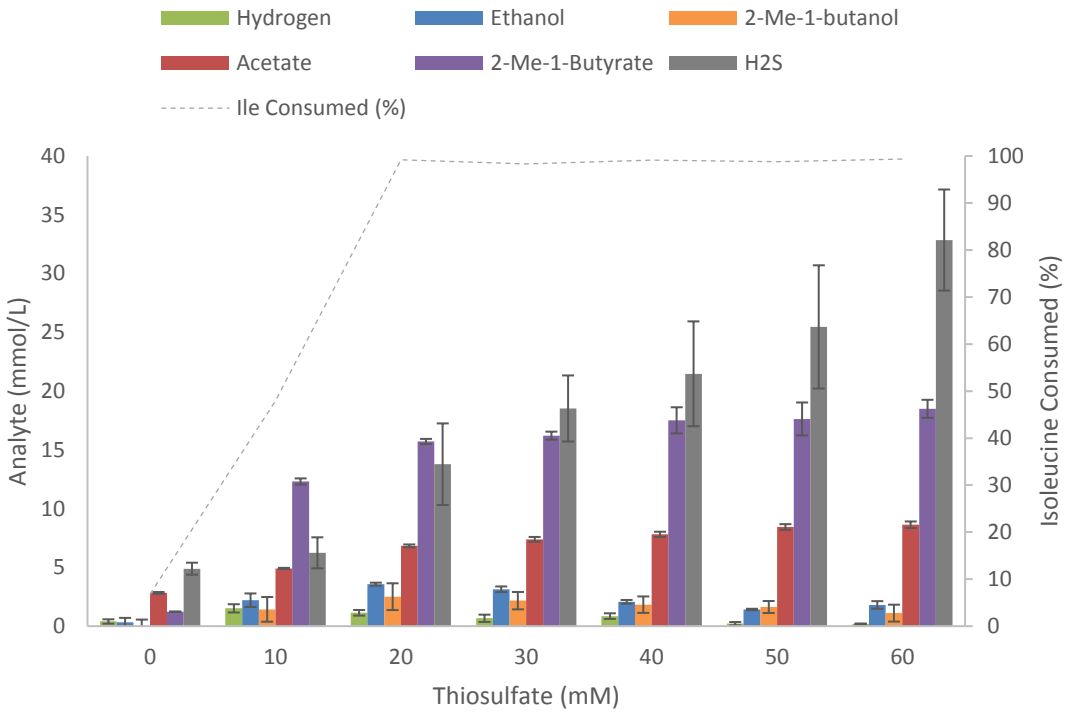

Figure 4. Influence of initial thiosulfate concentrations on end product profile of isoleucine (20 mM) fermentation. Values represent the average of triplicates ± standard deviation.

Degradation of isoleucine without any electron accepter resulted in small quantities of both 2-methyl-1-butyrate and 2-methyl-1-butanol with less than 10% of isoleucine degraded (Figure 4). The addition of 10 mM of thiosulfate increased the amount of the amino acid degraded (9.5 mM; 48%) and resulted in higher amounts of the 2-methyl-1-butyrate and 2-methyl-1-butanol, or 12.31 and 1.43 mM, respectively. By increasing the thiosulfate to 20 mM and above, isoleucine was almost completely degraded. However, at thiosulfate concentrations of 20 mM and higher, the balance of reduced to oxidized end products shifts with the ratio of the alcohols to acid shifting in favor of BCFA formation; at 20 mM thiosulfate addition the ratio of the acid over the alcohol was 6.25 but changed to 16.65 at 60 mM initial thiosulfate concentration. Not surprisingly, the concentration of hydrogen gradually decreasing with increasing thiosulfate concentrations. Concentration of H_2S varied from 4.81 (at 10 mM thiosulfate) to 32.85 mM (at 60 mM thiosulfate) and end concentration of thiosulfate was always below 0.5 mM.

3.3 Kinetic experiments

To better understand the change in BCOH/BCFA ratio over the course of BCAA fermentation, the strain was individually cultivated on all three BCAAs supplemented with thiosulfate and monitored for 7 days. During growth on leucine in the presence of 20 mM of thiosulfate, the strain reaches a maximum optical density of 0.66 after 12 h (Figure 5). However, the optical density decreases from 12 h to 24 h and starts to increase again reaching a maximum of 0.60 after 60 h in tandem with the degradation of the BCAAs and BCFA formation. Production of ethanol and acetate (from the yeast extract) also accumulates during the first 12 h while the formation of 2-methyl-1-butyrate and 2-methyl-1-butanol starts to increase after 10 h and gradually increases until end of experiment (max concentration of the acid and alcohol at 168 h is 22.7 mM and 1.07 mM, respectively). Thus, the ratio of the BCFA over the BCOH at final sampling point is 21.2. It is interesting to note that the thiosulfate added was much more rapidly degraded as compared to the amino acid degradation rate and was less than 0.5 mM after 60 h. Similarly, degradation pattern of valine and isoleucine (Supplementary Fig. 1 and 2) show similar patterns of amino acid and thiosulfate consumption and end product formation as for leucine degradation. The ratio of the BCFA/BCOH for valine and isoleucine at end of fermentation is 21.2 and 12.9, respectively.

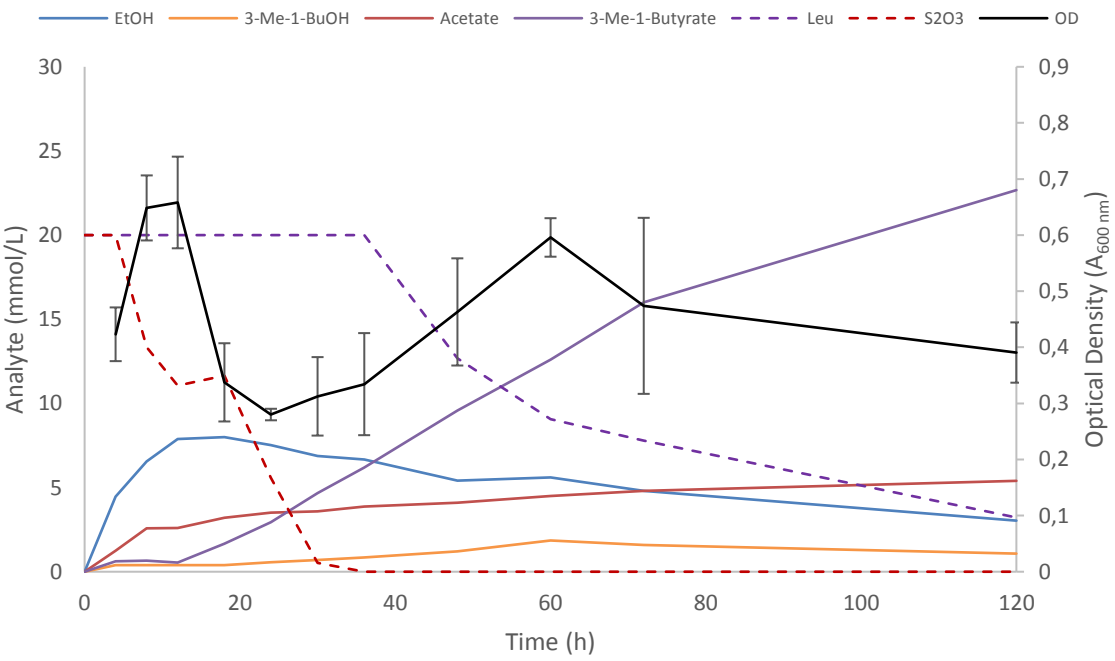

Figure 5. Fermentation kinetics of *T. pseudoethanolicus* grown on 20 mM leucine in the presence of thiosulfate (20 mM). Values represent the average of triplicates ± standard deviation.

3.4 NMR Studies with ^{13}C2-labled Substrates

The fermentation of ^{13}C2 leucine (20 mM; peak at 53.6 ppm) without (Figure 6A) and with thiosulfate (Figure 6B) was performed. In case no electron acceptor was added, no labelled end-products were produced. In the presence of thiosulfate a mixture of 3-methyl-1-butyrate and 3-methyl-1-butanol were produced as evidenced by peaks at 183.5 ppm and 60.3 ppm, respectively (Figure 6B).

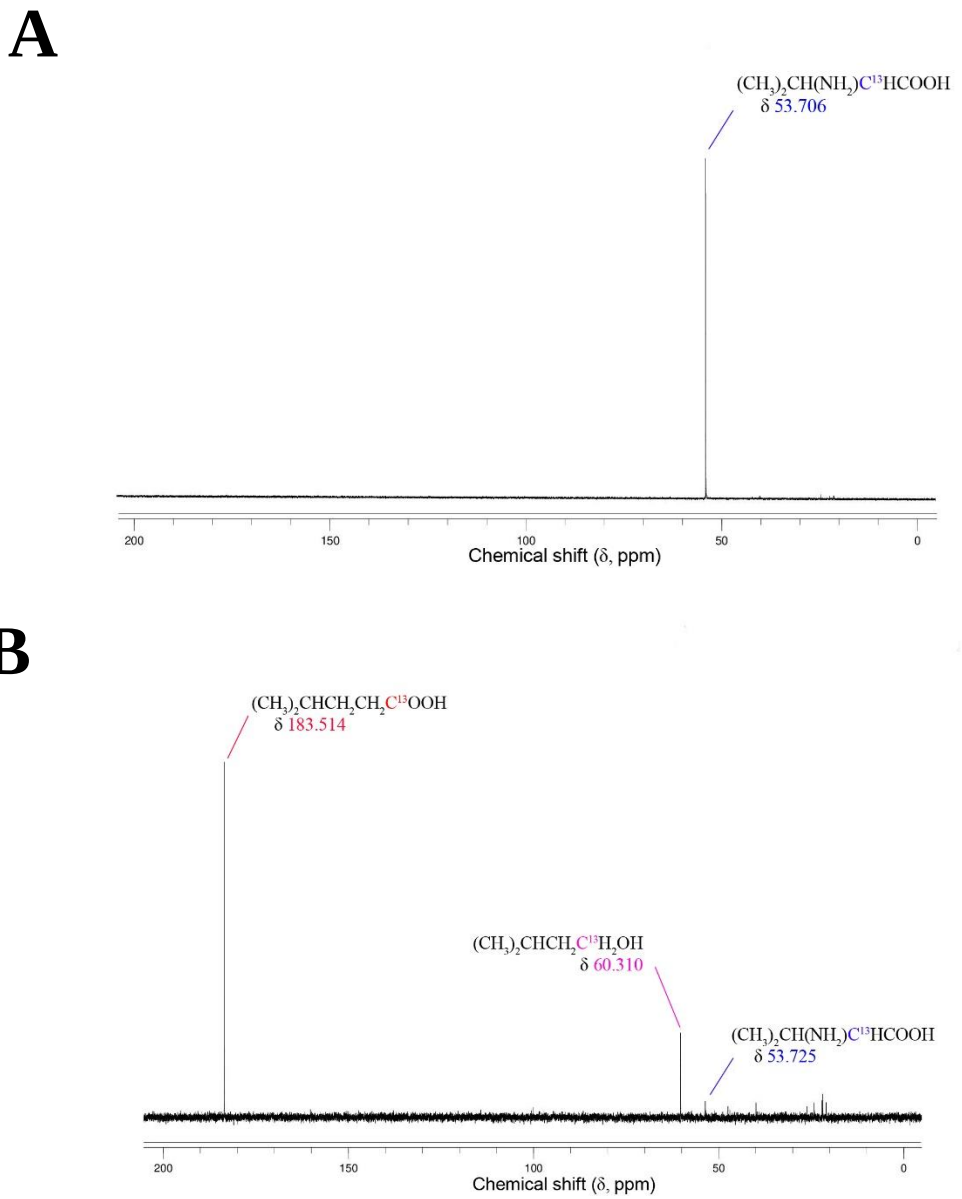

Figure 6. ^{13}C NMR spectrogram of fermentation of $^{13}C_2$ Leucine (20 mM) by *T. pseudoethanolicus* without (A) and with (B) the addition of thiosulfate.

In order to demonstrate the conversion of 3-methyl-1-butyrate to 3-methyl-1-butanol, *T. pseudoethanolicus* was cultivated in the presence of ^{13}C1-labled 3-methyl-1-butyrate with either glucose or leucine only and with leucine and thiosulfate a source of reducing potential (Figure 7A-C).

The reduction of 3-methyl-1-butyrate in the presence of glucose results in a new peak at 60.3 ppm that can be attributed to the formation of 3-methyl-1-butanol (Figure 7A). The same occurs when leucine is added to a ^{13}C2-labled 3-methyl-1-butyrate suggesting that the presence of the carboxylic acid as an electron donor is sufficient to accept the electrons liberated during leucine catabolism (Figure 7B). Similarly, leucine and ^{13}C2-labled 3-methyl-1-butyrate with the addition of thiosulfate produces relatively more 3-methyl-1-butanol (60.3 ppm) based on peek intensity as well as a new unidentified peak at 53.6 ppm (Figure 7C).

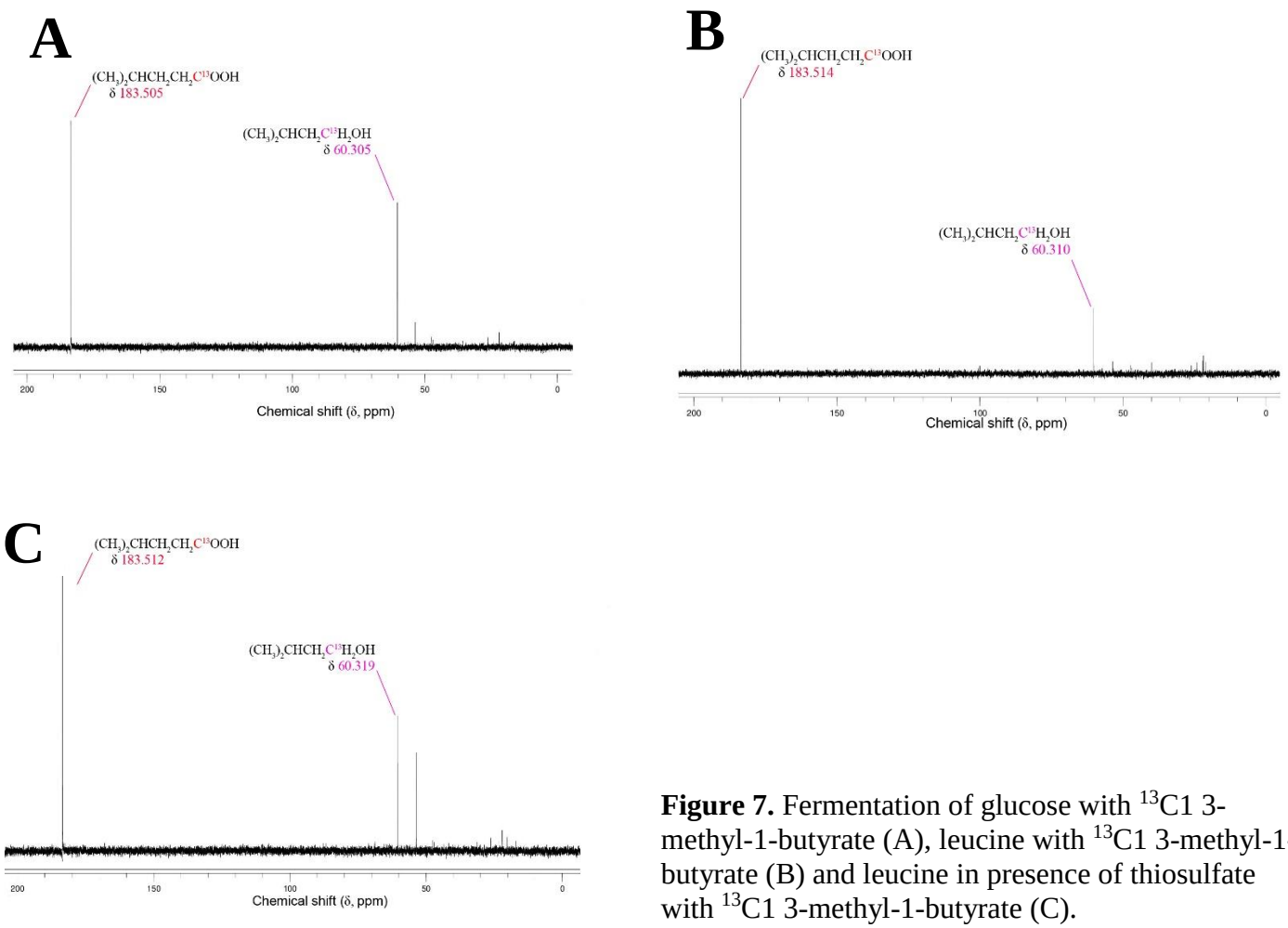

Figure 7. Fermentation of glucose with ^{13}C1 3-methyl-1-butyrate (A), leucine with ^{13}C1 3-methyl-1-butyrate (B) and leucine in presence of thiosulfate with ^{13}C1 3-methyl-1-butyrate (C).

To better illustrate the time required to observe the formation of 3-methyl-1-butanol, a time-course experiment using ^{13}C2-labeled leucine and thiosulfate was monitored over the course of 14 days (Figure 8). A peak attributable to 3-methyl-1-butanol becomes noticeable after 9 days of fermentation and slowly increased over the duration of the experiment.

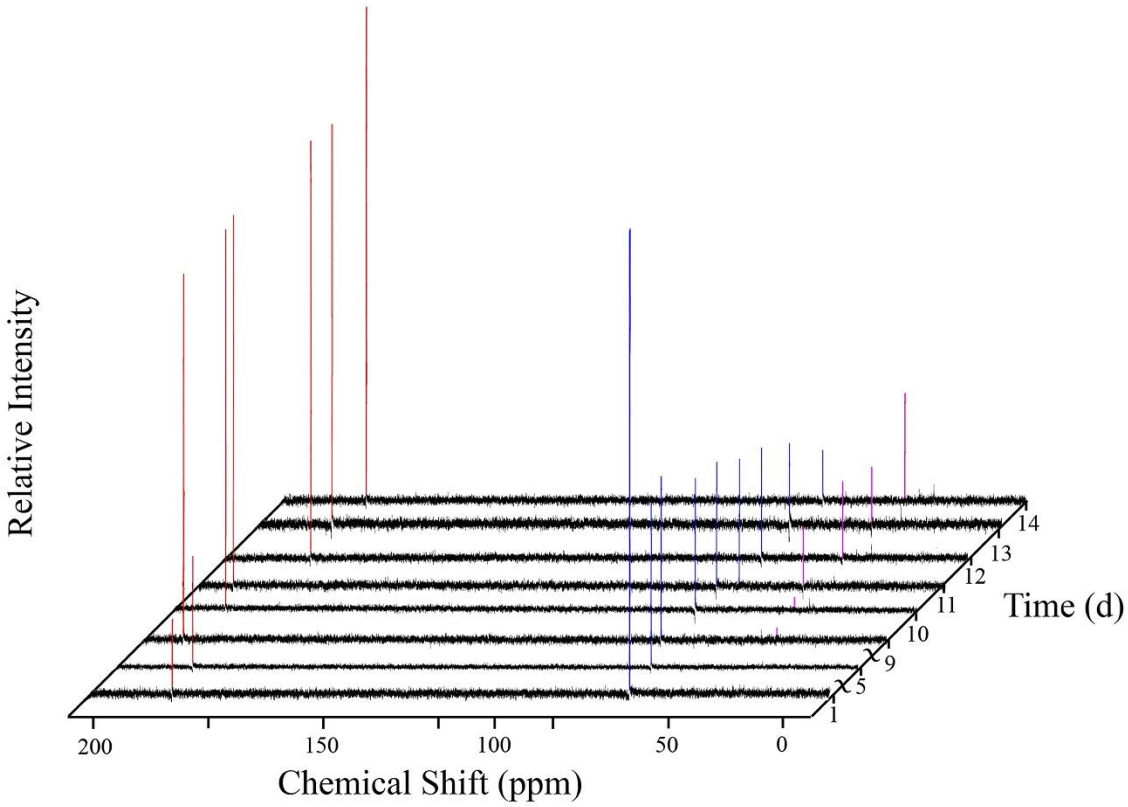

Figure 8. Time course of ^{13}C2 leucine (20 mM) fermentation in the presence of thiosulfate (20 mM) by *T. pseudoethanolicus* using glucose as the source of reducing potential. Blue, red, and magenta peaks correspond to the ^{13}C-labled carbon atom of leucine, 3-methyl-1-butrayte, and 3-methyl-1-butanol, respectively.

3.5 Enzymatic Assays

Cells harvested from the late exponential phase under two conditions (leucine with thiosulfate, leucine with thiosulfate and 3-methyl-1-butyrate) were evaluated for their ADH and AOR activities for C2-C6 substrates using both NAD^+ and $NADP^+$ as cofactors. Cells cultured on leucine and thiosulfate showed similar activities as compared to cells cultivated on a mixture of leucine, thiosulfate, and 3-methyl-1-butyrate. The apparent AOR activities on 3-methyl-butyraldehyde and hexaldehyde, however, we noticeably lower (less than 50 U/mg protein) on the latter conditions while NADP-linked ADH activity for C6-C8 alcohols was not detectable when only leucine and thiosulfate were present.

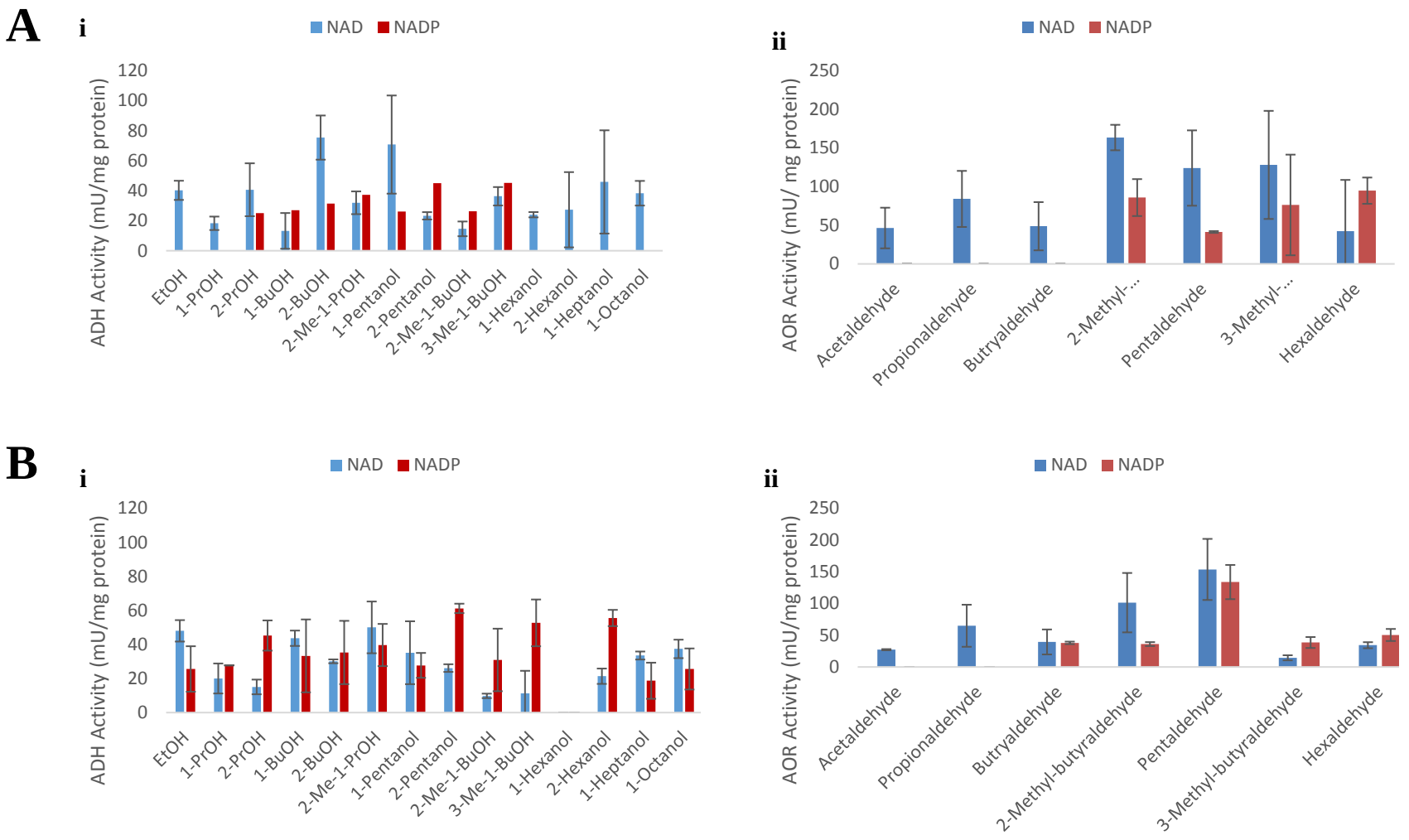

Figure 9. Specific activities of alcohol (**i**) and aldehyde oxidation (**ii**) in cell-free extracts of *T. pseudoethanolicus* after 24 hours grown on isoleucine (20 mM) in the presence of 20 mM of thiosulfate (**A**) and isoleucine (20 mM) + thiosulfate (20 mM) + 3-methyl-1-butyrate of thiosulfate (**B**). Values represent the average of triplicate fermentations with standard deviation represented as error bars.

4 DISCUSSION

Recent investigations in our laboratory revealed that during degradation by *Thermoanaerobacter* and *Caldanaerobacter* species of BCAA under methanogenic conditions, the BCAAs were degraded to their corresponding, one carbon less, BCFAs (Scully et al., 2015), as also previously observed by others (Fardeau et al., 1997; Faudon et al., 1995). However, when these bacteria were cultivated with a chemical electron acceptor (thiosulfate) they produced not only the BCFA from the BCAA, but also BCOH (Scully & Orlygsson, 2014; Scully & Orlygsson, 2015). A detailed work on *Thermoanaerobacter* strain AK85 showed that the length of the incubation time was a crucial factor of the ratio between the produced fatty acid over the alcohol (**Paper IV**, Scully and Orlygsson, 2019); if the strain was cultivated only for one week, the majority of the end products form BCAA degradation was the BCFA, but if the cultures were allowed to incubate for another week, the major end products were, in most cases, the BCOH. This suggested that the BCFA could indeed be converted to its corresponding BCOH, most likely in the stationary growth phase, and this was demonstrated by using isotopically labelled fatty acids (**Paper IV**).

This study examines some of the physiological aspects of the BCAA metabolism of *T. pseudoethanolicus*. Present investigation shows that the strain is producing a mixture of the BCFA and BCOH from their corresponding BCAA in the presence of thiosulfate (Figure 1) and that the concentration of the acid over the alcohols was always 3-4 times higher. It should be noted that this experiment lasted for seven days. In experiments done on the effect of pH, L-G phase ratios and increased concentration of thiosulfate (Figures 2-4) the cultures were allowed to grow for two weeks. : However, present study shows that by cultivating the strain for two weeks did not result in a conversion of the BCFA to the BCOH in the same extent as for observed for *Thermoanaerobacter* strain AK85 (Paper IV). With *T. pseudoethanolicus* this ratio was always in favor for the acid production (Figures 1-4). Thus, there seem to be a clear difference in the role of different electron scavenging systems determining the ratio of end products in these two *Thermoanaerobacter* strains. However, comparing the data from strain AK85 (**Paper IV**) to present investigation on *T. pseudoethanolicus* there is one major difference. The concentration of H_2S in case of strain AK85 was almost a factor of 10 times less as compared to *T. pseudoethanolicus*. Interestingly, in both strains, the concentration of thiosulfate is always close to zero, and in the kinetic experiments it is very rapidly taken up (Figure 5). It is difficult to interpret these results without knowing the sulfur metabolism of these two species but this will be focused upon in near future. The slow degradation kinetics of the BCAAs may suggest that they are not the preferred substrate when present with other components of the yeast extract being preferred leading to a diauxic growth pattern. Furthermore, the delayed end product formation from the BCAAs may suggest that their catabolism may have a role in starvation metabolism.

Further investigations towards showing the carbon flow from the BCAA were performed by using isotopically labelled leucine. *T. pseudoethanolicus* degrades ^{13}C1 leucine to a mixture of 3-methyl-butyrate and 3-methyl-butanol only in the presence of thiosulfate (Figure 6 A-B). Additional experiments to demonstrate the conversion of fatty acids to alcohols showed that when externally added ^{13}C1 3-methyl-1-butyrate was added to glucose, leucine with and without thiosulfate, in all cases the acid was converted to its corresponding alcohol (Figures 7A-C). Thus, as previously known, BCAA have been shown to be only degraded with thiosulfate addition to their culture or when co-cultured

with a hydrogen scavenging organism. Here we present that these amino acids can also use external fatty acids as electron acceptors as further supposed by the activities of ADH and AOR (Figure 9). The ability to reduce carboxylic acids to their corresponding alcohols has been recently demonstrated among *Thermoanaerobacter* strains, including *T. pseudoethanolicus* in which the presence of AOR-coding genes directly implicated (Hitschler et al., 2018) while later work done in our laboratory showed by using ^{13}C1-labeled carboxylic acids that *T. pseudoethanolicus* indeed converts carboxylic acids using glucose as a source of reducing potential (Scully et al. 2019). We therefore propose that the reduction of carboxylic acids play an important role in BCOH formation during BCAA fermentation as summarized in Figure 10. The production of BCOHs from an acyl-CoA intermediate may be similar to the Ehrlich pathway in yeast (Hazelwood, et al., 2008) or by carboxylic acid assimilation to an acyl-CoA which cannot be ruled out.

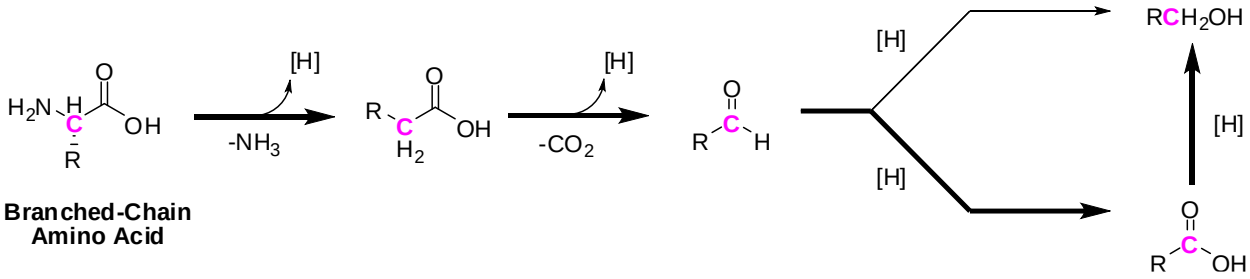

Figure 10. Proposed metabolic pathway for BCAA fermentation to a mixture of BCFAs and BCOHs.

The differences between *Thermoanaerobacter* and *Caldanaerobacter* strains with respect to their ability to utilize amino acids as well as their ability to convert carboxylic acids require further investigation.

Conclusion

Thermoanaerobacter pseudoethanolicus can degrade the branched-chain amino acids to a mixture of branched-chain fatty acids and alcohols in the presence of thiosulfate which is reduced to hydrogen sulfide. Providing additional thiosulfate shifts end product formation towards the more oxidized corresponding fatty acid while increasing the liquid-gas phase ratio increases the amount of branched-chain alcohol produced up to a liquid-gas phase ratio of 1:1. Altering the cultivation pH did not dramatically alter the ratio of branched-chain alcohol to branched-chain fatty acid formed within the established pH growth range. As previously observed with other *Thermoanaerobacter* strains, the fermentation of branched-chain amino acids is slow, even in the presence of thiosulfate, with the amount of branched-chain alcohol slowly increasing over time. The role of carboxylic acid reduction on branched-chain alcohol formation during branched-chain amino acid production is clear when ^{13}C-labled 3-methyl-1-butryate is present along with a source of reducing potential, in this case either glucose or leucine.

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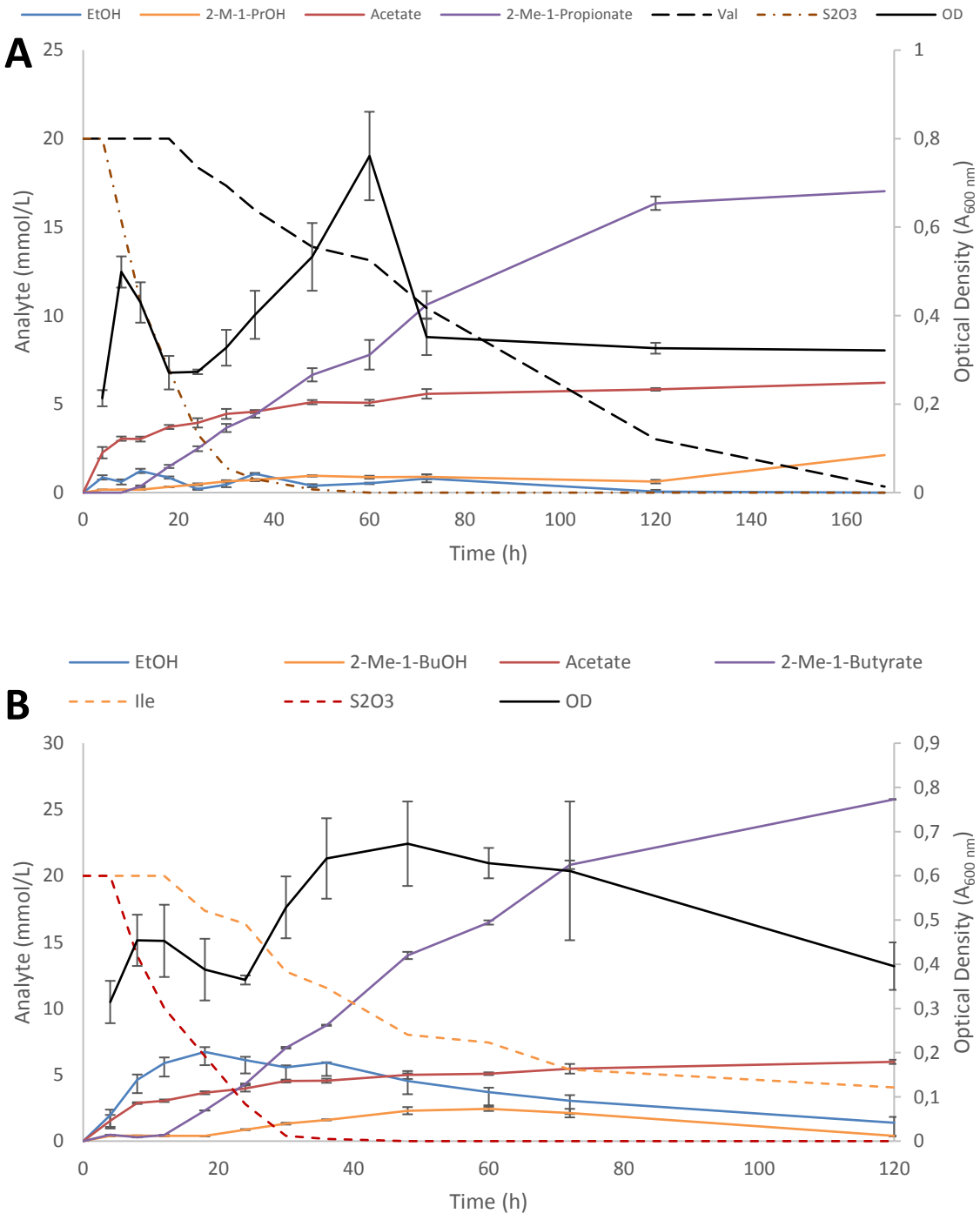
Supplementary Figures



Supplementary Figure 1. Fermentation kinetics of *T. pseudoethanolicus* grown on 20 mM of valine (A) or isoleucine (B) in the presence of thiosulfate (20 mM). Values represent the average of triplicates ± standard deviation.

Supplemental Table 1 - Influence of pH on the fermentation of isoleucine (20 mM) and thiosulfate (20 mM) by *Thermoanaerobacter pseudoethanolicus* after 14 days of cultivation. Values represent the average of triplicate measures ± standard deviation.

	Analyte (mmol/L)											
Initial pH	Ethanol	2-methyl-1-butanol	Acetate	2-methyl-1-butyrate	H_2	H_2S	S_2O_3	Ile	Ile degraded (%)	Carbon balance (%)	OD	pH
4.0	0.00±0.00	0.00±0.00	1.21±0.24	0.00±0.00	0.07±0.01	4.36±0.48	16.51	17.31±0.38	13.5	86.6	0.03±0.00	4.3±0.2
4.5	0.00±0.00	0.00±0.00	2.37±0.13	0.00±0.00	0.05±0.01	6.31±0.51	14.38	18.31±0.41	8.5	91.6	0.04±0.01	4.9±0.1
5.0	0.00±0.00	0.00±0.00	2.60±0.40	2.02±0.51	0.05±0.02	9.87±1.07	15.43	13.20±0.21	34.0	76.1	0.18±0.01	5.2±0.0
5.5	0.26±0.05	3.45±0.47	4.38±0.28	15.81±1.37	0.35±0.08	10.41±1.22	<0.50	0.34±0.07	98.3	98.0	0.21±0.03	5.8±0.1
6.0	0.63±0.08	4.21±0.19	8.21±0.22	16.25±0.34	0.41±0.09	11.58±0.72	<0.50	0.64±0.14	96.8	105.5	0.22±0.02	6.6±0.2
6.5	0.51±0.11	4.36±0.56	7.64±0.31	15.34±0.21	0.32±0.04	12.49±0.68	<0.50	1.39±0.38	93.1	105.5	0.22±0.03	6.8±0.0
7.0	0.67±0.06	3.94±0.39	7.91±0.49	16.71±0.67	0.34±0.07	12.08±2.41	<0.50	1.01±0.23	95.0	108.3	0.23±0.01	7.3±0.1
7.5	0.41±0.13	4.18±0.28	8.43±0.27	16.57±0.83	0.51±0.12	10.59±1.24	<0.50	1.27±0.27	93.7	110.1	0.24±0.02	7.6±0.1
8.0	0.33±0.03	4.09±0.40	8.13±0.13	16.38±0.49	0.39±0.05	11.89±0.33	<0.50	4.22±0.41	78.9	123.5	0.22±0.01	8.2±0.1
8.5	0.10±0.01	4.10±0.27	8.25±0.21	15.14±0.57	0.27±0.07	11.30±0.19	<0.50	3.61±0.62	82.0	114.3	0.18±0.02	8.8±0.0
9.0	0.24±0.13	1.84±0.14	7.84±0.39	11.12±0.74	0.05±0.02	13.21±0.36	<0.50	8.13±1.07	59.4	105.5	0.16±0.02	9.3±0.1

Supplemental Table 2 - Influence of liquid-gas phase ratio concentration on the fermentation of isoleucine (20 mM) and thiosulfate (20 mM|) by *Thermoanaerobacter pseudoethanolicus*. Values represent the average of triplicate measures ± standard deviation.

	Analyte (mmol/L)											
L-G	Ethanol	2-methyl-1-butanol	Acetate	2-methyl-1-butyrate	H_2	H_2S	S_2O_3	Ile	Ile degraded (%)	Carbon balance (%)	OD	pH
0.05	2.14±0.37	1.82±0.63	6.24±0.31	17.34±0.34	0.27±0.05	8.51±0.51	<0.50	0.56±0.17	97.22	98.6	0.24±0.01	7.3±0.2
0.34	1.97±0.27	1.91±0.22	5.76±0.66	18.11±0.67	0.57±0.11	9.35±0.23	<0.50	0.64±0.27	96.80	103.3	0.23±0.02	7.1±0.1
0.98	2.14±0.34	3.37±0.38	5.81±0.41	17.58±0.45	1.23±0.23	11.87±1.21	<0.50	0.79±0.14	96.05	108.7	0.27±0.04	7.4±0.1
2.08	2.43±0.39	3.01±0.54	4.98±0.37	16.34±0.32	1.49±0.17	10.67±0.87	<0.50	0.23±0.05	98.85	97.9	0.27±0.03	7.2±0.1
5.4	2.28±0.13	3.12±0.34	3.94±0.28	16.19±0.17	1.57±0.37	12.34±1.94	<0.50	2.15±0.43	89.25	107.3	0.25±0.04	7.3±0.0

Supplemental Table 3 - Influence of thiosulfate concentration on the fermentation of isoleucine (20 mM) by *Thermoanaerobacter pseudoethanolicus* after 14 days of cultivation. Values represent the average of triplicate measures ± standard deviation.

	Analyte (mmol/L)											
$[S_2O_3]$ (mM)	Ethanol	2-methyl-1-butanol	Acetate	2-methyl-1-butyrate	H_2	H_2S	S_2O_3	Ile	Ile degraded (%)	Carbon balance (%)	OD	pH
0	0.34±0.02	0.00±0.00	2.83±0.17	1.23±0.06	0.40±0.00	4.89±0.51	<0.50	18.63±2.74	6.85	99.3	0.20±0.01	7.2±0.1
10	2.21±0.34	1.43±0.34	4.91±0.27	12.31±0.07	1.52±0.35	6.24±1.32	<0.50	10.41±0.68	47.95	120.8	0.24±0.04	7.3±0.1
20	3.57±0.20	2.51±0.62	6.83±0.30	15.71±0.43	1.14±0.24	13.78±3.47	<0.50	0.16±0.04	99.20	91.9	0.23±0.02	7.1±0.0
30	3.14±0.35	2.17±0.24	7.39±0.47	16.21±0.21	0.67±0.31	18.52±2.81	<0.50	0.34±0.07	98.30	93.6	0.34±0.04	7.4±0.2
40	2.07±0.42	1.83±0.31	7.81±0.37	17.51±0.49	0.86±0.24	21.47±4.46	<0.50	0.17±0.08	99.15	97.6	0.29±0.03	7.3±0.1
50	1.42±0.20	1.63±0.38	8.44±0.21	17.63±0.72	0.21±0.14	25.46±5.24	<0.50	0.24±0.05	98.80	97.5	0.34±0.04	7.6±0.3
60	1.81±0.31	1.11±0.27	8.63±0.10	18.49±1.01	0.19±0.04	32.85±4.30	<0.50	0.13±0.11	99.35	98.7	0.32±0.04	7.5±0.2

Supplemental Table 4 - Fermentation kinetics of valine (20 mM) in the presence of thiosulfate (20 mM) by *Thermoanaerobacter pseudoethanolicus*. Values represent the average of triplicate measures ± standard deviation.

	Analyte (mmol/L)									
Time (h)	Ethanol	2-methyl-1-propanol	Acetate	2-methyl-1-propionate	H_2	S_2O_3	Val	% AA degraded	Carbon balance (%)	OD
4	0.86±0.12	0.00±0.00	2.26±0.32	0.00±0.00	0.32±0.02	20.00±0.00	20.00±0.00	0.0	100.0	0.21±0.02
8	0.60±0.14	0.00±0.00	3.05±0.12	0.00±0.00	0.45±0.13	15.37±0.98	20.00±0.00	0.0	100.0	0.50±0.04
12	1.23±0.12	0.00±0.00	3.03±0.14	0.36±0.05	1.07±0.28	10.78±0.53	20.00±0.00	0.0	101.8	0.43±0.05
18	0.85±0.06	0.32±0.02	3.71±0.11	1.48±0.09	1.14±0.22	6.96±0.36	20.00±0.00	0.0	109.0	0.27±0.04
24	0.20±0.04	0.47±0.05	3.94±0.26	2.48±0.14	1.59±0.40	3.31±0.46	18.37±0.39	8.2	106.6	0.27±0.01
30	0.44±0.14	0.65±0.06	4.45±0.28	3.65±0.23	1.44±0.18	1.39±0.40	17.34±0.24	13.3	108.2	0.33±0.04
36	1.07±0.05	0.71±0.06	4.58±0.09	4.40±0.17	2.48±0.37	0.77±0.18	15.96±1.09	20.2	105.4	0.40±0.05
48	0.39±0.09	0.95±0.04	5.11±0.12	6.66±0.37	2.23±0.63	0.19±0.07	13.89±3.62	30.6	107.5	0.53±0.08
60	0.52±0.04	0.87±0.08	5.08±0.16	7.79±0.84	1.94±0.31	0.00±0.00	13.14±2.95	34.3	109.0	0.76±0.10
72	0.80±0.22	0.89±0.14	5.58±0.27	10.61±0.77	2.31±0.49	0.00±0.00	10.42±0.94	47.9	109.6	0.35±0.04
120	0.11±0.05	0.62±0.10	5.83±0.07	16.35±0.38	1.83±0.42	0.00±0.00	3.02±0.63	84.9	100.0	0.33±0.01
168	0.00±0.00	2.12±0.17	6.21±0.14	17.03±0.41	1.07±0.37	0.00±0.00	0.34±0.07	98.3	97.5	0.32±0.02

Supplemental Table 5 - Fermentation kinetics of isoleucine (20 mM) in the presence of thiosulfate (20 mM) by *Thermoanaerobacter pseudoethanolicus*. Values represent the average of triplicate measures ± standard deviation.

	Analyte (mmol/L)									
Time (h)	Ethanol	2-methyl-1-butanol	Acetate	2-methyl-1-butyrate	H_2	S_2O_3	Ile	% AA degraded	Carbon balance (%)	OD
4	1.95±0.42	0.00±0.00	1.53±0.45	0.48±0.12	0.17±0.05	20.00±0.00	20.00±0.00	0.0	102.4	0.31±0.05
8	4.62±0.40	0.00±0.00	2.87±0.06	0.29±0.03	0.63±0.19	20.00±0.00	20.00±0.00	0.0	101.5	0.45±0.06
12	5.87±0.44	0.00±0.00	3.05±0.09	0.46±0.10	1.43±0.21	13.99±0.29	20.00±0.00	0.0	102.3	0.45±0.08
18	6.73±0.36	0.00±0.00	3.65±0.11	2.31±0.36	1.72±0.43	10.04±0.54	17.37±0.39	0.0	98.4	0.39±0.07
24	6.10±0.26	0.87±0.04	3.97±0.23	4.31±0.20	1.63±0.38	6.40±0.37	16.38±0.76	18.1	107.8	0.36±0.01
30	5.56±0.16	1.32±0.07	4.53±0.11	7.04±0.43	1.84±0.55	2.79±0.33	12.79±0.96	36.1	103.5	0.53±0.07
36	5.92±0.03	1.60±0.04	4.55±0.15	8.74±0.29	2.07±0.20	0.40±0.14	11.55±0.30	42.3	109.5	0.64±0.09
48	4.54±0.57	2.30±0.27	4.99±0.30	14.00±1.14	2.43±0.42	0.16±0.05	8.01±0.88	60.0	121.6	0.67±0.10
60	3.70±0.33	2.44±0.16	5.09±0.10	16.48±0.74	1.20±0.38	0.00±0.00	7.44±0.34	62.8	131.8	0.63±0.03
72	3.04±0.43	2.11±0.32	5.46±0.37	20.83±1.25	1.40±0.23	0.00±0.00	5.39±0.27	73.1	119.4	0.61±0.16
120	1.39±0.44	2.39±0.29	5.98±0.16	25.76±0.94	1.03±0.13	0.00±0.00	4.05±0.07	79.8	116.1	0.40±0.05
168	1.10±0.20	4.07±0.24	6.08±0.17	24.34±1.67	0.79±0.05	0.00±0.00	1.27±0.13	93.7	118.4	0.34±0.07

Supplemental Table 6 - Fermentation kinetics of leucine (20 mM) in the presence of thiosulfate (20 mM) by *Thermoanaerobacter pseudoethanolicus*. Values represent the average of triplicate measures ± standard deviation.

	Analyte (mmol/L)									
Time (h)	Ethanol	3-methyl-1-butanol	Acetate	3-methyl-1-butyrate	H_2	S_2O_3	Leu	% AA degraded	Carbon balance (%)	OD
4	5.38±0.93	0.00±0.00	1.25±0.16	0.63±0.15	0.34±0.12	20.00±0.00	20.00±0.00	0.0	103.1	0.42±0.07
8	6.56±0.44	0.00±0.00	2.57±0.64	0.65±0.12	0.78±0.33	20.00±0.00	20.00±0.00	0.0	103.3	0.65±0.06
12	7.89±0.31	0.00±0.00	2.59±0.21	0.54±0.05	1.07±0.24	13.34±1.13	20.00±0.00	0.0	102.7	0.66±0.05
18	8.00±0.10	0.00±0.00	3.21±0.25	1.66±0.40	1.40±0.47	11.06±0.19	20.00±0.00	0.0	108.3	0.34±0.09
24	7.52±0.10	0.57±0.01	3.51±0.53	2.94±0.79	1.66±0.23	11.65±3.61	20.00±0.00	0.0	114.7	0.28±0.02
30	6.87±0.19	0.70±0.07	3.59±0.17	4.68±0.93	1.83±0.56	5.58±1.77	20.00±0.00	0.0	113.4	0.31±0.03
36	6.68±0.14	0.85±0.07	3.88±0.15	6.20±0.86	2.25±0.38	0.53±0.19	20.00±0.00	0.0	108.6	0.33±0.11
48	5.41±0.45	1.21±0.10	4.10±0.28	9.58±0.98	2.41±0.47	0.00±0.00	20.00±0.00	0.0	110.2	0.46±0.06
60	5.60±0.24	1.85±0.80	4.50±0.66	12.61±3.44	2.87±0.69	0.00±0.00	12.68±0.97	36.6	132.5	0.60±0.13
72	4.80±0.04	1.59±0.13	4.80±0.26	16.01±1.90	1.63±0.43	0.00±0.00	9.07±1.50	54.7	133.4	0.47±0.11
120	3.04±0.42	1.07±0.47	5.40±0.33	22.67±1.61	1.28±0.18	0.00±0.00	7.79±0.47	61.1	127.6	0.39±0.06
168	2.06±0.37	1.43±0.34	5.71±0.27	23.37±1.32	1.32±0.21	0.00±0.00	3.22±0.24	83.9	120.1	0.41±0.02

VI

Genus-wide Study of *Thermoanaerobacter* and *Caldanaerobacter*: Protein and Amino Acid Metabolism

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Abstract

Protein is a ubiquitous carbon and nitrogen source in the environment. Unlike studies on the ability of thermophilic anaerobes to degrade carbohydrates, protein and amino acids are rare leaving a knowledge gap as to how potential bioprocessing strains, such as the highly ethanologenic *Thermoanaerobacter ethanolicus*, catabolize these compounds. The ability of the 14 type strains of *Thermoanaerobacter* and 5 *Caldananaerobacter* species to degrade various proteins and amino acids was investigated with and without thiosulfate as an electron acceptor. Six *Thermoanaerobacter* strains and two *Caldanaerobacter* strains were positive on beef extract when added without thiosulfate although twelve strains were positive with inclusion of thiosulfate. Similarly, degradation of single amino acids were heavily dependent on the addition of external electron acceptor (thiosulfate). Without thiosulfate addition, only six amino acids (glycine, serine, threonine, alanine, cysteine, and methionine) could be degraded by a handful of tested strains. In the presence of thiosulfate, a much broader spectrum of amino acid utilization was observed. The branched-chain amino acids were degraded to a mixture of their branched-chain fatty acids and alcohols but the concentration of the fatty acid was always considerable higher. Only two strains, both *Caldanaerobacter* could utilize aromatic amino acids, producing a mixture of their corresponding, one carbon shorter aromatic fatty acid and alcohol. Kinetic experiments by two species of *Thermoanaerobacter* and one species of *Caldanaerobacter* on the three branched-chain amino acid revealed that, in the presence of thiosulfate, valine was degraded to a mixture of 2-methyl-1-propionate and 2-methyl-1-propanol; isoleucine to 2-methyl-1-butyrate and 2-methyl-1-butanol; leucine to 3-methyl-1-butyrate and 3-methyl-1-butanol.

1. Introduction

Species within the genus of *Thermoanaerobacter* and *Caldanaerobacter* have received considerable attention due to their biotechnological potential for the production of biofuels due to their broad substrate spectrum, especially among the sugars present in lignocellulosic biomass, as well as the utility of their heat-stable enzymes including pecinase, xylanses, and alcohol dehydrogenases. A number of species, such as *Thermoanaerobacter pseudoethanolicus* and *Thermoanaerobacter mathranii*, are highly ethanologenic (Chades, Scully, Ingvadottir, & Orlygsson, 2018; Lacis & Lawford, 1988; Larsen, Nielsen, & Ahring, 1997; Scully & Orlygsson, 2015b; Taylor et al., 2009) which has led to a great deal of emphasis on the carbohydrate metabolism of these strains. However, there has been significantly less attention has been given to the amino acid metabolism of thermophilic bacteria including those within *Caldanaerobacter* and *Thermoanaerobacter*, with only several studies on *Thermoanaerobacter* species having been examined (Fardeau et al., 1997; Faudon et al., 1995; Scully & Orlygsson, 2014; Scully et al., 2015; Scully & Orlygsson, 2015; **Paper IV and V**). Furthermore, most characterization papers describing new species within these genera, let alone other strains falling with in Class *Clostridia*, do not systematically explore amino acid and protein metabolism and if present, often neglect data concerning amount of end product formation which leaves signifigant knowledge gaps and moves away from the historical classification of clostrial strains as being saccharolytic or proteolytic, or some combination thereof. Given the abundance of protein and amino acids in the biosphere, understanding the role of these species within the environment is critical to identifying their role in nutrient cycling as well as developing their biotechnological potential.

The genera of *Caldanaerobacter* and *Thermoanaerobacter* fall into Clusters V in the phylogenetic interrelationship of *Clostridium* (Collins et al., 1994). The type strain, *Thermoanaerobacter ethanolicus*, was isolated from a geothermal feature in Yellowstone National Park (USA) in the early 1980s (Wiegel & Ljungdahl, 1981). The taxonomy of the genus of *Thermoanaerobacter* was refined 25 years ago (Lee, et al., 1993) although many new species have been described or renamed pulling many members of the now defunct genus of "*Thermoanaerobium*" although more recent rearrangements have occurred based on analysis of the 16S RNA gene. For instance, *T. subterraneus* and *T. yonseiensis* were recently assigned to a new genus, *Caldanaerobacter* (Fardeau et al., 2004). The genus *Caldanaerobacter* currently contains 2 species and 4 subspecies whereas *Thermoanaerobacter* contains 16 species and 5 subspecies according to LPSN (Parte, 2014). "*Thermoanaerobacter keratinophilus",* described by (Riessen & Antranikian, 2001), is not recognized as validly described although it is notable for its ability to degrade keratin. All species within *Thermoanaerobacter* and *Caldanaerobacter* are obligate anaerobes; ferment various carbohydrates and amino acids to ethanol, acetate, lactate, alanine, hydrogen, and carbon dioxide (Wagner & Wiegel, 2008). Species within the two genera originate from various habitats, including canned foods (Carlier et al., 2006), geothermal features (Jessen & Orlygsson, 2012; Larsen et al., 1997; Onyenwoke et al., 2007; Juergen Wiegel & Ljungdahl, 1981), subsurface oil reservoirs (Cayol et al., 1995; Kondratieva et al., 1989), deep sea vents (Sokolova et al., 2001), compost piles (Fong et al., 2006), and anaerobic digesters (Tomás et al., 2013).

Proteins are ubiquitous biomolecules in nature ranging in their physical properties and susceptibility to hydrolysis as exemplified by the easily degraded soluble protein casein to much more recalcitrant structural proteins such as collagen and keratin. Historically, classifying *Clostridia* as either saccharolytic or proteolytic has been an intrinsic aspect of their characterization. That said, investigations of the ability to degrade proteins and the subsequent fermentation of the liberated amino acids has been neglected with the exception of the ability of several mesophilic *Clostridia* species, particularly those of agricultural or clinical relevance. A clear definition of what constitutes being "proteolytic" is lacking although it is generally accepted that the ability to hydrolyse proteins to oligopeptide or amino acids fits this definition. The proteases of many clostridial species have also been investigated, particularly those acting on collagen and its hydrolysate, gelatin, and have been noted particularly among species such as *Clostridium histolyticum* (Jung, Matsushita, Katayama, & Minami, 1999; Soru & Zaharia, 1972; Takahashi & Seifter, 1972), as well as those producing clostripain (Gille, Imhoff, & Keil, 1979; Gros & LaBouesse, 1960; Labousesee & Gros, 1960; Mitchell, 1977; Mitchell & Harringon, 1968). Some efforts to categorise proteolytic *Clostridia* in the context of their ability to ferment specific amino acids or use Stickland reactions was put forth by Mead (Mead, 1971) which was later expanded by Elsden and co-workers (Elsden & Hilton, 1978, 1979; Elsden et al., 1976) although much of this work was again limited to *Clostridium* species of clinical relevance. Of the 20 proteogenic amino acids, there are many potential degradation routes known among *Clostridia* as reviewed in the literature (Barker, 1981; McInerney, 1988) while there have been no newer general overviews of clostridial amino acid metabolism. However, some recent reviews on specific aspects of glutamate catabolism (Buckel, 2001) and its role in stress response (Feehily & Karatzas, 2013), include the pathways used by *Clostridium sticklandii* (Sangavai & Chellapandi, 2017), and more generalized works examining the role of amino acid metabolism by anaerobes in the gut (Neis, Dejong, & Rensen, 2015).

Reduced amino acids are often oxidatively degraded to their corresponding keto acids which are further oxidatively decarboxylated to their, one carbon shorter, fatty acids. Several amino acids are degraded without net oxidation/reduction mechanism and some amino acids use reductive degradation pathways (McInerney, 1988). Branched-chain amino acids (BCAAs) are examples of amino acids that are oxidatively deaminated (Andreesen, Bahl, & Gottschalk, 1989; Fardeau et al., 1997). These amino acids can only be fermented if the electrons produced from the initial deamination step are scavenged due to the unfavorable thermodynamics involved (Fardeau et al., 1997; Örlygsson, 1994). The $\Delta G°'$ for the degradation of the BCAAs is between +4.2 and +9.7 kJ/mol (Fardeau et al. 1997). Addition of external electron acceptor like thiosulfate (which can be reduced to H_2S or S^0) to the medium or co-cultivating the amino acid degrader with hydrogen scavenging bacteria, such as a methanogen or sulfate reducer, drives the reaction forward resulting in the amino acids being degraded to their corresponding branched-chain fatty acids (BCFAs) in addition to CO_2 and ammonium (Fardeau et al., 1997). Amino acid metabolism of *Thermoanaerobacter brockii* has been investigated in some detail. Under hydrogen scavenging conditions, leucine, isoleucine, and valine are degraded to 3-methyl-1-butyrate, 2-methyl-1-butyrate, and 2-methyl-1-propionate, respectively. A recent investigation in our laboratory demonstrated that *T. brockii* and *Caldanaerobacter subterraneus* subsp.

yonseiensis degrades BCAAs to a mixture of the corresponding BCFAs and branched chain alcohols (BCOHs) when cultivated in the presence of thiosulfate but only to the corresponding BCFAs when co-cultured with a hydrogenotrophic methanogen (Scully & Orlygsson, 2014).

The aim of this study was to perform a direct comparisons of protein and amino acid degradation capabilities and end product formation of all members of the genera of *Thermoanaerobacter* and *Caldanaerobacter*, with the exception of *T. acetoethylicus* and *T. thermocopriae* which could not be obtained.

2. Methodology

Isotopically labeled substrates were obtained from Cambridge Isotope Laboratories (Tewksbury, MA, USA). Chicken feathers were sourced from a local poultry farmer and were dried and ground into a fine powder using a Waring blender. All other materials were obtained from Sigma-Aldrich.

2.1. Strains and Cultivation Conditions

Thermoanaerobacter and *Caldanaerobacter* strains were cultivated in Basal Mineral (BM) medium (Chades et al., 2018) using the Hungate technique (Hungate, 1969; T. L. Miller & Wolin, 1974). The final substrate concentration was 20 mM except for polymeric substrates in which the concentration was 2% (w/v). All other components of the medium were added separately in the form of filter sterilized (0.22 µm) solutions. All experiments were conducted at 65°C and at pH of 7.0 with a liquid-gas (L-G) ratio of 1:1 unless otherwise noted. In all cases, experiments were performed with three replicates.

The type strains of *Thermoanaerobacter* and *Caldanaerobacter* were obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ); *Thermoanaerobacter brockii* subsp. *brockii* (DSM 1457), *Thermoanaerobacter brockii* subsp. *finnii* (DSM 3389), *Thermoanaerobacter brockii* subsp. *lactiethylicus* (DSM 9801), *Thermoanaerobacter italicus* (DSM 9252), *Thermoanaerobacter ethanolicus* (DSM 2246), *Thermoanaerobacter kivui* (DSM 2030), *Thermoanaerobacter mathranii* subsp. *mathranii* (DSM 11426), *Thermoanaerobacter pentosaceusus* (DSM 25963), *Thermoanaerobacter pseudoethanolicus* (DSM 2355), *Thermoanaerobacter siderophilus* (DSM 12299), *Thermoanaerobacter sulfurigenens* (DSM 17917), *Thermoanaerobacter sulfurophilus* (DSM 11584), *Thermoanaerobacter thermohydrosulfuricus* (DSM 567), *Thermoanaerobacter uzonensis* (DSM 18761), *Thermoanaerobacter wiegelii* (DSM 10319), *Caldanaerobacter subterraneus* subsp. *yonseiensis* (DSM 13777), *Caldanaerobacter subterraneus* subsp. *subterraneus* (DSM 13054), *Caldanaerobacter subterraneus* subsp. *pacificus* (DSM 12653), *Caldanaerobacter subterraneus* subsp. *tengcongensis* (DSM 15242), and *Caldanaerobacter uzonensis* (DSM 18923). Strains were preserved in rigorously degassed by sonication under vacuum BM medium supplemented

with 30% v/v glycerol and stored at -20°C. All cultivations were conducted at pH 7.0 at the organism's T_{opt} which is listed in **Supplementary Table 1**.

All inoculation stocks were taken from stock cultures stored in BM amended with 30% (v/v) glycerol which was rigorously degassed via sonication prior to the transfer of cells. Freezer stocks were maintained at (-20°C) cultures and reactivated on BM medium containing glucose (20 mM) immediately prior to use. Reactivated cultures were inoculated (2% (v/v)) from exponential growth phase to 25 mL serum bottles (liquid-gas ratio 1:1). Cultures were grown for 7 days and then analyzed.

2.2 Amino acid utilization patterns on casamino acids

Strains were cultivated according to the method described by (Elsden & Hilton, 1979) with modifications; BM medium was supplemented with 30 g/L Casamino Acids and 1 g/L tryptone with and without the addition of thiosulfate (40 mM). After 7 days of cultivation, the amino acids were analyzed by two-dimensional paper chromatography as described by (Silverman & Christenson, 1996) using 10x10 cm papers (Whatman). Briefly, 2 µL was spotted and the paper run with mobile phase I (pyridine/acetone/ammonia/dH_2O 40:30:5:20 v/v). The paper was air dried, turned 90° and run with mobile phase II (2-propanol/formic acid/dH_2O 66:15:15). After air drying the paper was sprayed with 0.2% (w/v) ninhydrin in acetone and heated at 105°C for 60 s. Spots were compared to fresh medium and standards prepared from the 20 proteogenic amino acids.

2.3 Degradation of proteins and amino acids in protein hydrolysates

For the action on milk, milk hydrolysis reactions were judged visually in BM supplemented 10% (w/v) with skim milk powder in 18x150 mm Hungate tubes. Similarly, action on meat was determined visually with BM amended with cooked meat medium (12.5% (w/v)). Experiments were performed +/- thiosulfate (40 mM) and evaluated after 7 days.

2.4 Degradation of proteins

All strains were cultivated in BM supplemented with various proteins (20 g/L) for 14 days in Hungate tubes (18x50 mm). Proteins used were collagen (from bovine Achilles tendon), keratin (from chicken feather), tryptone, and gelatin (bovine skin). After cultivation, degree of protein hydrolysis was determined by analyzing decrease of protein concentration in the culture bottle as well as analyzing end-product formation.

2.5. Utilization of single mmino acids

All strains were cultivated on all the 20 proteogenic amino acids (20 mM) as single substrates in 25 mL serum bottles with and without thiosulfate (40 mM). Heat-stable amino acids were added to the medium prior to autoclaving while the remaining amino acids were added from syringe filtered stock bottles. End-product formation was then determined from supernatants of cultures after 7 days of cultivation.

2.6. Kinetic experiments on Branched-chain Amino Acids

Kinetic experiments on the fermentation of valine, leucine, and isoleucine (20 mM) in the presence of thiosulfate (20 mM) were performed in 125 mL serum bottles with a L-G of 1:1 at 65°C for *T. siderophilus*, *T. uzonensis*, and *Caldanaerobacter subterraneus* subsp. *yonseiensis*. Periodically, 1 mL samples were taken for analysis and 0.2 mL of headspace gas was removed for analysis of hydrogen.

2.7. Analytical methods

Hydrogen was analyzed by Perkin Elmer Auto System XL gas chromatograph equipped with a thermo-conductivity (TCD) detector as previously described (Orlygsson & Baldursson, 2007). Alcohols and volatile fatty acids were measured by gas chromatography using a Perkin-Elmer Clarus 580 gas chromatograph equipped with a flame ionization detector (FID) as previously described (Orlygsson & Baldursson, 2007). Headspace pressure was determined using a handheld pressure meter (Pendotech). Amino acids were analyzed as previously described (Scully & Orlygsson, 2014). The qualitative identification of aromatic end products was performed by TLC according to (Elsden et al., 1976) with minor modification; 1 mL of cell-free culture supernatant was acidified with 5 µL of concentrated sulfuric acid followed by the addition of 200 µL of diethylether. 20 µL of the resultant ether layer was spotted onto an aluminum-backed silica plate (Merck Silica gel 60 F_{254}) and run using mobile phase (1-hexane/diethyl ether/acetic acid 100:100:1). Plates were visualized using a UV lamp (254 nm) and acidic end products were visualized by spraying with 0.2% (w/v) bromocresol green in ethanol.

Hydrogen sulfide was analyzed according to the method described by Cord-Ruwisch (Cline, 1969). Thiosulfate was analyzed according to Westley (Westley, 1987) modified for use in microplates. Indole tests were performed according to (Tindall, Sikorski, Smibert, & Krieg, 2007) with strains grown in BM containing 20 mM tryptophan.

Cell concentration was determined by measuring absorbance at 600 nm by a Perkin Elmer Lambda-25 UV-Vis spectrophotometer in a cuvette with a pathlength of 1 cm. Cell dry weight (CDW) was correlated to OD by filtering cultures of known $OD_{600\ nm}$ through 0.22 µm filter paper which was then dried for 2 hours at 105°C.

API ZYM assays (bioMérieux, France) were performed for each strain in triplicate from overnight glucose-grown (20 mM) cultures in accordance with the manufacture's directions with the following modification: strips were inoculated with 50 µL of culture and incubated in a humidified bag at 65°C for 4 hours.

Protein was determined using the Lowry method (Copeland, 1994) modified for microplates. Briefly, 25 µL of sample was added to a microplate well followed by 250 µL of freshly prepared Lowry reagent and incubated at ambient temperature for 20 minutes. 25 µL of Folin´s reagent was then added and the plate briefly mixed on a microplate shaker (150 rpm, 30 s) and read at 540 nm on a Bioscreen C (GrowthCurves, Ltd, Finland). A standard curve prepared from bovine serum albumin (0.1 to 1.4 mg/mL) was used.

The degree of protein hydrolysis (DH) was analysed as described by (Peinado, Koutsidis, & Ames, 2015) and references therein. OPA reagent was freshly prepared and consisted of a solution containing 7.620 g of sodium tetraborate, 200 mg of sodium dodecyl sulphate, 150 mL of dH_2O to which 4 mL of ethanol containing 160 mg *o*-phthaldialdehyde (OPA) and 176 mg of dithioerythritol in ethanol was added before filling to a V_f of 200 mL. 50 µL of diluted sample or standard were added together with 150 µL of OPA reagent and incubated for 2 min. The absorption was read at 340 nm using a Shimadzu UV-1800 spectrophotometer in a quartz cuvette (*l*=1 cm). DH was calculated according to equations below where h_{tot} is specific to the raw material, h is meqv of serine/g protein; serine-NH_2 is meqv serine-NH_2/g protein; a and b dependent on the specific raw material, and for the present study were estimated as a=1.00, b=0.4, x = g sample, P=protein in sample; 0.1 is the sample volume (L).

$$DH = \frac{h}{h_{tot}} \cdot 100\%$$

$$h = \frac{(Serine - NH2) - b}{a}$$

$$Serine - NH2 = \frac{Abs_{sample} - Abs_{blank}}{Abs_{standard} - Abs_{blank}} \cdot 0.9516 \cdot 0.1 \cdot \frac{100}{X} \cdot P$$

2.8. Enzyme assays

Assays for non-specific protease, gelatinase, and collagenase activity were performed by transferring 20 µL of fermentation broth to an Eppendorf tube containing 5.0 mg of substrate (casein, gelatin, or collagen, respectively) to which 1 mL of 50 mM TES buffer (pH 7.5) containing 360 µM $CaCl_2$ was added. Tubes were incubated for 5 hours at 65°C and then quenched with 100 µL of 60% (w/v) trichloroacetic acid. 10 µL of solution was transferred to a microtiter plate followed by the addition of 50 µL of ninhydrin-citric acid solution (4% (w/v) ninhydrin in 2-methoxyethanol containing 7.2 mM $SnCl_2$ mixed 1:1 with 200 mM sodium citrate (pH 5.5) followed by 100 µL of 50% (v/v) 1-propanol. Plates were then read at 600 nm using a Bioscreen C (Growthcurves Ltd, Finland) and the amount of liberated leucine was calculated from a standard curve (0.1 to 10 mM) of L-leucine. The volumetric enzyme activity was calculated according to the equation below:

$$Unit \cdot mL^{-1} = \frac{\mu mol\ Leu\ liberated}{min}$$

Trypsin activity was determined according to the method described by (Kristjansson, 2001) with minor modifications. Briefly, 5 µL of solution was added to a microplate followed by the addition of 475 µL of 100 mM Tris-HCl (pH 8.2) containing 10 mM $CaCl_2$. 20 µL of 12.5 mM BAPNA was then added. The plate was incubated at 60°C and the absorbance at 405 nm was read every 10 min for 120 min. Molar extinction coefficient of *p*-nitrophenol 8480 M^{-1} cm^{-1} was used as the volumetric activity calculated according to the equation below.

3. Results and Discussion

Present investigation is towards pan genus study of protein and amino acid metabolism of *Thermoanaerobacter* and *Caldanaerobacter* species. Majority of species within both genera were investigated on their capability to degrade proteins and single amino acids, in the presence and absence of thiosulfate. There are many knowledge gaps of protein and amino acid metabolism of thermophilic bacteria. The original classification of Clostridia species of being saccharolytic, proteolytic and lipolytic is outdated and often misleading and e.g. many species are both saccharolytic and proteolytic. It is well known that many anaerobic microorganisms can utilize various compounds present in yeast extract, not necessarily meaning they are proteolytic.

Early studies on protein and amino acid metabolism were often done with mesophilic ruminal species of *Clostridia* (Elsden & Hilton, 1978, 1979; Elsden et al., 1976; Mead, 1971) revealing that amino acids are degraded via many redox reactions where amino acids can act both as electron donors and acceptors. Both the type of amino acids degraded and the type of microorganisms determine which degradation pathways are used. One of the bottleneck in degradation of amino acids is the complex nutritional requirements of the microorganism used, e.g. degradation of many amino acids requires a syntrophic relationship between amino acid degrading microorganism and a hydrogen consuming bacterium; in rumen by hydrogenotrophic methanogenesis. Although our knowledge concerning amino acid metabolism is mainly derived from mesophiles, some efforts have been made towards investigating thermophiles with this respect. *Thermoanaerobacter* and *Caldanaerobacter* represent two major genera of thermophilic Clostridia. Considerable work has been done on the former concerning amino acid metabolism, mainly showing the importance of electron scavenging systems for the degradation of the BCAA (Fardeau et al., 1997; Faudon et al., 1995). The present investigation focuses upon the protein and amino acid metabolism by the majority of species, and subspecies within both *Thermoanaerobacter* and *Caldanaerobacter*. To our knowledge this is the first time, such a whole pan genomic study is performed on thermophilic bacteria in general.

3.1 Characterization of enzyme chemistries and action on cooked meat and skim milk medium

To assess the proteolytic nature as well as which common enzymes chemistries were present among *Thermoanaerobacter* and *Caldanaerobacter* type strains, each strain was analyzed using an API ZYM strip, for which data can be found in Supplemental Table 1, and common assays for proteolysis involving cultivation of the strains on cooked meat medium and skim milk medium. Of particular note is that all strains show clear phosphatase activity, namely acid phosphatase and naphthol-AS-BI-phosophohydrolase, and C4 esterase activity. Two strains, *T. mathranii* subsp. *mathranii* (DSM 11426) and *T. sulfurigenens* (DSM 17917), show clear indications of trypsin activity while *C. subterraneus* subsp. *yonseiensis* (DSM 13777) demonstrates distinct chymotrypsin activity. It should be noted that *Caldanaerobacter subterraneus* subsp. *yonseiensis* produces "thermicin", a heat-stable subtilisin-like serine protease which is active against some forms of collagen (Jang et al., 2002) which could be responsible for this activity.

Additionally, traditional determinants of proteolysis such as the degradation of skim milk, action on cooked meat (CM) medium with and without the addition of thiosulfate was evaluated for most species of both *Thermoanaerobacter* and *Caldanaerobacter* as summarized in Table 1. No strains produced noticeable reactions on skim milk nor did they produce a positive indole reaction although action on CM medium was apparent for some strains. Representative examples of the observed action on CM are presented in Figures 1 and 2 for three *Thermoanaerobacter* and three *Caldanaerobacte*r species with results ranging from no apparent action (negative), some softening of the particles (positive), to visible deterioration of meat particles and discoloration (double positive).

Eight out of 19 strains tested showed some softening of the meat particles in the assay performed without thiosulfate addition (6 of 14 *Thermoanaerobacter* and 2 of 5 *Caldanaerobacter* strains) as summarized in Figure 1B-C and Figure 2 B-C. Increased activity was observed when the strains were cultivated in the presence of thiosulfate. A total of 12 strains out of 19 were positive and 9 strains showed visible deterioration of meat particles and discoloration (exemplified in Figures 1C and 2C).

The apparent chemistries apparent on the API ZYM strips as well as the action on cooked meat medium in the prescnece of thiosulfate suggest that *Themoanaerobacter* and *Caldanaerobacter* strains may play several roles in the environment, pariticularly with regard to the cycling of phosphate and organic nitrogen. While action on cooked meat medium was observed, the lack of action on skim milk may suggest that proteases, if present, do not prefer highly soluble (i.e. highly charged) proteins such as casein, the dominant protein in skim milk.

Table 1. General proteolytic features of *Thermoanaerobacter* and *Caldanaerobacter* species; ++ = strongly positive, + = positive, w = weak, - = negative.

	T. *brockii* subsp. *brockii* (DSM 1457)	*T. brockii* subsp. *finnii* (DSM 3389)	*T. ethanolicus* (DSM 2246)	*T. italicus* (DSM 9252)	*T. kivui* (DSM 2030)	*T. mathranii* subsp. *mathranii* (DSM 11426)	*T. pentosaceusus* (DSM 25963)	*T. pseudoethanolicus* (DSM 2355)	*T. siderophilus* (DSM 12299)	*T. sulfurigignens* (DSM 17917)	*T. sulfurophilus* (DSM 11584)	*T. thermohydrosulfuricus* (DSM 567)	*T. uzonensis* (DSM 18761)	*T. wiegelii* (DSM 10319)	*C. subterraneus* subsp.*pacificus* (DSM 12653)	*C. subterraneus* subsp. *subteraneus* (DSM 13054)	*C. subterraneus* subsp. *tengcongensis* (DSM 15242)	*C. subterraneus* subsp. *yonseiensis* (DSM 13777)	*C. uzonensis* (DSM 18923)
Indole test	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Trypsin	-	-	-	-	-	+	-	-	-	-	+	-	-	-	-	-	-	-	-
Chymotrypsin	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
Action on skim milk	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Action on skim milk (+thiosulfate)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Action on cooked meat	+	w	-	+	-	+	-	-	w	+	+	-	+	-	-	-	+	+	-
Action on cooked meat (+thiosulfate)	++	++	-	++	-	++	+	-		+	++	-	++	-	-	+	++	++	-

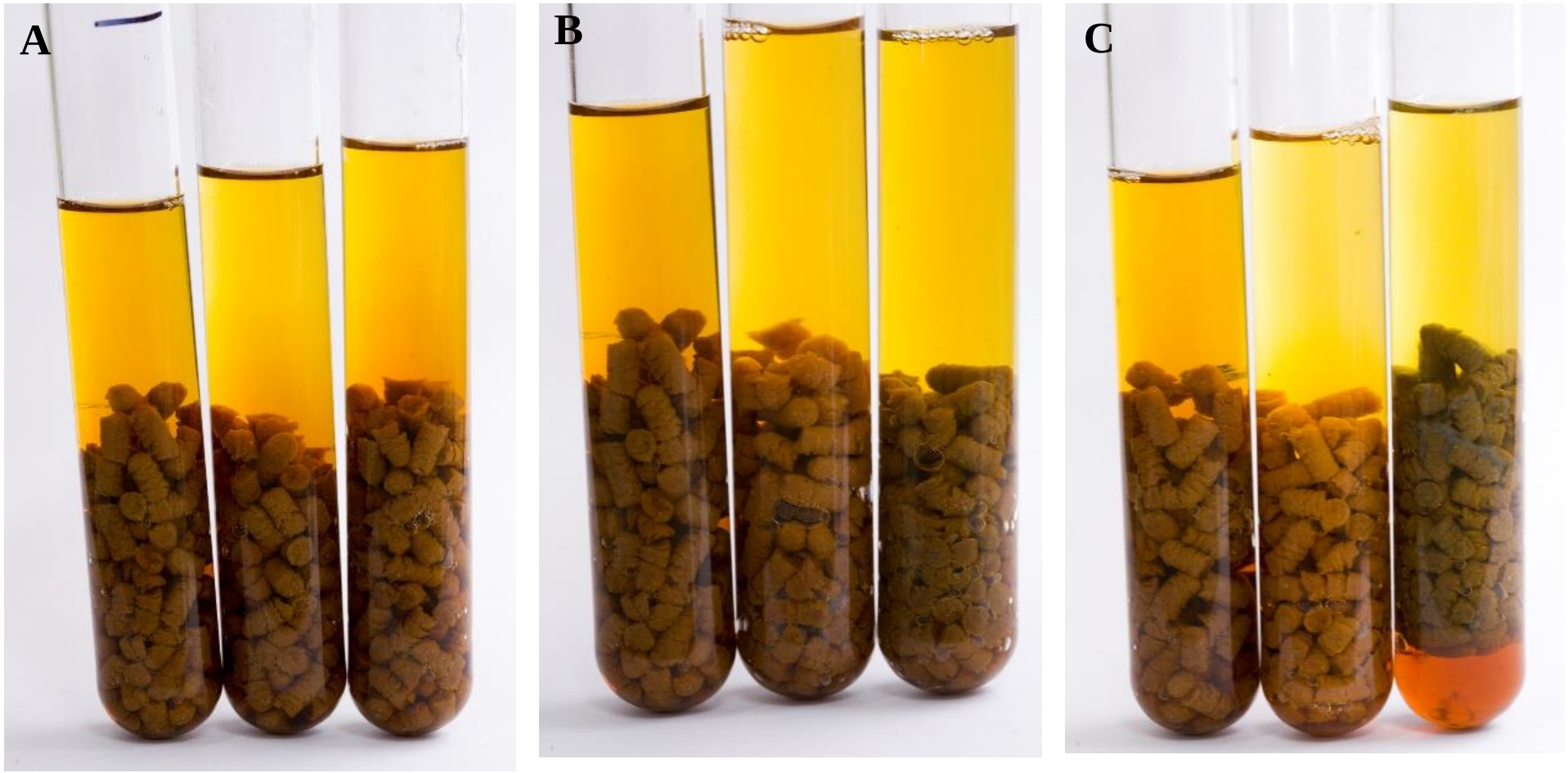


Figure 1 – Action of *T. ethanolicus* (A), *T. sulfurigignens* (B), *T. sulfurophilus* (C). Tubes left to right: control (not inoculated), no thiosulfate, 40 mM thiosulfate

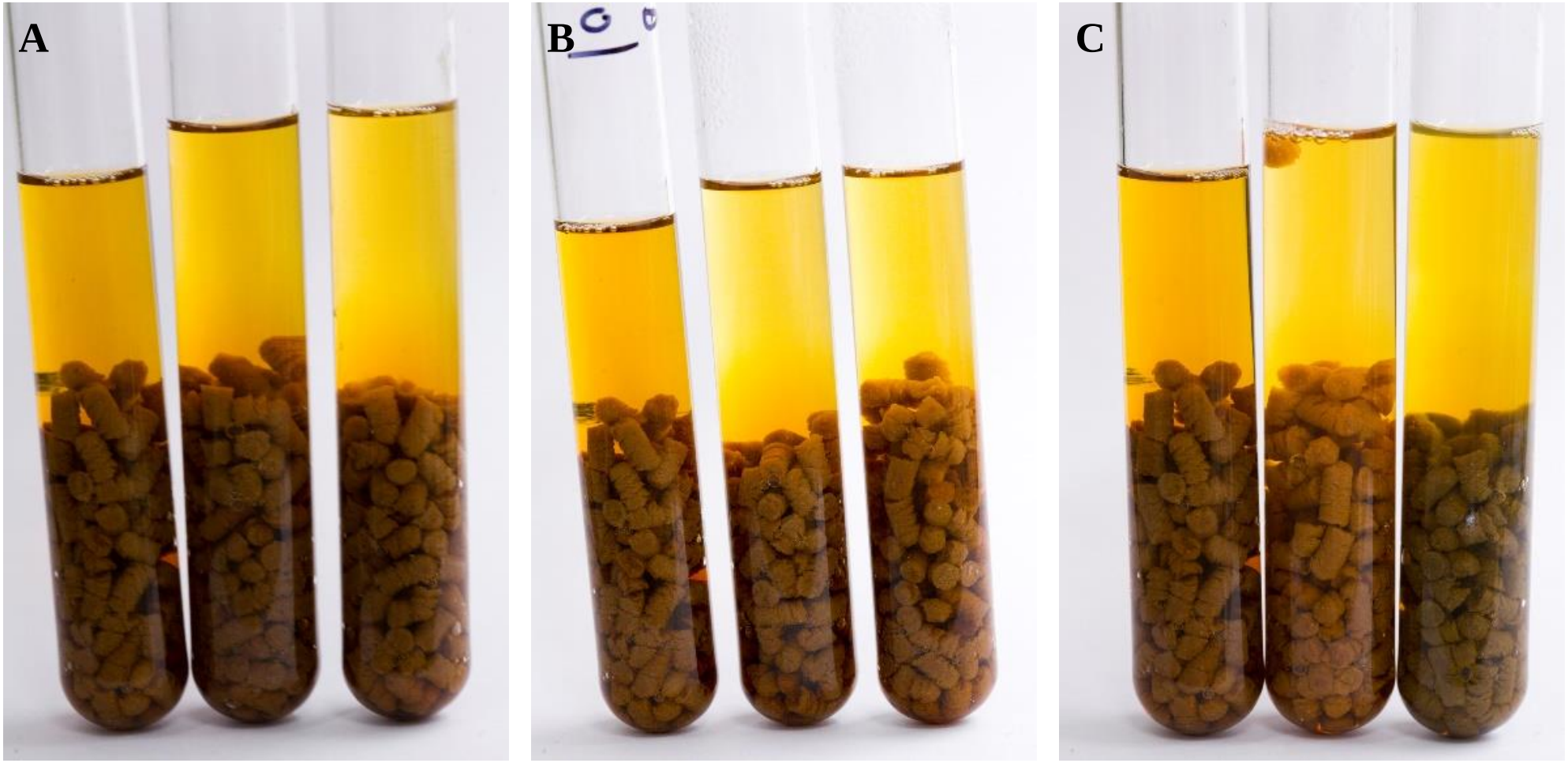


Figure 2 – Action of *C. subterraneus* subsp. *pacificus* (A), *Caldanaerobacter subterraneus* subsp. *subterraneus* (B), and *Caldanaerobacter subterraneus subsp. yonseiensis* (C) on cooked meat medium. Tubes left to right: control (not inoculated), no thiosulfate addition, 40 mM thiosulfate.

3.2. Degradation and fermentation of proteins

In order to gain a better understanding of the proteolytic nature of *Thermoanaerobacter* and *Caldanaerobacter* species, individual strains were cultivated on several selected proteins (casein, tryptone, gelatin, collagen, and keratin) which represent various states of protein hydrolysis with and without the addition of thiosulfate. To assess the proteolytic nature of the strains, end product formation as well as several metrics to examine changes to the proteins present were examined as well as general protease activity (measured by activity of caseinase, gelatinase, and collagenase) were evaluated. Additionally, the change in the degree of hydrolysis and the change in the amount of soluble protein were quantified. The skim milk medium from the previous experiment was included.

Three representative examples from *Thermoanaerobacter* are provided in Figure 3 with Figure 4 containing examples from *Caldanaerobacter*. The remaining strain data can be found in Supplemental Materials. The type strain of *Thermoanaerobacter, T. ethanolicus*, showed very low protease activities, changes in degree of hydrolysis and protein concentration, and low amounts of volatile end products from the proteins examined (Figure 3A). *T. sulfurigignens* (DSM 17917) and *T. sulfurophilus* (DSM 12299) (Figure 3B and C, respectively) showed higher protease, gelatinase, and collagenase activities after 7 days and higher end product formation yielding 47 and 37 mM of acetate from skim milk in the presence of thiosulfate, respectively and traces of propionate, butyrate, and 3-methyl-1-butryate.

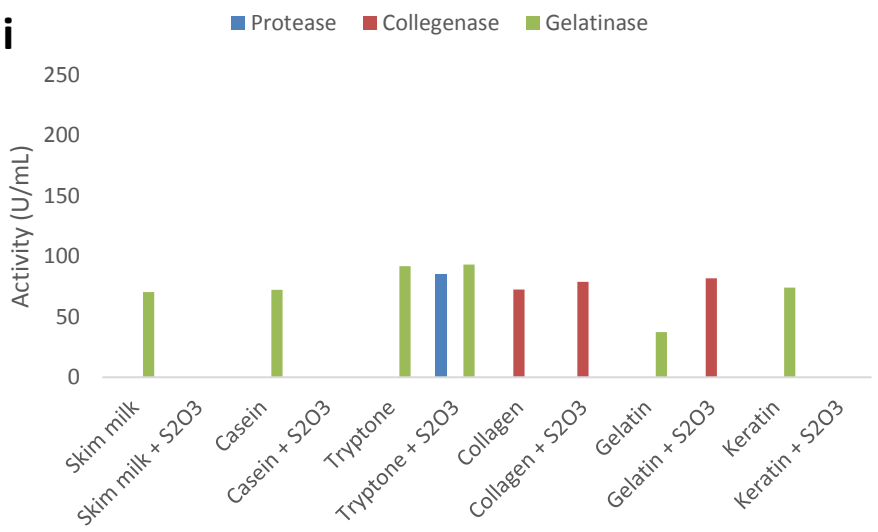
The vast majority of strains examined demonstrated some non-specific protease activity when cultivated on skim milk, casein, or tryptone with no more than 250 U/mL being detectable. Generally speaking, all *Caldanaerobacter* strains examined tended towards higher overall protease activity as compared with *Thermoanaerobacter* strains. There was no apparent change in the degree of hydrolysis or protein concentration in the skim milk, further supporting the previous qualitative observations that all strains do not show action towards skim milk medium. That stated, some strains demonstrated enhanced end product formation over yeast extract controls from skim milk. For example, both *Thermoanaerobacter sulfurigignes* (DSM 17917) and *Thermoanaerobacter sulfurophilus* (DSM 11584), produced acetate (47.3 and 37.1 mM) and traces of other short-chain fatty acids when cultivated on skim milk in the presence of thiosulfate.

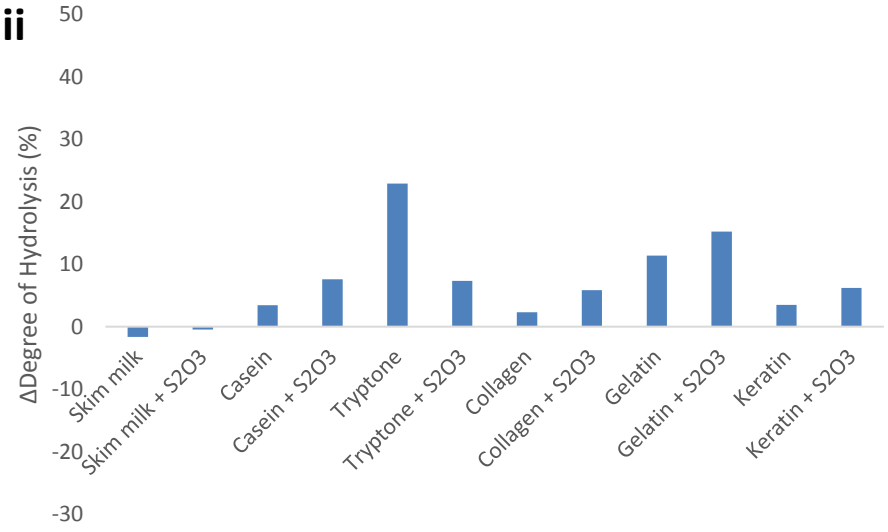
The ability to degrade collagen and gelatin (partially hydrolyzed collagen) are often reported features of *Clostridia*. The vast majority of *Thermoanaerobacter* and *Caldanaerobacter* strains examined showed little or no activity or end product formation from either collagen or gelatin. Two notable exception include *T. brockii* subsp. *brockii* (DSM 1457) and *T. brockii* subsp. *finnii* (DSM 3389) when they were cultivated on collagen, showing 248 and 790 U/mL versus 198 U/mL and 763 U/mL on collagen and collagen with thiosulfate were detected, respectively. This suggests that both of these strains are collagenolytic which is further supported by increased degree of hydrolysis (DH) and soluble protein. However, the observed gelatinase activity on gelatin and

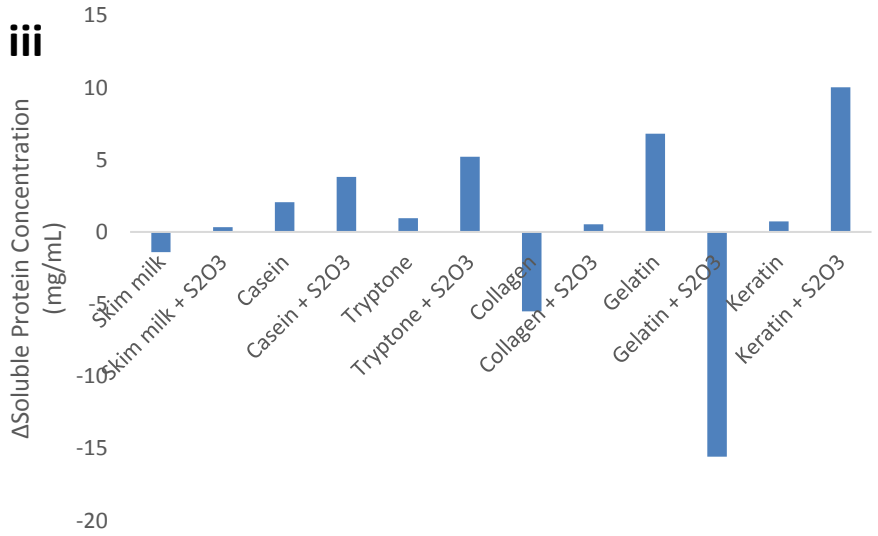
collagen-grown cultures by other species tested was always less than 250 U/mL (data not shown).

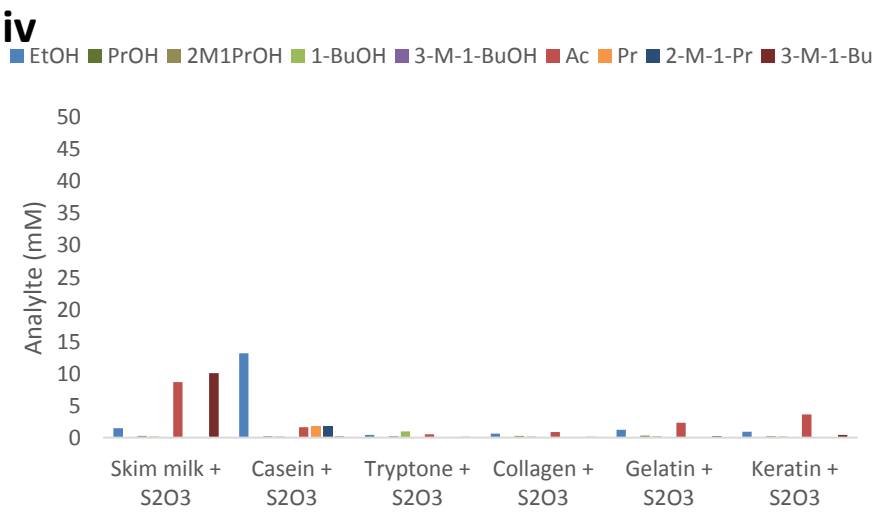
Alterations in the composition and concentration of the soluble protein were also examined using changes in the degree of hydrolysis and changes in the soluble protein concentration, respectively. These metrics must be used with caution and in parallel with end product formation as changes especially in light of the fact that thiosulfate may improve utilization of some amino acids. Decreases in the degree of hydrolysis can be interpreted as a consumption of the liberated amino acids while an increase indicates liberation of free amino acids. The largest changes in DH were typically observed on the protein sources with a higher degree of hydrolysis (i.e. tryptone and gelatin). The strains exhibiting the largest change in DH were *T. brockii* subsp. *finnii* (DSM 3389) which showed a net positive change of 47.4% while *T. kivui* (DSM 2030) showed a -25.8% DH on tryptone in the presence of thiosulfate suggesting that they are both using the liberated amino acids.

A

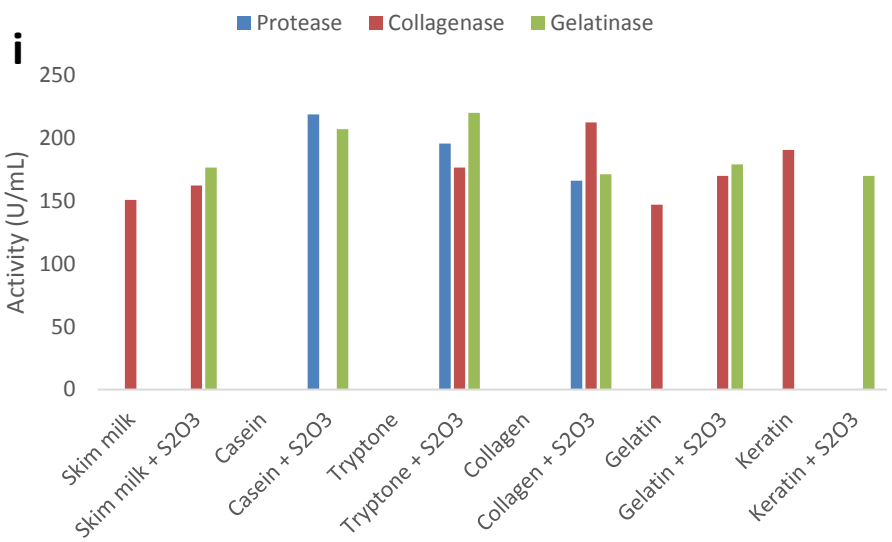

i
Protease
Collegenase
Gelatinase
Activity (U/mL)
250
200
150
100
50
0
Skim milk
Skim milk + S2O3
Casein
Casein + S2O3
Tryptone
Tryptone + S2O3
Collagen
Collagen + S2O3
Gelatin
Gelatin + S2O3
Keratin
Keratin + S2O3


ii
ΔDegree of Hydrolysis (%)
50
40
30
20
10
0
-10
-20
-30
Skim milk
Skim milk + S2O3
Casein
Casein + S2O3
Tryptone
Tryptone + S2O3
Collagen
Collagen + S2O3
Gelatin
Gelatin + S2O3
Keratin
Keratin + S2O3


iii
ΔSoluble Protein Concentration (mg/mL)
15
10
5
0
-5
-10
-15
-20
Skim milk
Skim milk + S2O3
Casein
Casein + S2O3
Tryptone
Tryptone + S2O3
Collagen
Collagen + S2O3
Gelatin
Gelatin + S2O3
Keratin
Keratin + S2O3


iv
EtOH
PrOH
2M1PrOH
1-BuOH
3-M-1-BuOH
Ac
Pr
2-M-1-Pr
3-M-1-Bu
Analyte (mM)
50
45
40
35
30
25
20
15
10
5
0
Skim milk + S2O3
Casein + S2O3
Tryptone + S2O3
Collagen + S2O3
Gelatin + S2O3
Keratin + S2O3

B


i
Protease
Collagenase
Gelatinase
Activity (U/mL)
250
200
150
100
50
0
Skim milk
Skim milk + S2O3
Casein
Casein + S2O3
Tryptone
Tryptone + S2O3
Collagen
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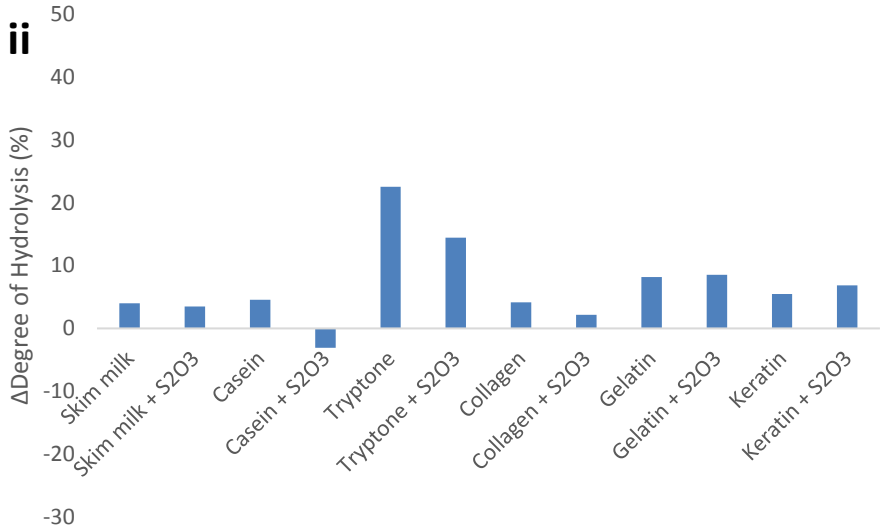
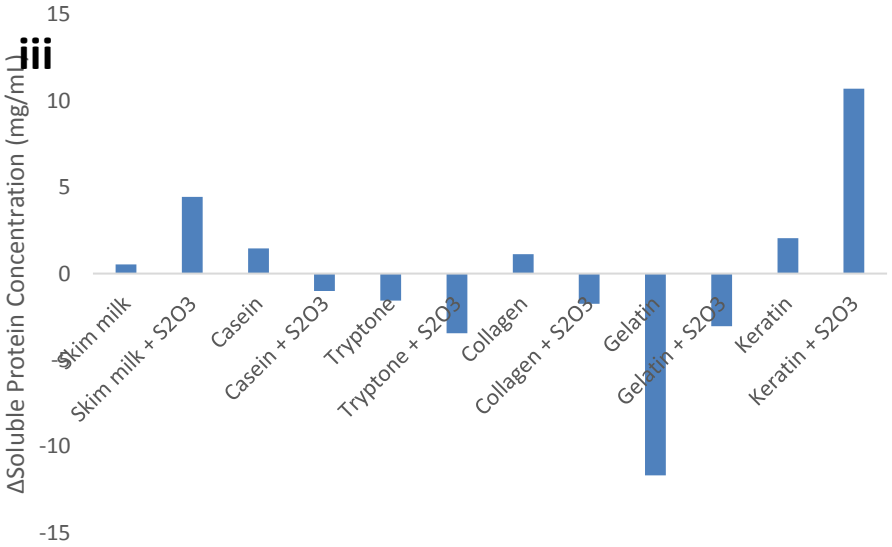
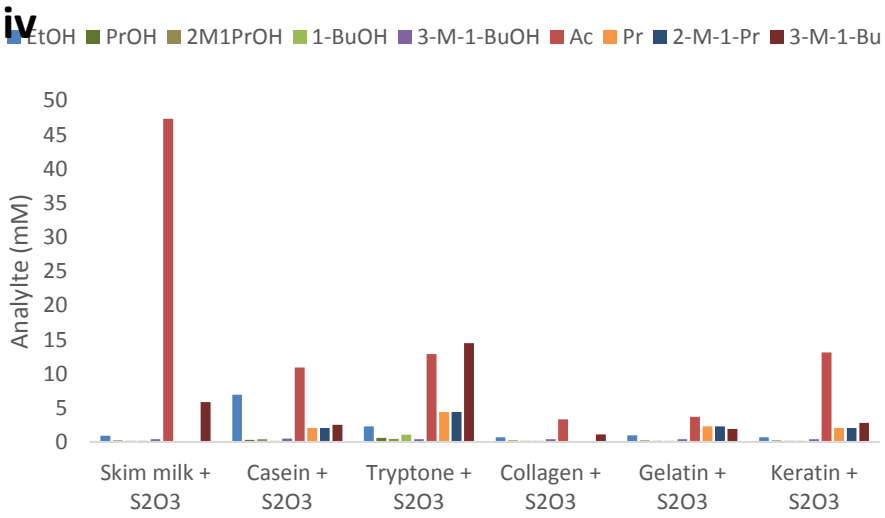
Gelatin
Gelatin + S2O3
Keratin
Keratin + S2O3


ii
ΔDegree of Hydrolysis (%)
50
40
30
20
10
0
-10
-20
-30
Skim milk
Skim milk + S2O3
Casein
Casein + S2O3
Tryptone
Tryptone + S2O3
Collagen
Collagen + S2O3
Gelatin
Gelatin + S2O3
Keratin
Keratin + S2O3


iii
ΔSoluble Protein Concentration (mg/mL)
15
10
5
0
-10
-15
Skim milk
Skim milk + S2O3
Casein
Casein + S2O3
Tryptone
Tryptone + S2O3
Collagen
Collagen + S2O3
Gelatin
Gelatin + S2O3
Keratin
Keratin + S2O3


iv
EtOH
PrOH
2M1PrOH
1-BuOH
3-M-1-BuOH
Ac
Pr
2-M-1-Pr
3-M-1-Bu
Analyte (mM)
50
45
40
35
30
25
20
15
10
5
0
Skim milk + S2O3
Casein + S2O3
Tryptone + S2O3
Collagen + S2O3
Gelatin + S2O3
Keratin + S2O3

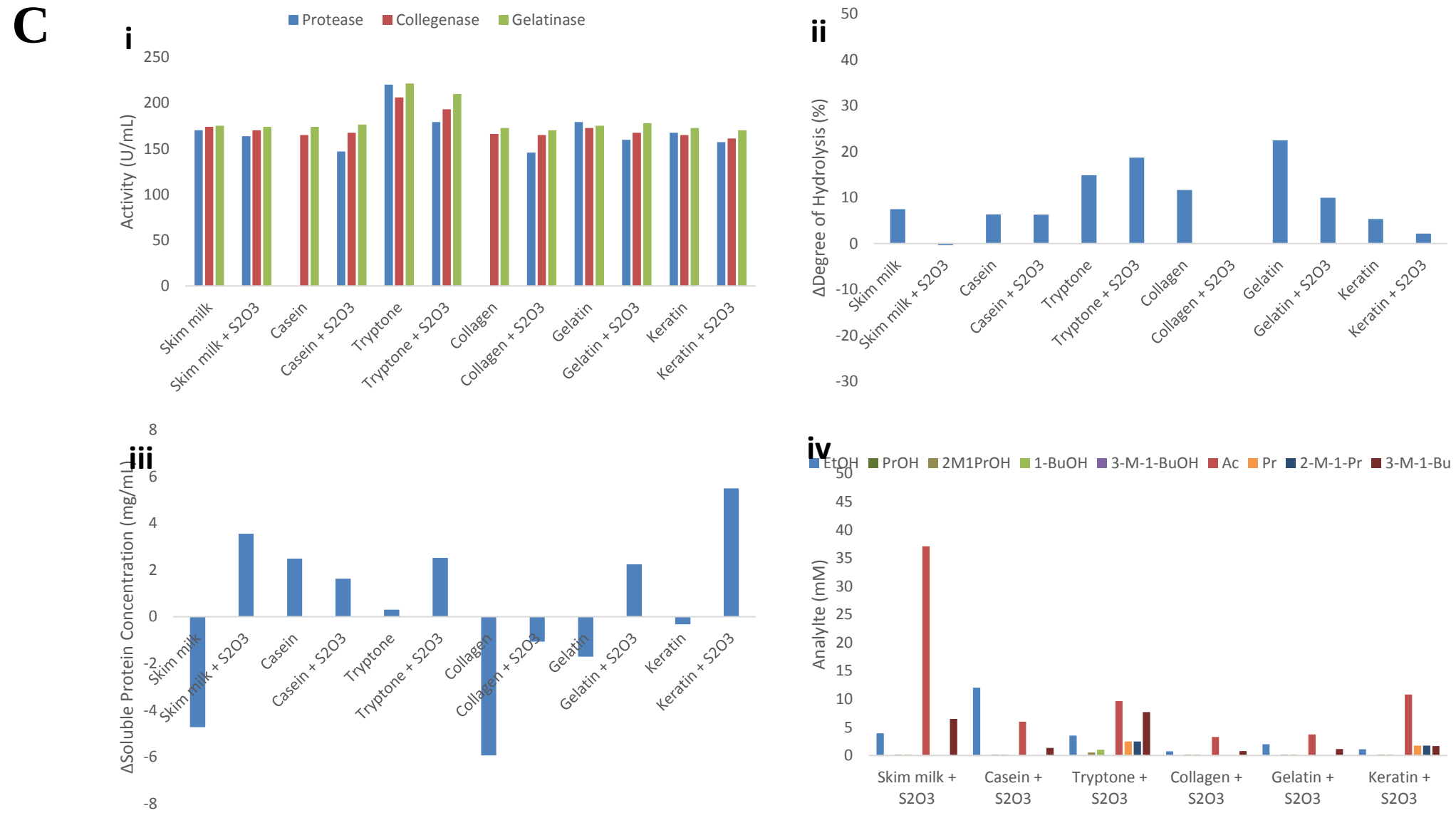
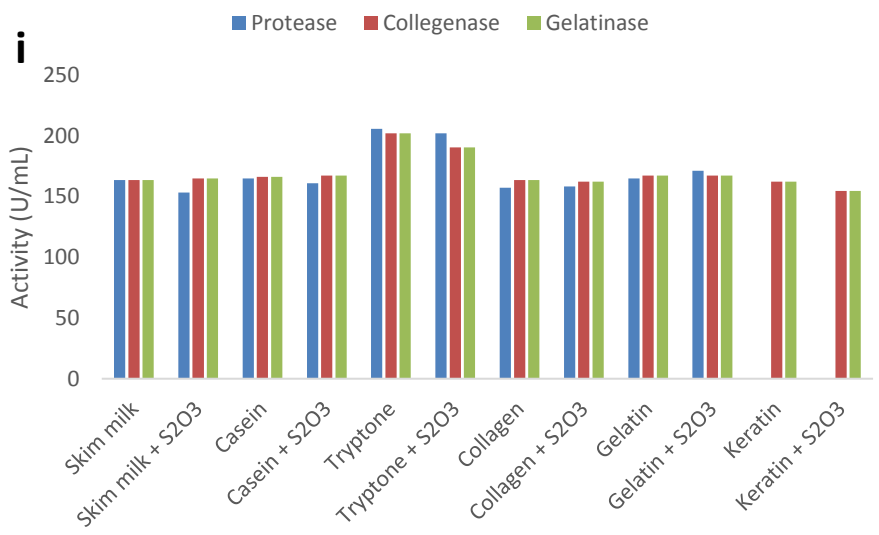
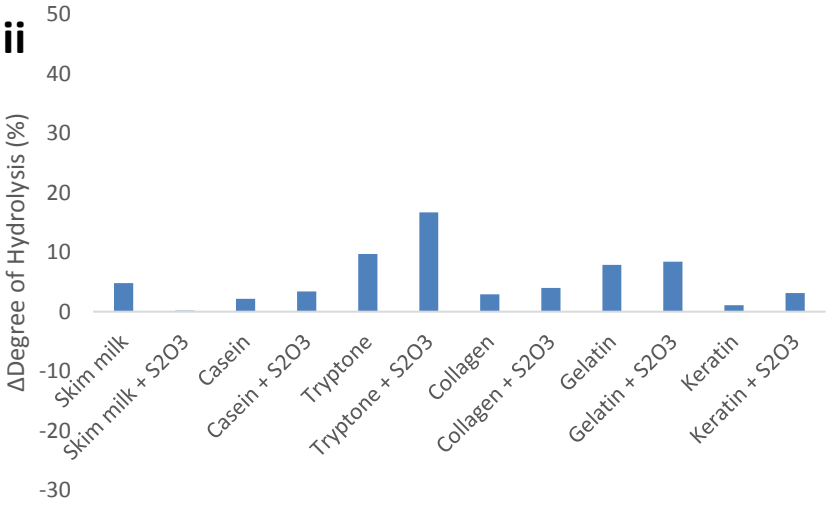
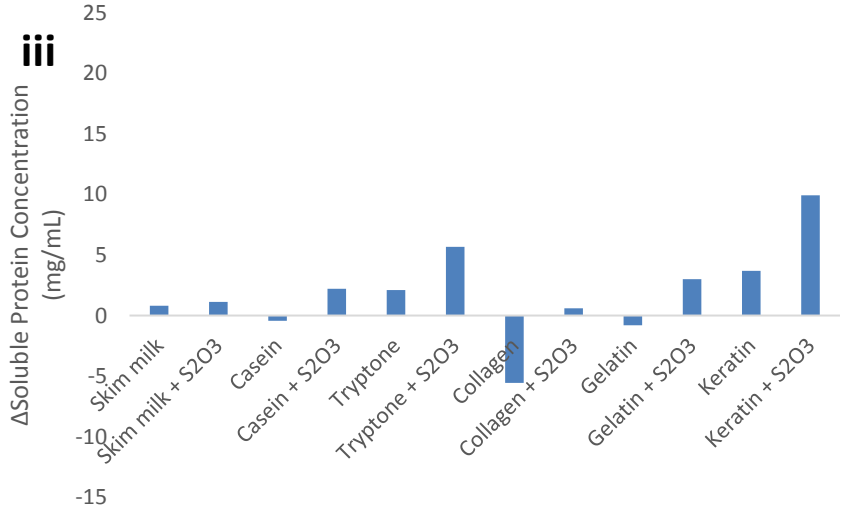


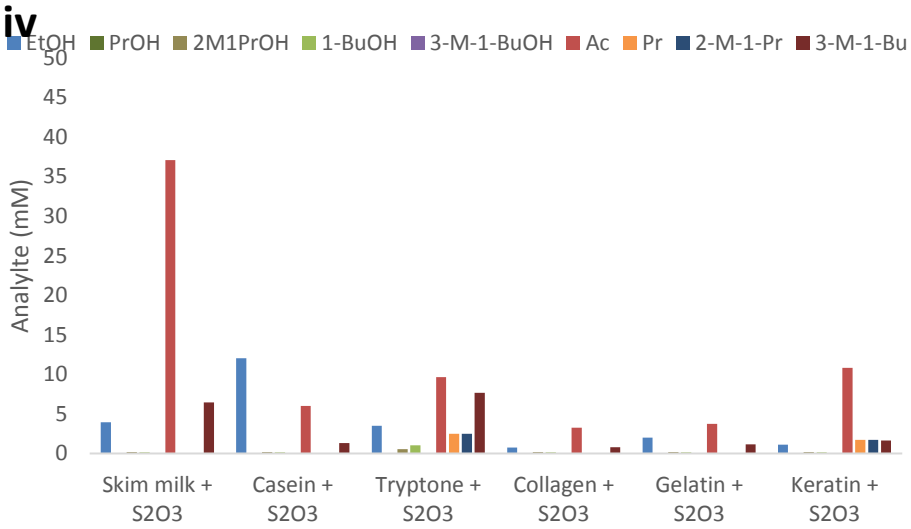
Figure 3 - Fermentation of selected proteins by *T. ethanolicus* (A), *T. sulfurigignens* (B), *T. sulfurophilus* (C) with or without thiosulfate (40 mM) addition after 7 days. Panel (i) enzyme activites, (ii) change in degree of hydrolysis, (iii) change in protein concentration), (iv) fermentation end products.

A

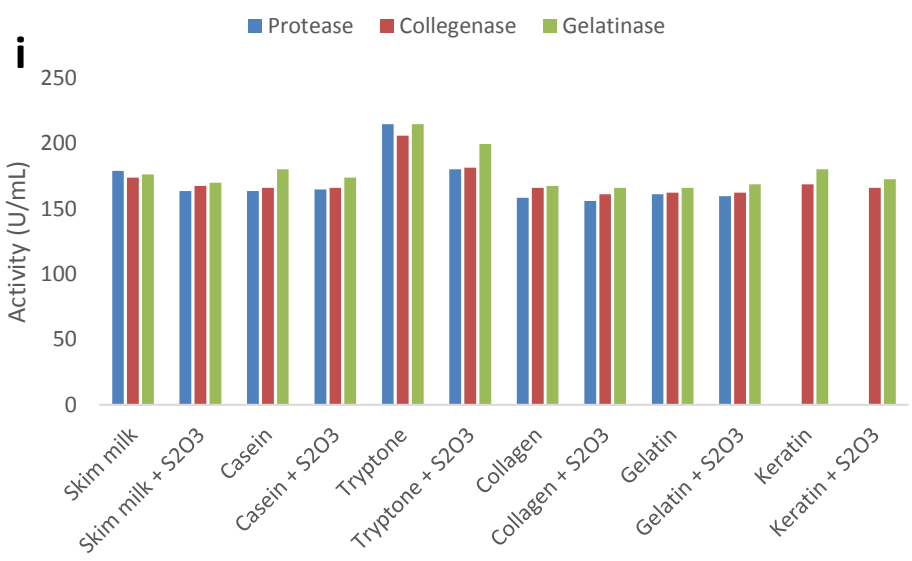

i
Protease
Collegenase
Gelatinase
Activity (U/mL)
250
200
150
100
50
0
Skim milk
Skim milk + S2O3
Casein
Casein + S2O3
Tryptone
Tryptone + S2O3
Collagen
Collagen + S2O3
Gelatin
Gelatin + S2O3
Keratin
Keratin + S2O3

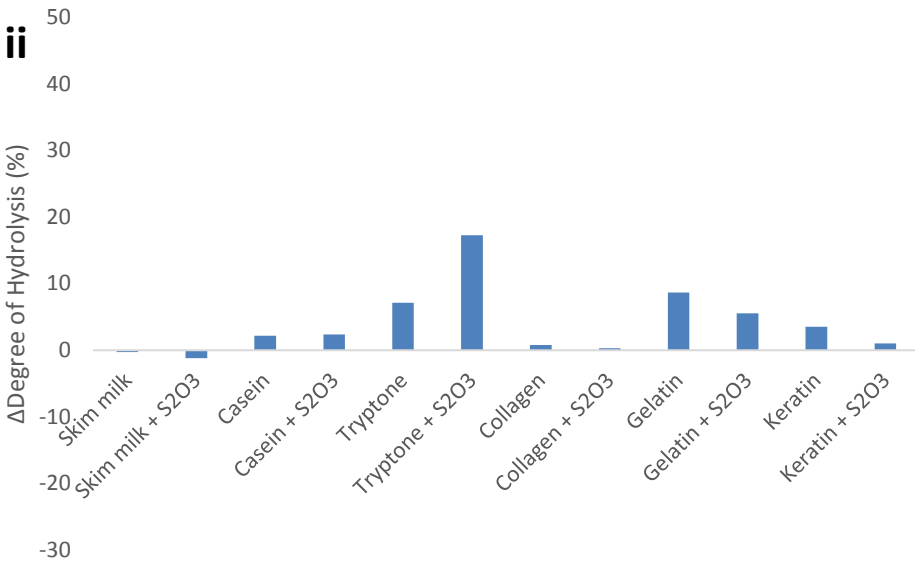

ii
ΔDegree of Hydrolysis (%)
50
40
30
20
10
0
-10
-20
-30
Skim milk
Skim milk + S2O3
Casein
Casein + S2O3
Tryptone
Tryptone + S2O3
Collagen
Collagen + S2O3
Gelatin
Gelatin + S2O3
Keratin
Keratin + S2O3

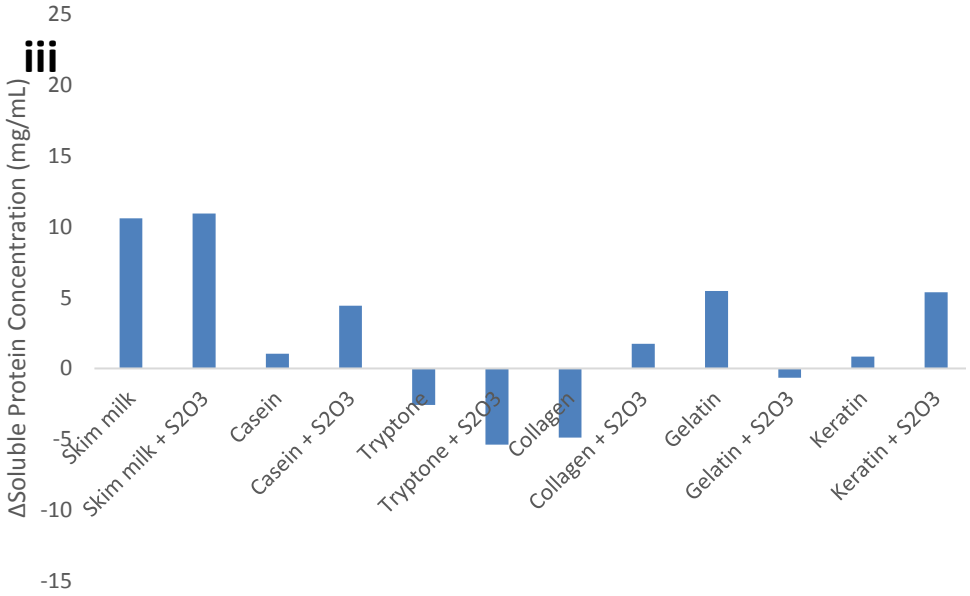

iii
ΔSoluble Protein Concentration (mg/mL)
25
20
15
10
5
0
-5
-10
-15
Skim milk
Skim milk + S2O3
Casein
Casein + S2O3
Tryptone
Tryptone + S2O3
Collagen
Collagen + S2O3
Gelatin
Gelatin + S2O3
Keratin
Keratin + S2O3

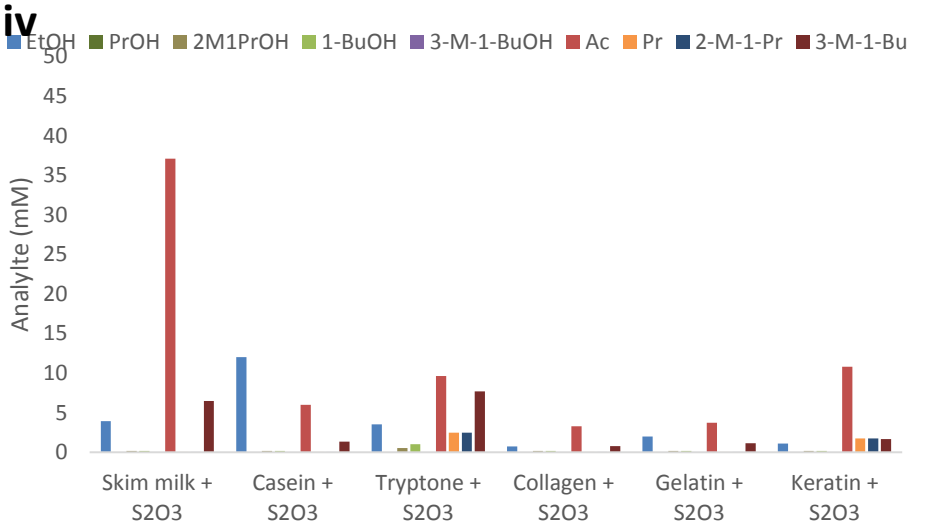

iv
EtOH
PrOH
2M1PrOH
1-BuOH
3-M-1-BuOH
Ac
Pr
2-M-1-Pr
3-M-1-Bu
Analyte (mM)
50
45
40
35
30
25
20
15
10
5
0
Skim milk + S2O3
Casein + S2O3
Tryptone + S2O3
Collagen + S2O3
Gelatin + S2O3
Keratin + S2O3

B


i
Protease
Collegenase
Gelatinase
Activity (U/mL)
250
200
150
100
50
0
Skim milk
Skim milk + S2O3
Casein
Casein + S2O3
Tryptone
Tryptone + S2O3
Collagen
Collagen + S2O3
Gelatin
Gelatin + S2O3
Keratin
Keratin + S2O3


ii
ΔDegree of Hydrolysis (%)
50
40
30
20
10
0
-10
-20
-30
Skim milk
Skim milk + S2O3
Casein
Casein + S2O3
Tryptone
Tryptone + S2O3
Collagen
Collagen + S2O3
Gelatin
Gelatin + S2O3
Keratin
Keratin + S2O3


iii
ΔSoluble Protein Concentration (mg/mL)
25
20
15
10
5
0
-5
-10
-15
Skim milk
Skim milk + S2O3
Casein
Casein + S2O3
Tryptone
Tryptone + S2O3
Collagen
Collagen + S2O3
Gelatin
Gelatin + S2O3
Keratin
Keratin + S2O3


iv
EtOH
PrOH
2M1PrOH
1-BuOH
3-M-1-BuOH
Ac
Pr
2-M-1-Pr
3-M-1-Bu
Analyte (mM)
50
45
40
35
30
25
20
15
10
5
0
Skim milk + S2O3
Casein + S2O3
Tryptone + S2O3
Collagen + S2O3
Gelatin + S2O3
Keratin + S2O3

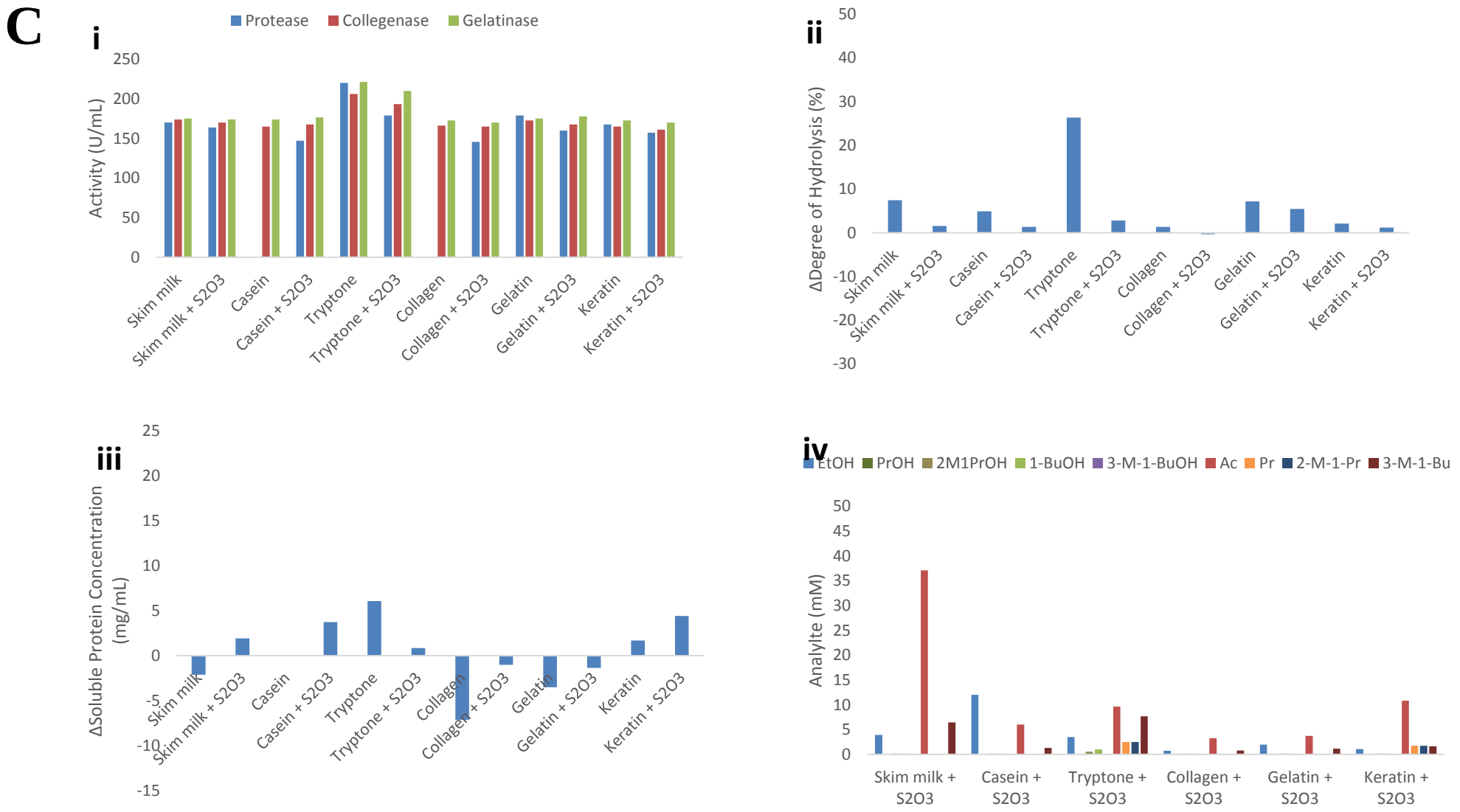


Figure 4 – Fermentation of selected proteins by *C. subterraneus* subsp. *pacificus* (A), *Caldanaerobacter subterraneus* subsp. *subterraneus* (B), and *Caldanaerobacter subterraneus subsp. yonseiensis* (C) with or without thiosulfate (40 mM) addition after 7 days. Panel (i) enzyme activities, (ii) change in degree of hydrolysis, (iii) change in protein concentration), (iv) fermentation end products.

3.3 Substrate spectra and end product formation from amino acids

Whether some strains may be considered proteolytic or not, that does not necessary reveal if they can degrade single amino acids. Since, from earlier work it has been clearly demonstrated that scavenging electrons produced for degradation of many amino acids is of great importance we decided to cultivate all strains on all 20 proteogenic amino acids without and with thiosulfate (Table 2). It is well known that species within both genera tested can use thiosulfate as an electron scavenging molecule. Thus, it is unsurprising that only two strains, *T. uzonensis* and *C. subterraneus* subsp. *tengcongensis*, could degrade more than three amino acids without the addition of thiosulfate (Table 2). Especially, neutral (serine, threonine) and oxidized (glycine) amino acids can be degraded without an electron scavenging system. Indeed, serine and threonine were degraded by nine and four strains without thiosulfate addition, respectively. Addition of thiosulfate, however, not surprisingly, increased the number of amino acids degraded and number of strains that showed positive reaction on single amino acid utilization. Many amino acids were only degraded in the presence of thiosulfate, e.g. the BCAAs and the ArAAs.

For simplicity the amino acids are classified as 1) nonpolar, aliphatic R-groups (glycine, alanine, proline, valine, leucine, isoleucine, methionine), 2) aromatic groups (phenylalanine, tyrosine, tryptophan), 3) polar, uncharged R-groups (serine, threonine, cysteine, asparagine, glutamine), 4) positively charged R-groups (lysine, histidine, arginine) and 5) negatively charged R-groups in Table 1.

Of the nonpolar, aliphatic amino acids glycine was degraded by *T. uzonensis* (DSM 18761) and *C. subterraneus* subsp. *tengcongensis* (DSM 15242) both with and without thiosulfate (Table 2). Alanine was degraded by *C. subterraneus* subsp. *tengcongensis* (DSM 15242) as the sole substrate but several strains showed weak positive response. Three strains could utilize alanine in the presence of thiosulfate *T. uzonensis* (DSM 18761), *C. subterraneus* subsp. *yonseiensis* (DSM 13777), and *T. sulfurophilus* (DSM 11584). Proline was degraded by only *C. subterraneus subsp. tengcongensis* (DSM 15242) with thiosulfate addition. Methionine was degraded by only *C. subterraneus* subsp. *yonseiensis* (DSM 13777) without thiosulfate but almost all (8 positive and 9 weakly positive) strains could degrade the amino acid with thiosulfate. End product formation from these four amino acids were acetate, propionate, carbon dioxide, and hydrogen (results not shown). BCAAs were degraded by all members of both genera in the presence of thiosulfate but not without an electron acceptor. End products from the BCAAs in the presence of thiosulfate were in all cases a mixture of their BCFA and BCOH (results not shown). The BCFAs and BCOHs are well known as important flavour compounds in cheeses (Kranenburg et al. 2002).

The three aromatic amino acids, phenylalanine, tyrosine and tryptophan were degraded by *C. subterraneus* subsp. *tengcongensis* (DSM 15242) and *C. subterraneus* subsp. *yonseiensis* (DSM 13777), but only in the presence of thiosulfate. End products, analysed by TLC, were a mixture of their corresponding aromatic fatty acids and alcohol (results not shown). The compound formed from aromatic amino acid degradation, e.g. phenylacetaldehyde, phenylacetic acid and indole-3-acetate are valuable compounds that may be used to scent perfume, dye textiles and synthesize antibiotics (Barden, 2011, Letizia et al. 2003, Luengo et al. 2001). To our knowledge no thermophilic bacterium has been shown to anaerobically degrade the aromatic amino acids to their corresponding fatty

acids and alcohols although this phenomenon is well-known amoung complex consortia of rumen microorganisms.

The polar, uncharged R-group amino acids, serine, threonine, cysteine, asparagine and glutamine were degraded by many of the bacteria tested. Serine was degraded by nine strains as a single substrate but by 12 strains in the presence of thiosulfate. Four and nine strains degraded threonine in the absence and presence of thiosulfate, respectively. Mainly, serine and threonine were degraded to their one chain shorter fatty acid, acetate and propionate, respectively (results not shown). Only *C. subterraneus* subsp. *tengcongensis* (DSM 15242) could degrade cysteine as a single substrate but eight strains degraded the amino acid (mainly to acetate, CO_2, and H_2; results not shown) in the presence of thiosulfate. Asparagine and glutamine were only degraded by handful of strains in the presence of thiosulfate.

Of the amino acids with R-positively charged groups (lysine, histidine, and arginine) only lysine was degraded (by nine strains) in the presence of thiosulfate but several strains showed a weak positive response. Finally, the two amino acids with negatively charged R group (aspartic acid and glutamic acid) were only degraded by handful of strains in the presence of thiosulfate. *C. subterraneous* subsp. *tengcongensis* (DSM 15242) was the only strain degrading glutamic acid but four strains could degrade aspartic acid. End products from these two amino acids were mixture of acetate, butyrate, ethanol, CO_2 and H_2 (results not shown).

Table 2 Degradation of amino acids by members of the genera *Thermoanaerobacter* and *Caldanaerobacter*. + = growth as measured by more than 20% end-product formation as compared with control samples (yeast extract only). w = weak growth as analysed by less than 20% of end-product formation compared with control samples.

	T. *brockii* subsp. *brockii* (DSM 1457)	*T. brockii* subsp. *finnii* (DSM 3389)	*T. ethanolicus* (DSM 2246)	*T. italicus* (DSM 9252)	*T. kivui* (DSM 2030)	*T. mathranii* subsp. *mathranii* (DSM 11426)	*T. pentosaceusus* (DSM 25963)	*T. pseudoethanolicus* (DSM 2355)	*T. siderophilus* (DSM 12299)	*T. sulfurigignens* (DSM 17917)	*T. sulfurophilus* (DSM 11584)	*T. thermohydrosulfuricus* (DSM 567)	*T. uzonensis* (DSM 18761)	*T. wiegelii* (DSM 10319)	*C. subterraneus* subsp.*pacificus* (DSM 12653)	*C. subterraneus* subsp. *subteraneus* (DSM 13054)	*C. subterraneus* subsp. *tengcongensis* (DSM 15242)	*C. subterraneus* subsp. *yonseiensis* (DSM 13777)	*C. uzonensis* (DSM 18923)
Nonpolar R-group																			
Glycine	-	-	-	-	-	-	-	-	-	-	w	-	+	-	-	w	+	-	-
Glycine+ S_2O_3	-	-	-	-	-	-	-	-	-	-	w	-	+	-	-	-	w	-	-
Alanine	-	w	-	-	-	-	-	-	-	-	w	w	w	-	-	w	+	-	-
Alanine+ S_2O_3	-	w	-	-	-	-	w	-	-	-	+	w	+	-	-	w	w	+	-
Proline	-	-	-	-	-	-	-	-	-	-	-	-	w	-	-	-	w	w	-
Proline+ S_2O_3	-	-	-	-	-	-	-	-	-	-	-	-	w	-	-	-	+	w	-
Valine	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Valine+ S_2O_3	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Leucine	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-
Leucine+ S_2O_3	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Isoleucine	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-
Isoleucine+ S_2O_3	+	+	+	+	+	+		+	+	+	+	+	+	+	+	+	+	+	+
Methionine	-	-	-	-	-	-	-	-	-	-	-	-	-	w	-	-	w	+	-
Methionine+ S_2O_3	-	-	w	w	w	w	+	w	w	+	+	w	+	+	w	+	+	+	w
Aromatic R-group																			
Phenylalanine	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Phenylalanine+ S_2O_3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-
Tryptophan	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Tryptophan+ S_2O_3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-
Tyrosine	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Tyrosine+ S_2O_3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-

Table 2 (continued) Degradation of amino acids by members of the genera *Thermoanaerobacter* and *Caldanaerobacter*. + = growth as measured by more than 20% end-product formation as compared with control samples (yeast extract only). w = weak growth as analysed by less than 20% of end-product formation compared with control samples.

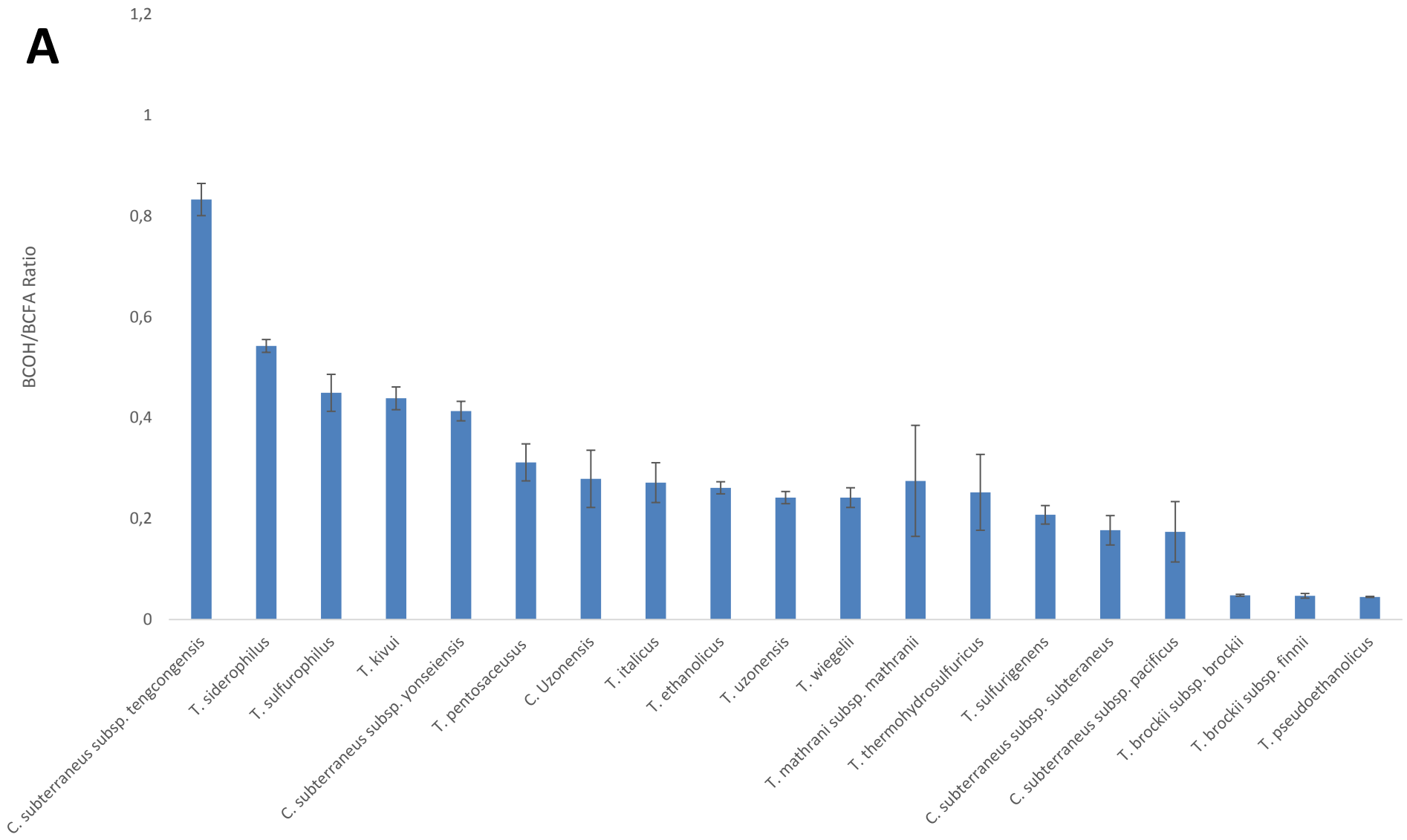
	T. brockii subsp. *brockii* (DSM 1457)	*T. brockii* subsp. *finnii* (DSM 3389)	*T. ethanolicus* (DSM 2246)	*T. italicus* (DSM 9252)	*T. kivui* (DSM 2030)	*T. mathranii* subsp. *mathranii* (DSM 11426)	*T. pentosaceusus* (DSM 25963)	*T. pseudoethanolicus* (DSM 2355)	*T. siderophilus* (DSM 12299)	*T. sulfurigignens* (DSM 17917)	*T. sulfurophilus* (DSM 11584)	*T. thermohydrosulfuricus* (DSM 567)	*T. uzonensis* (DSM 18761)	*T. wiegelii* (DSM 10319)	*C. subterraneus* subsp.*pacificus* (DSM 12653)	*C. subterraneus* subsp. *subteraneus* (DSM 13054)	*C. subterraneus* subsp. *tengcongensis* (DSM 15242)	*C. subterraneus* subsp. *yonseiensis* (DSM 13777)	*C. uzonensis* (DSM 18923)
Polar, uncharged R-group																			
Serine	+	+	w	-	-	-	-	+	w	+	+	-	+	-	-	+	+	+	-
Serine+ S_2O_3	+	+	+	-	-	w	+	+	+	+	+	-	+	-	w	+	+	+	-
Threonine	-	w	w	-	-	-	-	-	w	+	+	-	+	-	-	w	+	-	-
Threonine+ S_2O_3	+	w	+	-	-	-	-	w	+	+	+	-	+	-	w	+	+	+	-
Cysteine	-	-	-	w	-	-	w	-	w	-	w	-	+	-	-	-	-	-	-
Cysteine+ S_2O_3	-	+	-	+	-	-	+	-	+	+	w	-	+	+	w	-	+	+	-
Asparagine	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Asparagine+ S_2O_3	-	-	-	w	w	+	w	w	-	w	+	-	w	+	w	-	+	+	-
Glutamine	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Glutamine+ S_2O_3	-	-	-	w	+	-	w	+	w	w	+	+	+	+	-	-	+	+	-
Positive charged R group																			
Lysine	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Lysine+ S_2O_3	-	-	-	+	+	+	+	+	-	+	+	+	w	w	-	w	+	w	w
Histidine	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Histidine+ S_2O_3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Arginine	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Arginine + S_2O_3	-	-	-	-	-	w	-	w	-	-	-	-	-	-	-	-	-	-	-
Negatively charged R group																			
Aspartic acid	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Aspartic acid+ S_2O_3	-	-	-	+	-	-	+	w	-	w	w	-	w	-	-	-	+	+	-
Glutamic Acid	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	w	-	-
Glutamic Acid+ S_2O_3	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-

3.4. Degradation of branched-chain amino acids

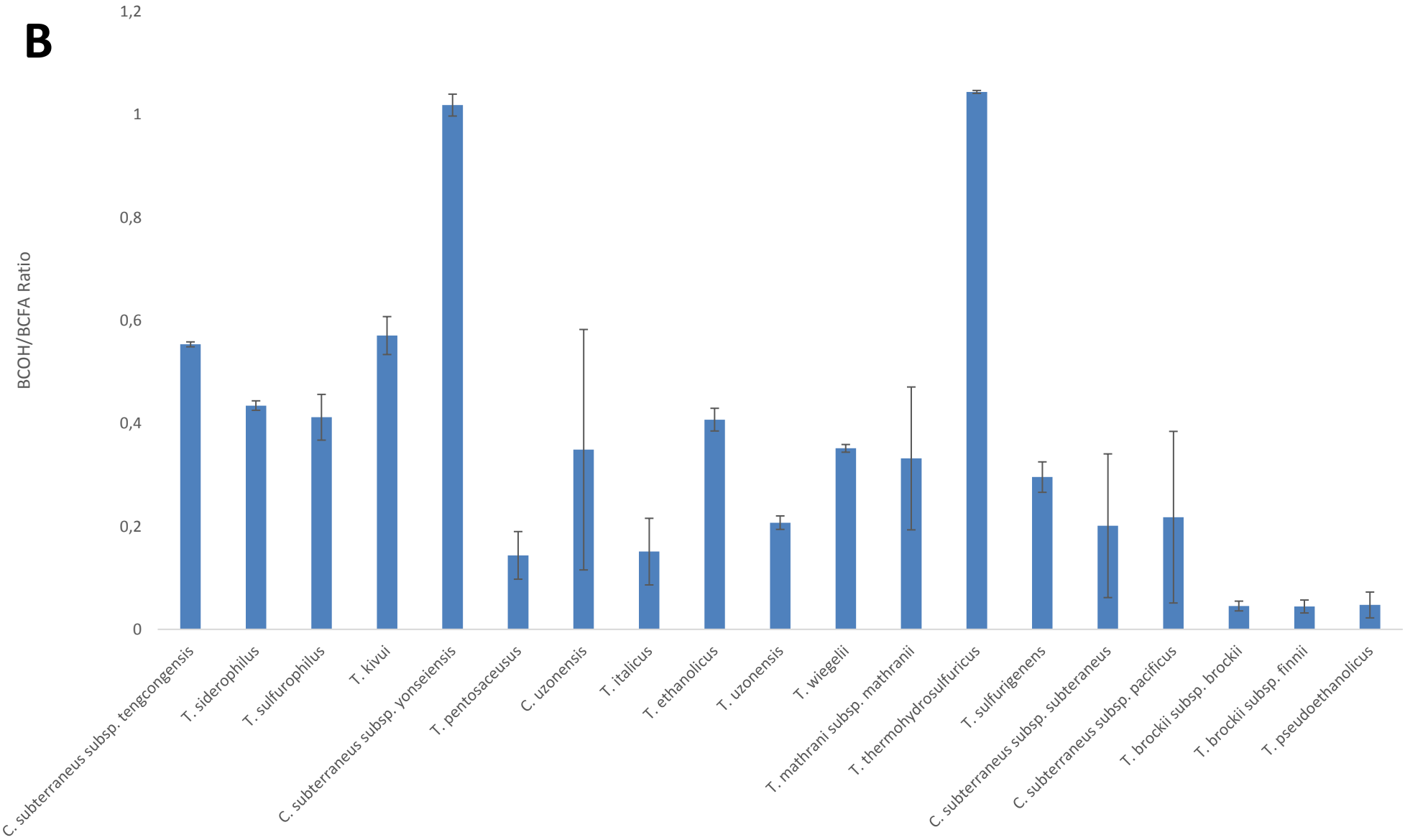
In present study a special attention was on the degradation of BCAAs (leucine, isoleucine, and valine). These amino acids are reduced and therefore, when degraded under anaerobic conditions, usually oxidized to their corresponding fatty acid. All strains tested in this investigation could not degrade any of the three BCAA when used as a single substrate (Table 1). This is in agreement to the fact that the oxidative deamination of these amino acids to their corresponding keto acids is thermodynamically highly unfavorable ($\Delta G^{\circ\prime}$ values between +51.5 to +57 kJ/mol). However, by scavenging the electron produced during degradation of BCAAs, it has been shown that they can be converted to their corresponding fatty acids (Fardeau et al., 1997; Scully & Orlygsson, 2014). The electron scavenging system can be either inorganic (addition of thiosulfate) or by co-cultivating the amino acid degrading bacteria in a co-culture with a hydrogen scavenging microorganism as exemplified by the work with *Thermoanaerobacter* strain AK90 (Scully & Orlygsson, 2015). In present investigation, thiosulfate was added together with separate BCAAs to all species investigated. In all cases this resulted in degradation of most of the BCAAs to not only their corresponding BCFAs but also to BCOHs (Figure 5A-C). Interestingly differences in the ratios of BCOH to BCFA appear for the genus with apparent differences between the resultant BCOH and BCFA from leucine and isoleucine in particular (Figure 5A-B). When valine was used as the carbon source however, the ratio of BCOH formation over the fatty acid was very similar between all species investigated (between 0.09 to 0.27), with the highest forming alcohol producer being *T. thermohydrosulfuricus* (DSM 567) with a molar ratio of 0.27 (Figure 5A). However, a more clear difference was observed on both of the six carbon amino acids. *Caldanaerobacter subterraneus* subsp. *subterraneus* (DSM 13054) produced almost the same amount of 3-methyl-1-butanol and 3-methyl-1-butyrate from leucine (Figure 5B) whereas *T. pseudoethanolicus* (DSM 2355) produced the alcohol only in minor amounts. Similar spectrum was shown for isoleucine degradation, *Caldanaerobacter subterraneus* subsp. *subterraneus* (DSM 13054) being the best alcohol producer but *T. pseudoethanolicus* (DSM 2355) the worst (Figure 5C). In general, *Caldanaerobacter* species produced more of the alcohol as compared with *Thermoanaerobacter* species. The ratio of BCOH over the BCFA was 0.37 and 0.26 during leucine degradation for *Caldanaerobacter* and *Thermoanaerobacter*, respectively. The values for valine were 0.20 and 0.17 and 0.37 and 0.27 for isoleucine for *Caldanaerobacter* and *Thermoanaerobacter*, respectively.

Until recently, it was unclear if the bacteria within these two genera were oxidizing the keto acid from its corresponding amino acid to a mixture of the acid and the alcohol or if the bacteria were first producing the acid and then converting it to their corresponding alcohol. The latter would suggest the fatty acid is acting as an electron acceptor for the alcohol formation. In recent investigation in our laboratory, *Thermoanaerobacter* strain AK85 was shown to produce more of the BCOH compared with the BCFA which is opposite to present investigation on tested *Thermoanaerobacter* and *Caldanaerobacter* strains. *T. pseudoethanolicus* (DSM 2355) has also been investigated in some detail recently (**Paper V**) showing that the concentration of the BCFA over the BCOH was always higher. Indeed, *T. pseudoethanolicus* (DSM 2355) is the strain producing least of the BCOH in present study (Figure 5A,C). These contradictory results may be explained by the fact that, in case of strain AK85, the strain was cultivated for two weeks instead of one week (*T. pseudoethanolicus* and present study). Thus, it seems likely that some of the strains are able to convert their BCFA to their corresponding BCOH. This has since then

been reported by using radiolabeled fatty acids showing that they are converted by both *Thermoanaerobacter* and *Caldanaerobacter* species.


A
1,2
1
0,8
0,6
0,4
0,2
0
BCOH/BCFA Ratio
C. subterraneus subsp. tengcongensis
T. siderophilus
T. sulfurophilus
T. kivui
C. subterraneus subsp. yonseiensis
T. pentosaceusus
C. Uzonensis
T. italicus
T. ethanolicus
T. uzonensis
T. wiegelii
T. mathrani subsp. mathranii
T. thermohydrosulfuricus
T. sulfurigenens
C. subterraneus subsp. subteraneus
C. subterraneus subsp. pacificus
T. brockii subsp. brockii
T. brockii subsp. finnii
T. pseudoethanolicus

B


1,2
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BCOH/BCFA Ratio
C. subterraneus subsp. tengcongensis
T. siderophilus
T. sulfurophilus
T. kivui
C. subterraneus subsp. yonseiensis
T. pentosaceusus
C. uzonensis
T. italicus
T. ethanolicus
T. uzonensis
T. wiegelii
T. mathrani subsp. mathranii
T. thermohydrosulfuricus
T. sulfurigenens
C. subterraneus subsp. subteraneus
C. subterraneus subsp. pacificus
T. brockii subsp. brockii
T. brockii subsp. finnii
T. pseudoethanolicus

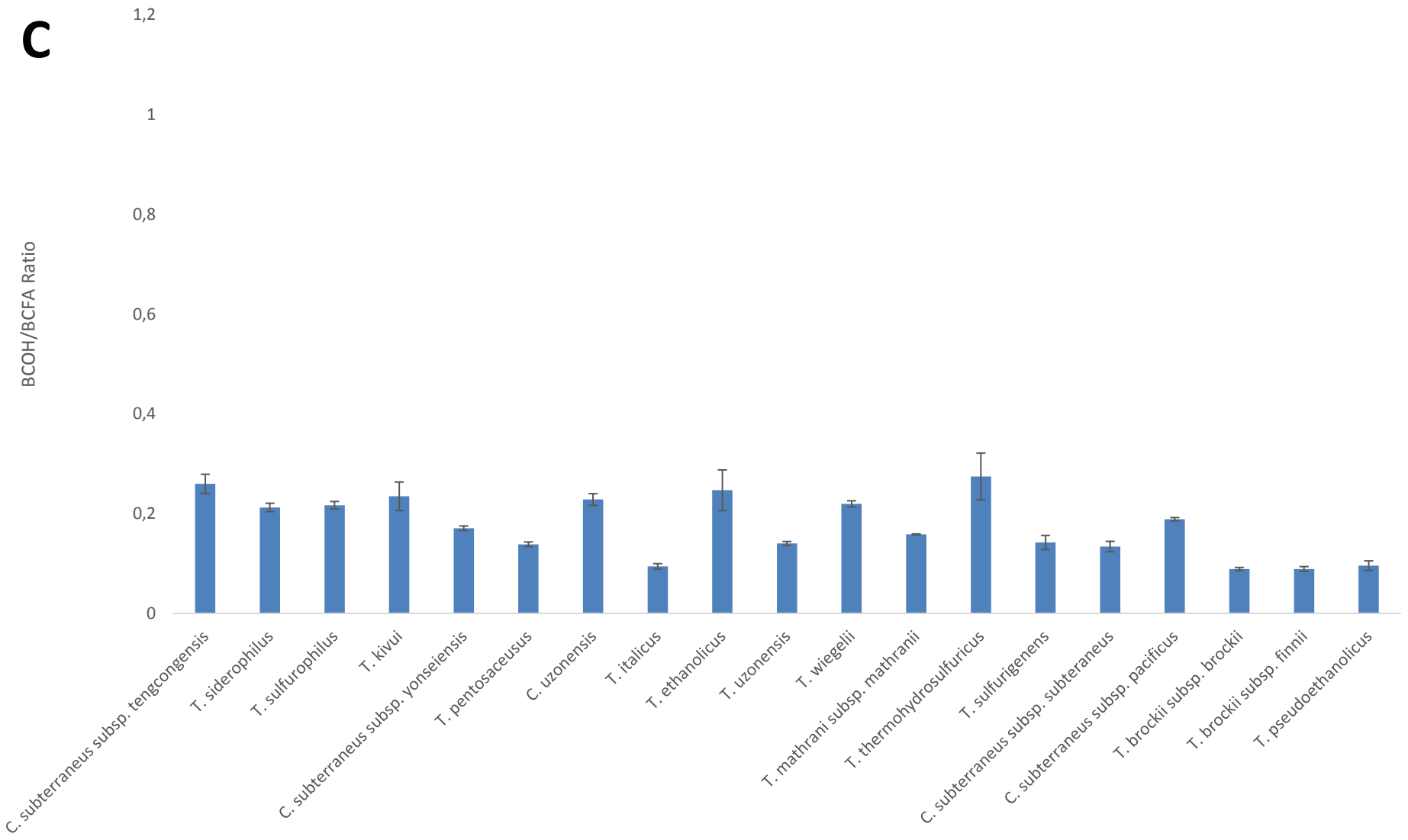

Figure 5 - Ratio of the formation of BCOH and BCFA from BCFA (A = leucine, B = isoleucine, and C = valine) (20 mM) in the presence of thiosulfate (20 mM).

3.5 Kinetic experiments on branched-chain amino acid fermentation by selected strains

To further investigate the subtle differences in the BCAA metabolism of *Thermoanaerobacter* and *Caldanaerobacter* species, three strains (*T. siderophilus* (DSM 12299), *T. uzonensis* (DSM 18761), *C. subterraneus* subsp. *subterraneus* (DSM 13054)) were selected from each subgroup and evaluated on leucine (Figure 6A-C), isoleucine, and valine.

Degradation of leucine by *T. siderophilus* (DSM 12299) in the presence of thiosulfate (20 mM of each) resulted in the formation of 3-methyl-1-butyrate (14.3 mM) and 2-methyl-1-butanol (4.2 mM) (Figure 6A). Other end products were ethanol and acetate, presumably products from the yeast extract. Leucine was completely degraded (not shown) and carbon balance for the degradation was 92.5%. Interestingly, production of both the BCFA and BCOH increased when the strain reached stationary phase of growth. Degradation of isoleucine and valine similarly resulted in a mixture of their corresponding BCFA and BCAA. Carbon balances for isoleucine and valine were 89.3 and 80.0%, respectively (results not shown). In all cases the concentration of the BCFA was considerably higher as compared with the BCOH.

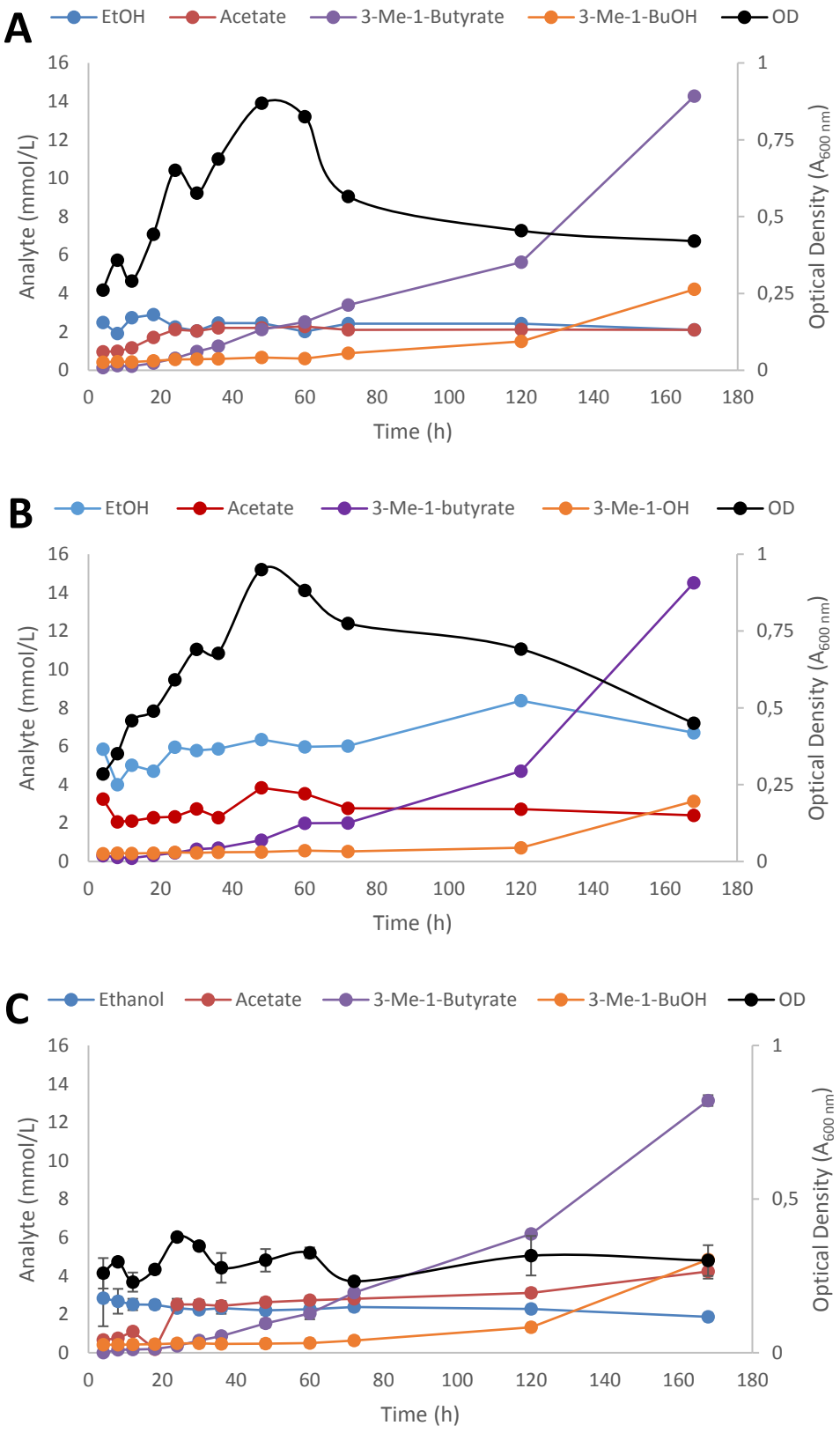

Figure 6 - Fermentation kinetics of *Thermoanaerobacter siderophilus* (A), *Thermoanaerobacter uzonensis* (B) and *C. subterraneus* subsp. *subterraneus* (C) grown on 20 mM leucine in the presence of thiosulfate (20 mM).

Degradation of leucine by *Thermoanaerobacter uzonensis* (DSM 18761) showed a very similar pattern as by *T. siderophilus* (DSM 12299); leucine was completely degraded (not shown) to a mixture of 3-methyl-1-butyrate (14.5 mM) and 3-methyl-1-butanol (3.24 mM) (Figure 6B) resulting in a carbon balance of 88.0%. Similarly, degradation of isoleucine and valine resulted in a mixture of their BCFAs and BCOHs with carbon balances being 100 and 85.8% (results not shown). As before, in all cases the concentration of the BCFA was considerably higher as compared with the BCOH.

Degradation of leucine by *C. subterraneus* subsp. *subterraneus* (DSM 13054) also showed similar spectra as for the other two species investigated. Leucine was completely degraded to a mixture of 3-methyl-1-butyrate (13.3 mM) and 3-methyl-1-butanol (4.85 mM) (Figure 6C) rendering a carbon balance of 89.9%. Also the other two BCAAs (valine and isoleucine) were degraded to mixtures of their corresponding BCFA and BCOH. As before, the concentration of the fatty acid was always considerably higher as compared with the alcohol (results not shown)

4. Conclusion

Degradation of proteins and amino acids by thermophilic bacteria within the genera of *Thermoanaerobacter* and *Caldanaerobacter* shows that the addition of an electron scavenging compound (thiosulfate) is of great importance. Various proteins (cooked meat medium, skim milk, casein, collagen, gelatin, and keratin) were not degraded to any extent unless provided with thiosulfate. Also, only six amino acids were degraded without thiosulfate addition by a handful of tested strains but a much broader substrate spectra was observed when the strains were cultivated with thiosulfate. For instance, the branched-chain amino acids were, with thiosulfate addition, degraded to a mixture of their corresponding, one carbon shorter, branched-chain fatty acid and alcohol. Also, two *Caldanaerobacter* strains (*C. subterraneus* subsp. *subterraneus* and *C. subterraneus* subsp. *yonseinesis*) were by far the best overall amino acid degraders of all tested strains. These two strains were the only strains degrading the aromatic amino acids to their corresponding aromatic fatty acids and alcohols.

Conflict of interests

The authors declare that there are no conflicts of interests.

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Supplementary Table 1 – Growth characteristics of type strains of selected thermophilic *Clostridia*; optimum conditions are **bolded** where available.

Strain	16S Ascension No.	DSM Number	Growth Conditions			Reference
			$T_{min/opt/max}$ (°C)	$pH_{min/opt/max}$	NaCl (%)	
Thermoanaerobacter						
T. acetoethylicus	L09163	2359	40/**65**/80	5.5/ND/8.5	ND	(Ben-Bassat & Zeikus, 1981; Rainey & Stackebrandt, 1993)
T. brockii subsp. *brockii*	L09165	1457	35/**65-70**/85	5.5/**6.5-7.5**/9.5	ND	(Zeikus et al., 1979)
T. brockii subsp. *finnii*	CP002466[a]	3389	40/**65**/75	ND/**6.5-6.8**/ND	ND	(Cayol et al., 1995; Schmid, et al., 1986)
T. brockii subsp. *lactiethylicus*	U14330	9801	40/**55-60**/75	5.0/**7.0**/8.5	0/**1**/4	(Cayol et al., 1995; Kondratieva et al., 1989)
T. italicus	AJ250846	9252	45/**70**/78	NR/**7.0**/NR	0/NR/1	(Kozianowski et al., 1997)
T. ethanolicus	L09162	2246	37/**69**/78	4.4/**5.8-8.5**/9.8	ND	(Juergen Wiegel & Ljungdahl, 1981)
T. kivui	L09160	2030	50/**66**/72	5.3/**6.4**/7.3	ND	(Leigh, Mayer, & Wolfe, 1981; Leigh & Wolfe, 1983)
T. mathranii subsp. *mathranii*	Y11279	11426	50/**70-75**/75	4.7/**7.0**/8.8	ND[c]	(Larsen et al., 1997)
T. mathranii subsp. *Alimentarius*	AY701758	Not Dep.	45/ND/70	NR	NR	(Carlier et al., 2006)
T. pentosaceusus	GU176611	25963	50/**70**/80	5.5/**7.0**/8.5	0/ND/4	(Tomás et al., 2013)
T. pseudoethanolicus	CP000924[a]	2355	ND/**65**/ND	ND	ND	(Onyenwoke, et al., 2007; Zeikus, Ben-Bassat, & Hegge, 1980)
T. siderophilus	AF120479	12299	39/**69-71**/78	4.8/**6.3-6.5**/8.2	0/ND/3.5	(Slobodkin et al., 1999)
T. sulfurigenens	AF234164	17917	34/**63-67**/72	4.0/**5.0-6.5**/8.0	ND[b]	(Y.-J. Lee et al., 2007)
T. sulfurophilus	Y16940	11584	55-60	ND	ND	(Bonch-Osmolovskaya et al., 1997)
T. thermocopriae	L09167	Not Dep.	47/**60**/74	6.0/**6.5-7.3**/8.0	ND	(Jin et al., 1988)
T. thermohydrosulfuricus	L09161	567	37/**67-69**/78	5.5/**6.9-7.5**/9.2	ND	(Lee, et al., 1993)
T. uzonensis	EF530067	18761	32.5/**61**/69	4.2/**7.1**/8.9	ND	(Wagner et al., 2008)
T. wiegelii	X92513	10319	38/**65-68**/78	5.5/**6.8**/7.2	ND	(Cook, et al., 1996)
Caldanaerobacter						
C. subterraneus subsp. *yonseiensis*	AF212925	13777	50/**75**/85	4.5/**6.5**/9.0	NR	(Kim, et al., 2001)
C. subterraneus subsp. *subterraneus*	AF195797	13054	40/**65**/75	6.0/**7.5**/8.5	0/**0**/3	(Fardeau et al., 2004; Fardeau et al., 2000)
C. subterraneus subsp. *pacificus*	AF174484	12653	50/**70**/80	5.8/**6.8-7.1**/7.6	ND/**2-2.5**/ND[d]	(Sokolova et al., 2001)
C. subterraneus subsp. *tengcongensis*	AF209708	15242	50/**75**/80	5,5/**7.0-7.5**/9.0	0/**0,2**/2.5	(Xue, et al., 2001)
C. uzonensis	EF195126	18923	50/**68-70**/75	4.8/**6.8**/8.0	0/**0.5**/2	(Kozina, et al., 2010)

[a]Whole genome sequence, [b]Strain tolerates 1 M sodium thiosulfate, [c]growth not affected at 2% w/v NaCl; [d]requires seawater for growth; ND-Not determined, NR – Not reported

Supplemental Table 2 – API ZYM data for *Thermoanaerobacter* and *Caldanaerobacter* species.

Activity	*T. brockii* ssp.*brockii*	*T. brockii* ssp.*finnii*	*T. ethanolicus*	*T. italicus*	*T. kivui*	*T. mathrani ssp. mathranii*	*T. pentosaceusus*	*T. pseudoethanolicus*	*T. siderophilus*	*T. sulfurigenes*	*T. sulfurophilus*	*T. thermohydrosulfuricus*	*T. uzonensis*	*T. wiegelii*	*C. subterraneus* subsp. *subteraneus*	*C.subterraneus* subsp. *pacificus*	*C.subterraneus* subsp. *tenaconqensis*	*C.subterraneus* subsp. *yonseiensis*	*C. uzonensis*
Alkaline phosphatase	-	-	-	-	-	-	-	+	+	+	+	-	-	+	+	+	+	+	+
Esterase (C4)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Lipase esterase (C8)	-	-	-	+	-	-	-	-	-	+	+	+	+	-	-	-	-	-	-
Lipase (C14)	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	+	-	-
Leucine arylamidase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Valine arylamidase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Cysteine arylamidase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Trypsin	-	-	-	-	-	+	-	-	-	+	-	-	-	-	-	-	-	-	-
α-Chymotrypsin	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
Acid phosphatase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Naphthol-AS-BI-phosophohydrolase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
α-Galactosidase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
β-Galactosidase	+	+	-	+	-	-	+	+	-	+	-	-	-	+	-	-	-	-	-
β-Glucuronidase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
α-Glucosidase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
β-Glucosidase	+	+	+	-	-	-	+	+	-	+	+	+	+	+	-	-	+	-	-
N-Acetyl-β-glucosaminidase	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-
α-Mannosidase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
α-Fucosidase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

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Anaerobes in the environment

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.

1. Introduction

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 the enantioselective biological reductions of ketones to their corresponding secondary alcohols [8,10,11]. However, the use of thermophilic *Clostridia* has not previously been investigated for their ability to produce higher-order alcohols (C3-C7) from short-chain fatty acids (SCFAs) although the addition of acetate to the fermentation medium is known to stimulate ethanol production [12]. Beyond the production of 1-butanol via the acetone-butanol-ethanol (ABE) process [13,14] and some limited work on the production 1-hexanol from the fermentation of syngas [15–17], there has been little focus on the production of C3 and larger primary alcohols. Historically, such alcohols are prepared using the oxo process or by the reduction of carboxylic acids using strong reducing agents, both of which have substantial drawbacks. There have been recent reports of *Clostridium saccharoperbutylacetonicum* N1-4 to convert 1-butyric acid to 1-butanol in the presence of glucose in the context of ABE fermentations [18,19] as well as by acetogens such as "*Clostridium ragsdaleii*" [20] and engineered *Clostridium autoethanogenum* [21] and in mixed culture with propionate-producing *Clostridium propionicum* and *Alkalibaculum bacchi* [22].

Recently, *Thermoanaerobacter* strains have been shown to produce branched-chain fatty acids and alcohols during the fermentation of branched-chain amino acids [23–25] although no wild type strains have been reported to produce 1-butanol or higher

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alcohols as a product of glucose fermentation while the mechanism behind branched-chain alcohols formation from amino acids remains unclear.

The present study investigates the ability of *Thermoanaerobacter pseudoethanolicus* (DSM 2355) to reduce C1-C8 short-chain fatty acids (SCFAs) to their corresponding alcohols using glucose as a carbon source and as a source of reducing potential. *T. pseudoethanolicus* was cultivated anaerobically with nitrogen in the gas phase in Hungate tubes (16 × 150 mm) containing 8.3 mL of Basal Mineral (BM) medium [26] containing 20 mM of glucose and 20 mM of the SCFA. Cultivations were performed at 65 °C and pH 7 in triplicate without agitation for 5 days. All materials were purchased from Sigma Aldrich except for ^{13}C-labelled compounds (Cambridge Isotope Laboratories, MA, USA).

Hydrogen was quantified by gas chromatograph equipped with a thermoconductivity detector while short-chain fatty acids and alcohols were quantified by gas chromatograph with a flame ionization detector as described previously [26]. The products were separated on a nitroterephthalic-acid-modified polyethylene glycol (DB-FFAP, J&W Scientific) 30 m capillary column, 0.32 i.d., 0.25 μm film thickness, using a temperature program of 40 °C, hold time 1 min, ramped to 240 °C (10 °C/min), hold time 10 min and a constant column head space pressure of 9.9 kPa. Optical densities were measured after 5 days of cultivation using a Shimadzu UV-1800 at 600 nm ($l = 1$ cm). The mass spectrometer ion source was set to 260 °C and the interface 250 °C, scanning took place once every 0.30 s in the range of 40–60 m/z. Peaks were identified using the NIST mass spectral database, versions 147 and 27, with an identity threshold cut-off of 80. Samples were filtered prior to injection using a 0.45 μm syringe filter. ^{13}C nuclear magnetic resonance (NMR) spectroscopy of fermentations containing ^{13}C1-labelled SCFAs were performed on a Bruker AV400 NMR spectrometer at 298 K after spiking with D_2O to obtain a signal lock (0.3 mL addition to 1 mL of aqueous sample).

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 (Fig. 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).

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% (Fig. 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 Fig. 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. Fig. 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.

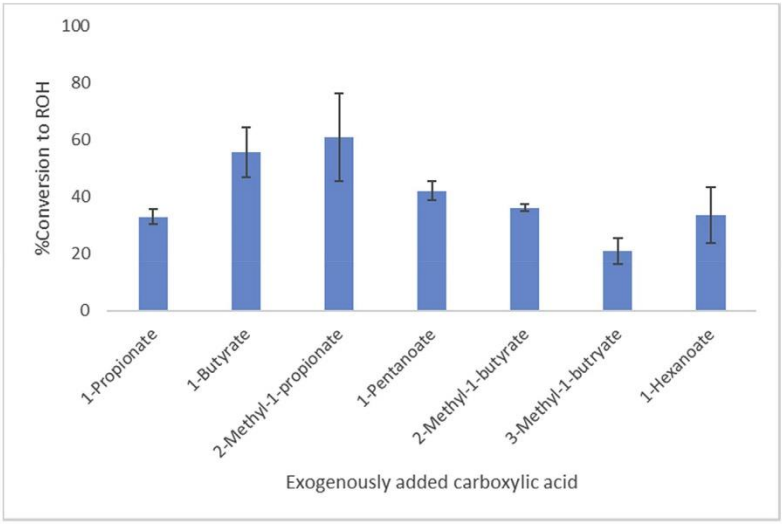

Fig. 1. Conversion of exogenously added SCFAs to fermentations containing 20 mM of glucose by T. pseudoethanolicus. Values represent the average of triplicates with error bars presented as standard deviation.

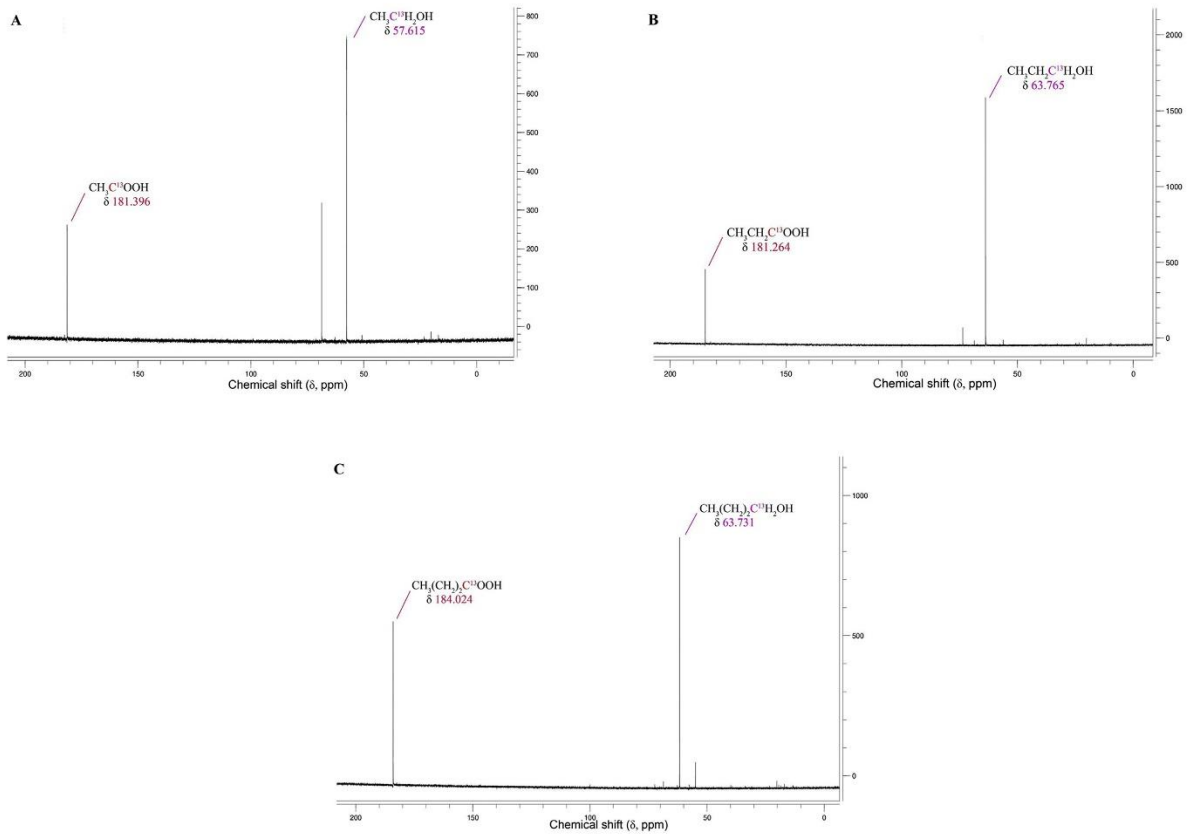

Fig. 2. ^{13}C NMR spectra of glucose fermentations (20 mM) by *T. pseudoethanolicus* with exogenously added $^{13}C1$-acetate (A), $^{13}C1$-propionate (B) and $^{13}C1$-butyrate (C) at 20 mM final concentration.

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-labelled 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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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.anaerobe.2019.03.004.

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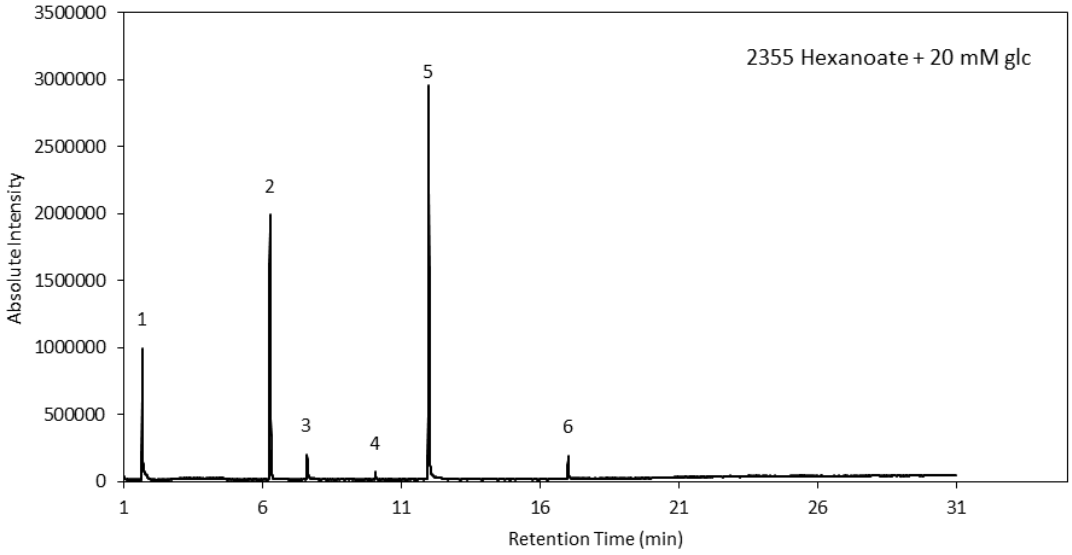
Supplementary Table 1 – Conversion of short-chain fatty acids to their corresponding alcohols by *Thermoanaerobacter pseudoethanolicus* on 20 mM glucose (control is without glucose); values represent average of triplicate ± standard deviation.

	Analyte (mmol/L)							
Substrate (20 mM) + exogenously added carboxylic acid (20 mM)	Hydrogen	Ethanol	Alcohol	Acetate	Carboxylic acid	Carboxylic acid conversion (%)	Optical Density (600 nm)	Carbon balance (%)
Control (yeast extract)	3.20±1.21	1.42±0.21	0.00±0.00	1.94±0.16	0.00±0.00	ND	0.16±0.04	ND
Glucose only	3.72±0.50	30.43±2.26	0.00±0.00	6.94±0.09	0.00±0.00	ND	0.34±0.14	93.4
Formate	2.50±0.15	23.23±4.64	ND	5.10±1.84	ND	ND	0.21±0.03	70.8
Acetate	1.45±0.04	25.70±3.28	ND	34.78±3.27	ND	ND	0.41±0.12	100.8
1-Propaionate	1.58±0.33	16.50±2.03	6.62±0.52 (1-propanol)	13.02±0.58	3.55±0.98	33.0	0.34±0.07	77.8
1-Butyrate	1.73±1.02	27.78±6.75	11.12±3.09 (1-butanol)	16.03±4.85	6.38±0.28	55.6	0.41±0.09	85.7
2-Methyl-1-propionate	1.24±0.22	20.88±6.06	12.20±3.79 (2-Me-1-PrOH)	20.24±4.77	10.24±1.17	61.0	0.38±0.09	73.6
1-Pentanoate	1.42±0.13	19.74±1.77	8.42±0.50 (1-pentanol)	16.91±1.68	11.59±0.75	42.1	0.30±0.07	94.4
2-Methyl-1-butryate	1.27±0.29	18.87±1.00	7.22±0.25 (2-Me-1-BuOH)	19.86±1.03	12.51±0.50	36.1	0.38±0.07	80.9
3-Methyl-1-butyrate	1.42±0.31	21.42±4.56	4.18±0.91 (3-Me-1-BuOH)	9.36±0.34	11.14±1.63	21.0	0.37±0.13	76.9
1-Hexanoate	1.15±0.31	31.73±5.70	6.69±1.96 (1-hexanol)	12.00±2.06	12.23±2.99	33.5	0.24±0.06	104.4

Supplementary Figure 1 – GC-MS chromatograph obtained using *Thermoanaerobacter pseudoethanolicus* demonstrating the formation of 1-hexanol from exogenous 1-hexanoic acid



Peak	Name	RT	% Area
1	Ethanol	1.662	12.0
2	1-Hexanol	6.27	36.5
3	Acetic Acid	7.601	2.9
4	2-Methyl-Butanoic Acid	10.067	0.7
5	Hexanoic Acid	11.984	44.0
6	Unidentified (“Diethyl Phthalate”)	17.008	2.3

VIII

Bioreduction of Organic Acids to Alcohols by *Thermoanaerobacter pseudoethanolicus*

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ABSTRACT *Thermoanaerobacter* and *Caldanaerobacter* species have gained increased interest due to their ability to produce bioalcohols from a wide range of substrates and been recently noted to reduce volatile fatty acids to their corresponding alcohols. Present investigation shows that *Thermoanaerobacter pseudoethanolicus* converts C2-C6 short-chain fatty acids (SCFAs) to their corresponding alcohols in the presence of glucose. The conversion yields varied from 21% of 3-methyl-1-butyrate to 57.9% of 1-pentanoate being converted to their corresponding alcohols. Culture conditions such as liquid-gas phase ratio have negligible impact on the conversion of the SCFAs to alcohols but directed the flow of electrons more towards the fatty acid conversion and less to ethanol production as compared with fermentation without acid addition. By increasing the initial glucose concentration, an increase in the conversion of SCFAs converted to their corresponding alcohols was observed. Inhibitory experiments on C2–C8 alcohols showed that C4 and higher alcohols are inhibitory to *T. pseudoethanolicus* suggesting that other culture modes may be necessary to improve the amount of fatty acids reduced to the analogous alcohol. The bioreduction of SCFAs to their corresponding further demonstrated using isotopically fatty acids and the conversion followed kinetically. Finally, increased activity of alcohol dehydrogenase and aldehyde oxidoreductase were observed in cultures *of T. pseudoethanolicus*, using both NADH and NADPH as cofactors, although the presence of the latter showed higher activity.

Keywords: butanol, hexanol, thermophiles, biocatalysis, carbonyl reduction

1. Introduction

Beyond biofuels, the sustainable production of chemical building blocks, such as aliphatic alcohols from renewable materials is a major goal of the circular bioeconomy. At present, the vast majority of alcohols are produced via the oxo process from petrochemical starting materials, although large quantities of 1-butanol have been produced via fermentation into the early to mid-20th Century (Jones, 2001). The production of bioethanol from first and second generation biomass is an expanding area of intense research. The production of 1-propanol, 2-propanol (Krouwel, Groot, & Kossen, 1983), 2-methyl-1-butanol (isobutanol), 1-hexanol (Phillips et al., 2015), and 1-octanol (Richter, Molitor, Diender, & Sousa, 2016) have been reported as fermentation end products of either carbohydrate or CO/CO_2 metabolism from species within the genus of *Clostridia*, although these fermentation routes have not reached industrial viability. Until recently, the production of C3 and higher alcohols has largely been restricted to mesophiles.

Thermophilic anaerobic bacteria within the genera of *Thermoanaerobacter*, *Thermoanaerobacterium* and *Caldanaerobacter* have been known for some time to be good ethanol and hydrogen producers (Cripps et al., 2009; Scully & Orlygsson, 2015; Taylor et al., 2009). The use of these bacteria are especially advantageous when complex biomass is used as a feedstock for fermentation, since they typically have a very broad substrate spectrum degrading various monosaccharides, disaccharides, and oligosaccharides to volatile end products (Scully and Orlygsson, 2014). It is well known that culture conditions are of great importance for end product formation. For example, manipulation of the liquid – gas phase ratio or adding electron scavenging molecules (such as thiosulfate) to the medium makes it possible to direct the end-products to more reduced (ethanol) or oxidized (acetate) formation during glucose or amino acid fermentation (M Fardeau, Faudon, & Cayol, 1996).

Instead of producing low molecular weight alcohols a more feasible approach would be to produce higher-order alcohols since their energy density increases linearly with increasing carbon number. The energy density of ethanol, propanol, and butanol are 26.8, 33.4, and 36.1 MJ kg^{-1}, respectively, as compared with 44.4 MJ kg^{-1} for isooctane. Higher order alcohols, such as butanol, are also more desirable as biofuels as they are less corrosive to existing infrastructure and are less hygroscopic than low molecular weight alcohols (Amiri & Karimi, 2019). Butanol production by fermentation has a long history using mesophilic *Clostridium* species with the acetone-butanol-ethanol (ABE) fermentation using *Clostridium acetobutylicum* and *Clostridium beijerinckii* (Dürre, 2008; Huang, Liu, & Gan, 2010; Moon et al., 2016; Sauer, 2016) and more recently with thermophiles like *Thermoanaerobacterium* strain M5 (Jiang et al., 2018). These bacteria produce acetate and butyrate in the acetogenic phase but due to the acid formation, the pH drops in the medium and the bacteria convert the acids to acetone and butanol. It is also known that some thermophilic bacteria like *Thermoanaerobacter* and *Caldanaerobacter* can produce a mixture of branched-chain fatty acids (BCFA) and alcohols (BCOH) from their corresponding branched-chain amino acids (BCAA). In this case, it is not clear if the bacteria first produce the acid before conversion to the alcohol or if the intermediate from the amino acid (α-keto acid) is both oxidized to its corresponding fatty acid and reduced to alcohol. A recent investigation in our laboratory on inhibitory effects of various volatile fatty acids on growth revealed that many *Thermoanaerobacter* species can reduce short-chain fatty acids (SCFAs) to their corresponding alcohols during growth on glucose

(Scully et al. 2019). It is suggested that *Thermoanaerobacter* species can utilize the reducing power generated by glucose oxidation to reduce the acids to alcohols. This has been show in studies with *Thermoanaerobacter pseudoethanolicus* and *Thermoanaerobacter* strain AK85 relative to its amino acid catabolism (Hitschler, Kuntz, Langschied, & Basen, 2018; Scully, et al., 2019; Scully & Orlygsson, 2019) as well as by autotrophic *Clostridia* such as "*Clostridium ragsdalei*" (Isom et al., 2015). The present study examines the ability of *T. pseudoethanolicus* to reduce C2 to C6 carboxylic acids with a particular empahsis on the role of culture conditions on the amount of carboxylic acid reduced, principally the role of liquid-gas phase ratio and the ratio of glucose to short-chain fatty acid. Additionally, the inhibitory impact of C2-C6 alcohols is also investigated.

2. Materials and Methods

2.1. General methods

All materials were obtained from Sigma Aldrich unless otherwise stated. Nitrogen gas used was of 5.0 quality (< 5 ppm O_2) obtained from AGA gas. Nucleotide cofactors were obtained from Megazyme with the exception of NADPH which was obtained from Sigma.

2.2 Culture Media and Organisms

The medium (per litre) consisted of: NaH_2PO_4 x $2H_2O$ 3.04 g, Na_2HPO4 x $2H_2O$ 5.43 g, NH_4Cl 0.3 g, NaCl 0.3 g, $CaCl_2$ x $2H_2O$ 0.11 g, $MgCl_2$ x $6H_2O$ 0.1 g, yeast extract 2.0 g, resazurin 1 mg, trace element solution 1 mL, vitamin solution (DSM 141) 1 mL, and $NaHCO_3$ 0.8 g. The trace element composition was as described earlier (Chades et al., 2018). The medium was prepared by adding the buffer to distilled water containing resazurin followed by boiling for 10 min followed by cooling under nitrogen flushing (<5 ppm O_2). The mixture was then transferred to serum bottles using the Hungate technique (Hungate, 1969; T. L. Miller & Wolin, 1974) and autoclaved for 60 min. All cultivations were performed in serum bottles at pH 7.0 at a liquid-gas phase ratio of 1:1 and without agitation unless otherwise stated. All experiments were conducted in triplicate. *T. pseudoethanolicus* (DSM 2355) was purchased from DSM. In all experiments, a 2% inoculation volume obtained from fresh cultures was used. After cultivation, the level of hydrogen produced was analysed by gas chromatography, the cells were then centrifuged (13000 rpm, 3 min) and the supernatant was stored at -40° prior to further analysis.

2.3 Conversion of fatty acids to alcohols in the presence of glucose

The strain was cultivated on glucose (20 mM) in the absence and presence of a range of different fatty acids. The acids (acetate, 1-propionate, 1-butyrate, 2-methyl-1-propionate, 2-methyl-1-butyrate, 3-methyl-1-butyrate, 1-pentanoate, and 1-hexanoate) were added to give a final concentration of 20 mM. The experiments were performed in Hungate tubes (16x150 mm) with equal volumes of liquid and headspace.

2.4 Kinetic experiments

Kinetic experiments on the fermentation of glucose, with and without added fatty acids (1-butyrate, 3-methyl-1-butyrate) were performed in 125 mL serum bottles with a L-G of 1:1 at 65°C for an incubation period of 120 h. Periodically, 1 mL samples was removed for the analysis of volatiles end products and 0.2 mL of headspace gas was removed for hydrogen analysis.

2.5 Effect of Liquid-gas phase ratio

The strain was cultivated in BM containing 20 mM of glucose (a) with addition of 1-propionate (b), 1-butyrate (c), 3-methyl-1-butyrate (d), and 2-methyl-1-butyrate (e). Five different L-G phases were used: 0.05, 0.34, 0.98, 2.08, and 5.40 in 57 mL serum bottles. End products were quantified after 5 days.

2.6 Inhibitory effects of C2-C6 alcohols on end product formation

To investigate the inhibitory effects of fatty acids and alcohols, the strain was cultivated on glucose in the presence of ethanol, 1-propanol, 2-propanol, 1-butanol, 2-methyl-1-propanol, 2-methyl-1-butanol, 1-pentanol, and 1-hexanol. The concentrations used were 0.0, 0.5, 1.0, 2.0, 3.0, 4.0, 5.0, and 7.0%. Experiments were performed in Hungate tubes (16x150 mm) with L-G phase of 1:1.

2.7 Effect of glucose and fatty acid ratio on end product formation

The strain was cultivated at different concentrations of glucose (0, 10, 20, 30, and 40 mM) in the presence of 1-propionate (20 mM), 1-butyrate (20 mM), or 2-methyl-1-butyrate (20 mM). The experiment was performed in 16x150 mm Hungate tubes with L-G phase of 1:1. The cultures were cultivated for 5 days.

2.8 NMR experiment

The strain was cultivated in the presence of 20 mM ^{13}C1-labled 3-methyl-1-butyrate and glucose (20 mM) in an 8.7 mL serum bottle (L-G 0.98) for 7 days and analyzed. ^{13}C NMR spectra were obtained using a Bruker AV400 at 298K after spiking with D_2O to obtain a signal lock (0.3 mL addition of D_2O to 1 mL of aqueous sample).

2.9 Enzyme assays

Cells for enzymatic assays were cultivated on 20 mM of SCFA + 20 mM glucose in 1 L serum bottles containing 500 mL of medium; cells were harvested after 18 hours of cultivation via centrifugation (4700 rpm, 0-4°C after dithionate addition to afford a final concentration of 5 mg/L) followed by washing three times with degassed 50 mM Tris-HCl (pH 7.5). Cells were suspended in 10 mL of 50 mM Tris-HCl (pH 7.5) to which an equal volume of glass beads (150-212 µm) were added followed by vortexing for 30s followed by cooling on an ice bath for 2 min; this was repeated a total of three times. Cell debris was removed by centrifugation and the supernatant transferred to nitrogen-flushed serum bottles. ADH and AOR activity assays were immediately performed using the NBT method of (Fibla & Gonzhlez-Duarte, 1993) as previously described by Scully and Orlygsson (**Paper V**) an the concentration of generated NAD(P)H were determined after 60 minutes and calculated according to the equation below.

$$ADH\ activity\ \left(\frac{mU}{mL}\right) = \frac{nmol\ NADH}{v \cdot t} = nmol\ NADHx2$$

2.10 Analytical methods

Hydrogen was analyzed with a Perkin Elmer Auto System XL gas chromatograph equipped with a thermo-conductivity (TCD) detector as previously described (Orlygsson and Baldursson 2007). Alcohols and volatile fatty acids were measured by gas chromatography using a Perkin-Elmer Clarus 580 gas chromatograph equipped with flame ionization detector (FID) as previously described (Orlygsson and Baldursson 2007). Optical density was determined using a Shimadzu UV-1800 at a wavelength of 600 nm (l=1 cm). Protein was quantified using the Lowry assay as described by (Copeland, 1994) with minor modifications as described by Scully and Orlygsson (2019). Bovine serum albumin was used as a standard at a concentration ranging from 0.1 to 1.4 mg/mL.

3. Results and Discussion

Recent investigations in our laboratory on the degradation capacity of *Thermoanaerobacter* species towards carboxylic acid containing waste materials revealed that most species within the genus can reduce carboxylic acids to their corresponding alcohols using glucose as the substrate. These findings were observed when carboxylic acids were added to a medium to investigate the inhibitory effects of them. Surprisingly, instead of being only inhibitory, most of the *Thermoanaerobacter* strains could convert the fatty acids to their corresponding alcohols. This phenomenon has now been published as a short communication for *T. pseudoethanolicus* (Scully et al., 2019) and further by one strain isolated in Iceland, *Thermoanaerobacter* strain AK85 (Scully and Orlygsson, 2019). The present investigation examines the ability of *T. pseudoethanolicus* to reduce carboxylic acids to their corresponding alcohols with an emphasis on the influence of culture conditions and the inhibitory impact of alcohols on fermentation as well as the activities of enzymes likely involved in the conversion.

3.1. Fatty acid conversion to alcohols

The strain was cultivated on 20 mM glucose in the absence or presence of 20 mM of various C1 to C8 SCFAs as shown in Figure 1. The catabolism of glucose without addition of fatty acids resulted in the production of ethanol as the main end product (35.1 ± 1.27 mM or 87.8% of the theoretical yield) with other end products being acetate (6.94 ± 0.09 mM) and hydrogen (3.72 ± 0.50 mmol/L). The end product formation from glucose, in the presence of fatty acids show a decrease in ethanol formation (35.1 mM in the glucose control as compared to 16.5 mM of ethanol when 1-propionate is included) and an increase in acetate (9.4 mM on 3-methyl-1-butyrate to 22.3 mM on 3-methyl-1-propinate) along with the conversion of the exogenously added carboxylic acid to the corresponding alcohol. The decrease in ethanol formation suggests that reducing equivalences from glucose oxidation is being redirected from ethanol towards the reduction of the SCFA while the remaining carbon from glucose metabolism is being shunted towards acetate allowing additional ATP formation, as evidenced by higher optical densities when SCFAs were provided (Figure 1). The conversion yields of the SCFAs examined varied; the addition of 1-pentanoate resulted in the highest conversion to its corresponding alcohol, 1-pentanol (57.9%; 11.58 mM) but only 21% (4.2 mM) of the 3-methyl-1-butyrate was converted to 3-methyl-1-butanol (Figure 1).

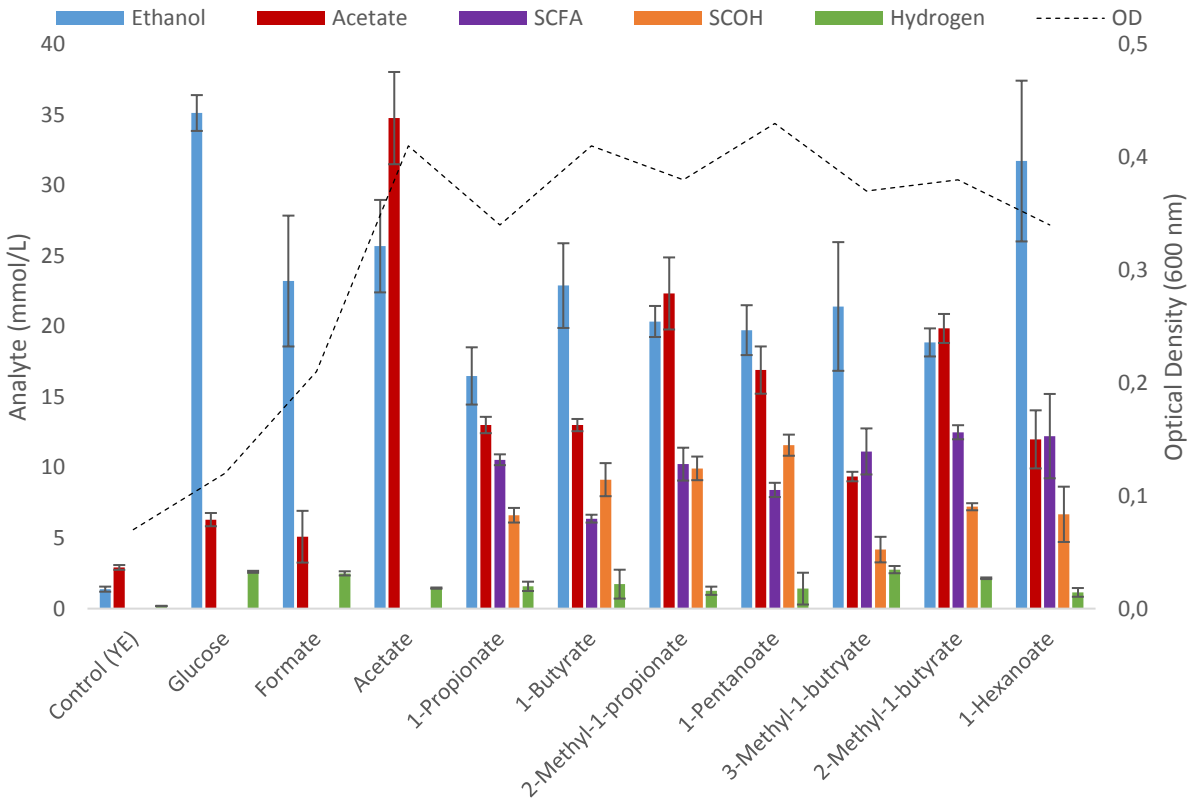
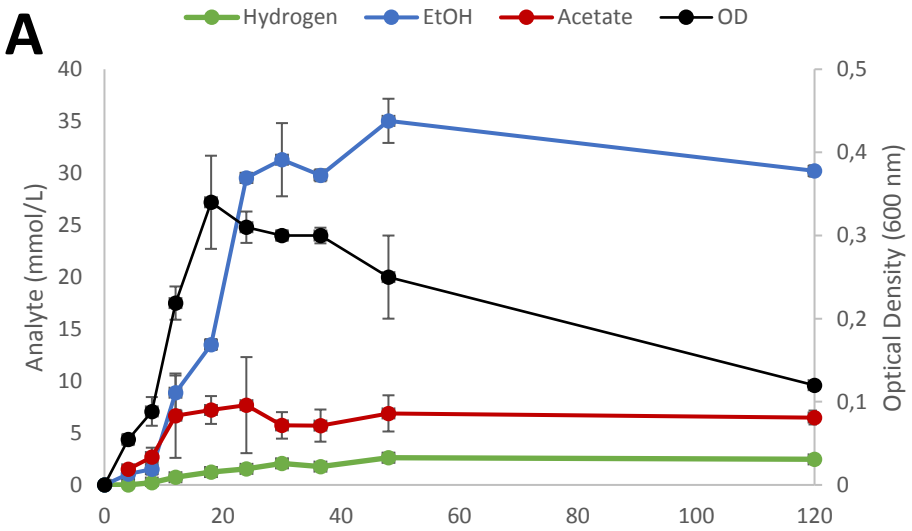


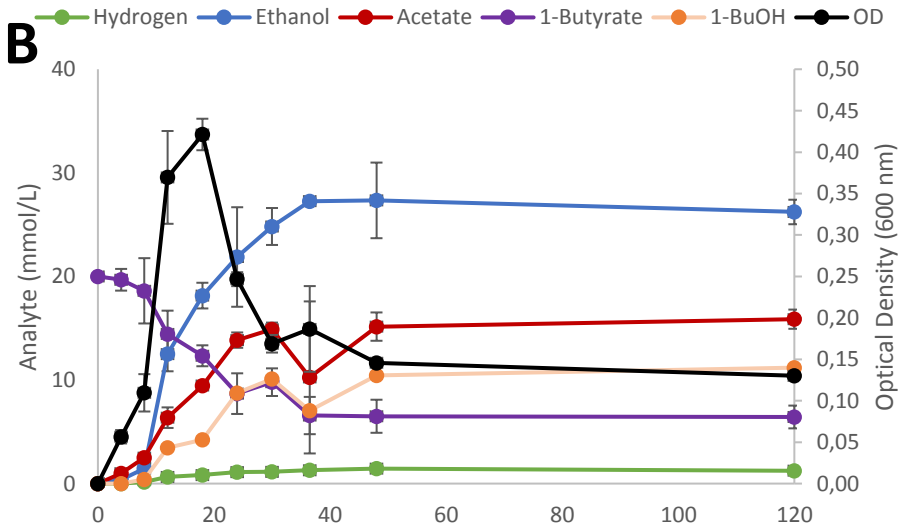
Figure 1. End product formation after 5 days from cultures of *T. pseudoethanolicus* containing glucose (20 mM) and of exogenously added carboxylic acid (20 mM). Values represent the average of triplicate fermentations with standard deviation presented as error bars.

3.2. Kinetic experiment

To better understand the conversion of SCFAs to alcohols by *T. pseudoethanolicus*, kinetic fermentation were performed on 20 mM glucose in the absence and presence of 1-butyrate and 3-methyl-1-butyrate (both 20 mM) as model compounds. During growth on glucose without acid addition, the strain reached a maximum OD after 18 h, producing 35.0 mM of ethanol, 7.7 mM of acetate, and 2.6 mmol/L of hydrogen (Figure 2A). The degradation of glucose in the presence of 1-butyrate (20 mM) reached a maximum OD within 18 h and once again ethanol, acetate, and hydrogen were produced. The concentration of ethanol (26.2 mM) was lower compared with the fermentation of glucose alone but the concentration of acetate was higher, or 15.9 mM. As before, 1-butyrate was reduced to 1-butanol; the amount of the acid consumed was 13.6 mM which resulted in production of 13.0 mM of the alcohol or a conversion of 65.0% (Figure 2B).

Similarly, the degradation of glucose in presence of 3-methyl-1-butyrate resulted in the same end products as before (ethanol, acetate, and hydrogen) in similar concentrations as during the growth on glucose in the presence of 1-butyrate. 3-Methyl-1-butyrate was converted to 3-methyl-1-butanol although to a lesser extent as compared with conversion of 1-butyrate to 1-butanol (Figure 2C). The concentration of 3-methyl-1-butyrate decreased from 20 mM to 13.0 mM and 6.2 mM of 3-methyl-1-butanol were produced. As with glucose, the maximum OD was also reached within 18 h suggesting that SCFA conversion occurs rapidly when reducing equivalence is available.





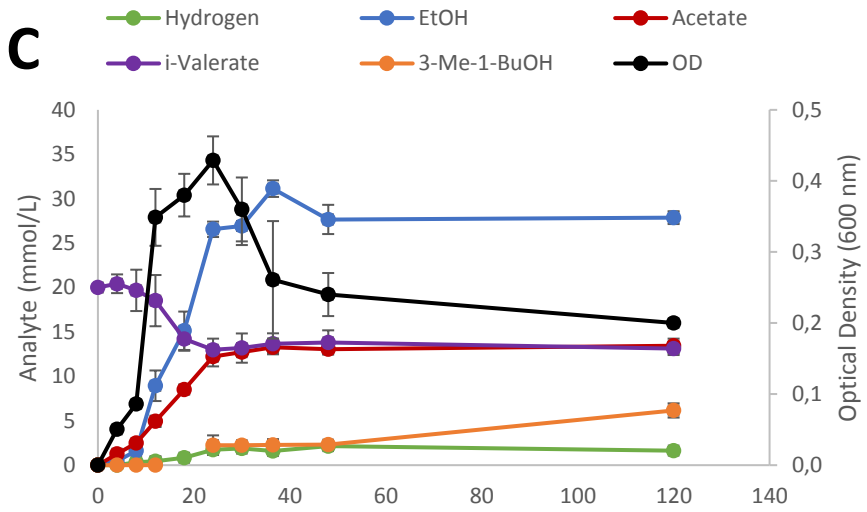


Figure 2 – Time-course studies of fermentation of 20 mM glucose (A), 20 mM 1-butyrate + 20 mM glucose (B), and 20 mM 3-methyl-1-butyrate + 20 mM glucose (C) by *T. pseudoethanolicus*.

3.3 Influence of liquid-gas phase ratio

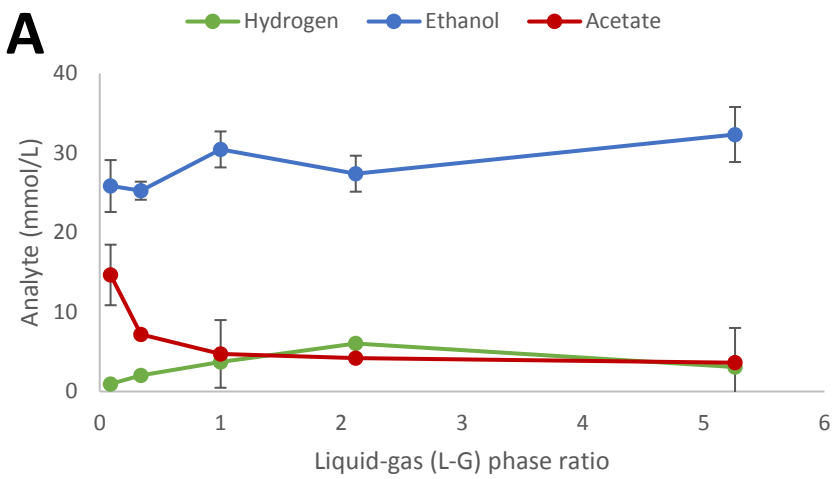
It is well known that the partial pressure of hydrogen (pH_2) strongly effects the ratio of oxidized and reduced end product formation with anaerobes in general (Brynjarsdottir, Wawiernia, & Orlygsson, 2012; J. E. J. Jessen & Orlygsson, 2012). Thus, at high pH_2, the tendency is to produce more reduced end products like ethanol and lactate but less of acetate and hydrogen. During growth on glucose alone, the strain produced a mixture of ethanol and acetate together with hydrogen (Figure 3A). At the lowest L-G phase used the ratio of ethanol and acetate was 1.76. During other conditions more ethanol and less acetate were produced with increasing L-G phase ratios (ethanol-acetate ratio varied from 3.51 (0.34 L-G phase ratio) to 8.95 (at 5.26 L-G phase ratio).

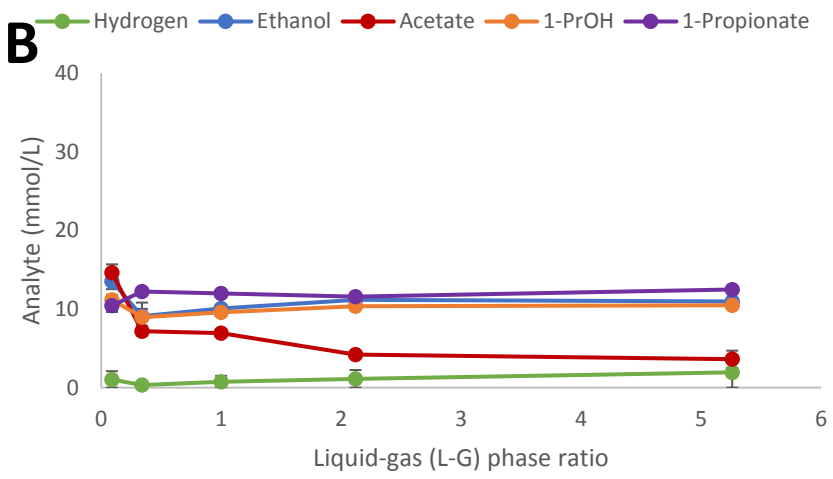
During growth of the strain on glucose in the presence of 1-propionate (20 mM) the strain produced similar amounts of ethanol (13.5 mM) and acetate (14.7 mM) at the lowest L-G phase ratio applied (Figure 3B). The concentration of ethanol in the presence of 1-propionate was however much less as compared with glucose alone but visa verse for acetate (Figure 3A). This indicates that the electrons are directed less to ethanol formation but more to the reduction of 1-propionate to 1-propanol. Ethanol concentrations were stable at higher L-G phase ratios (between 9.1 to 11.2 mM or about a third of the glucose control) whereas acetate production decreased with increasing L-G phase ratios (from 14.7 to 3.6 mM) clearly suggesting that reducing equivalence is being redirected to carboxylic acid reduction. The amount of 1-propionate that was converted to 1-propanol was similar under different L-G phase ratios and approximately 50% of the acid was converted to the alcohol.

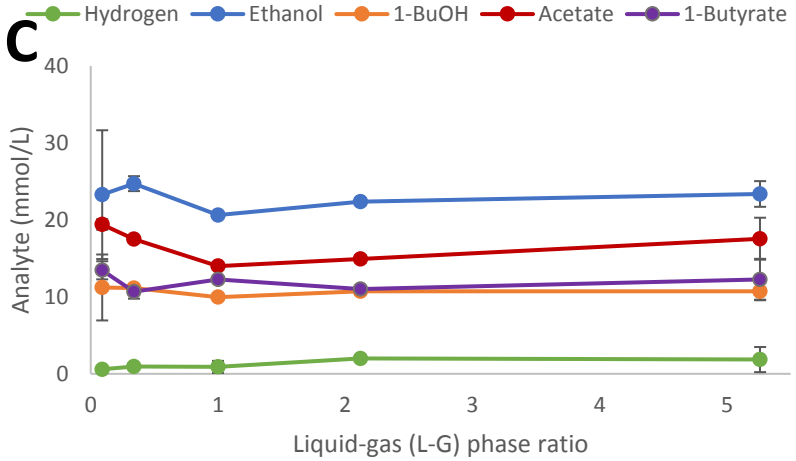
During growth on glucose in the presence of 1-butyrate the strain produced more ethanol as compared with addition of 1-propionate; between 20.6 mM to 24.7 mM of ethanol, which is approximately two-thirds of ethanol concentrations in the glucose control, and between 14.0 and 19.4 mM of acetate were produced (Figure 3C). 1-Butyrate was converted to similar amounts of 1-butanol at all L-G phase conditions (1-butanol concentrations ranged from 9.9 to 11.2 mM). Again, acetate concentrations were highest at low L-G phase ratios. The differences in the amount of 1-propanol and 1-butanol produced may suggest differences in the specificity of the alcohol dehydrogenases involved as has been previously reported for other clostridial solvent producers (Ismaiel, Zhu, Colby, & Chen, 1993; Lamed, Keinan, & Zeikus, 1981).

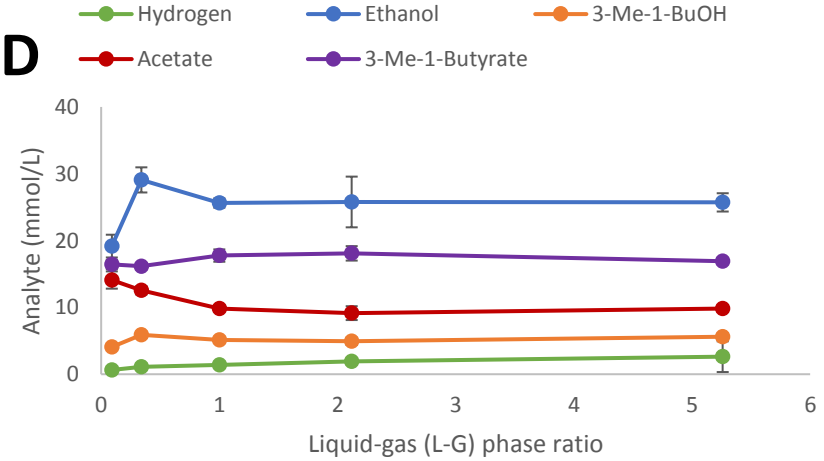
During the degradation of glucose in the presence of 3-methyl-1-butyrate and 2-methyl-1-butyrate, similar trends in ethanol and acetate were observed as before although ethanol concentrations were higher as compared with addition of 1-propionate and 1-butyrate (Figure 3D and 3E). However less of the BCFAs were converted to their corresponding alcohol. Maximum concentration of 3-methyl-1-butanol and 2-methyl-1-butanol were 5.9 mM and 8.1 mM, respectively.

Overall, the impact of L-G ratio on the amount of carboxylic acid converted to the corresponding fatty acid appears to be limited; the small quantities of hydrogen produced by *T. pseudoethanolicus* may limit the impact of hydrogen accumulation. Exogenously added hydrogen may be an option to supplement the reducing potential generated by glucose oxidation and drive further carboxylic acid reduction.


A
Hydrogen
Ethanol
Acetate
Analyte (mmol/L)
Liquid-gas (L-G) phase ratio


B
Hydrogen
Ethanol
Acetate
1-PrOH
1-Propionate
Analyte (mmol/L)
Liquid-gas (L-G) phase ratio


C
Hydrogen
Ethanol
1-BuOH
Acetate
1-Butyrate
Analyte (mmol/L)
Liquid-gas (L-G) phase ratio


D
Hydrogen
Ethanol
3-Me-1-BuOH
Acetate
3-Me-1-Butyrate
Analyte (mmol/L)
Liquid-gas (L-G) phase ratio

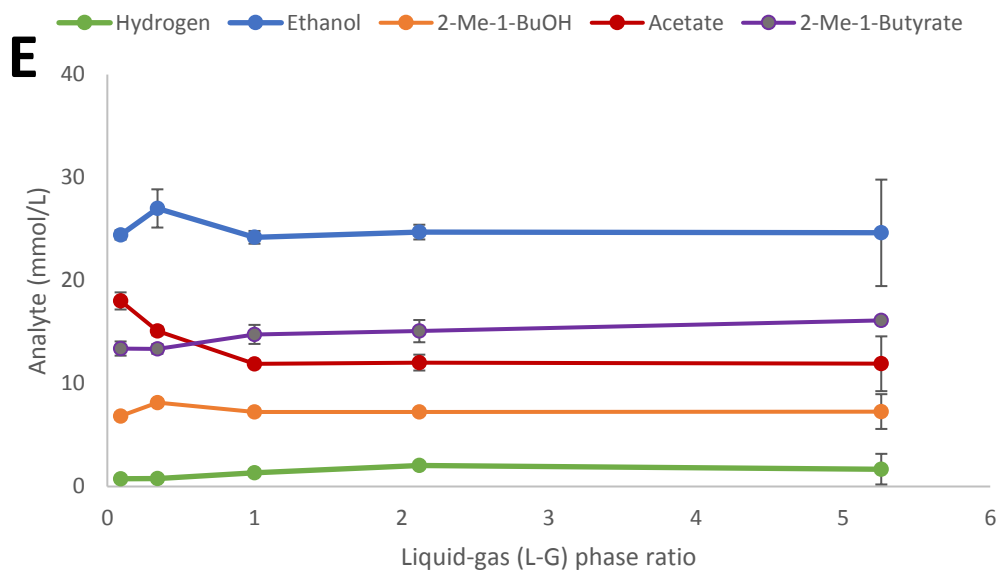
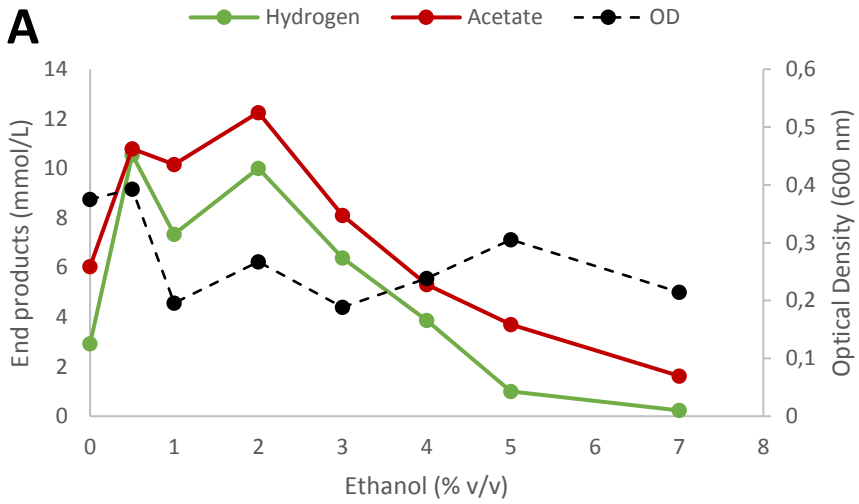


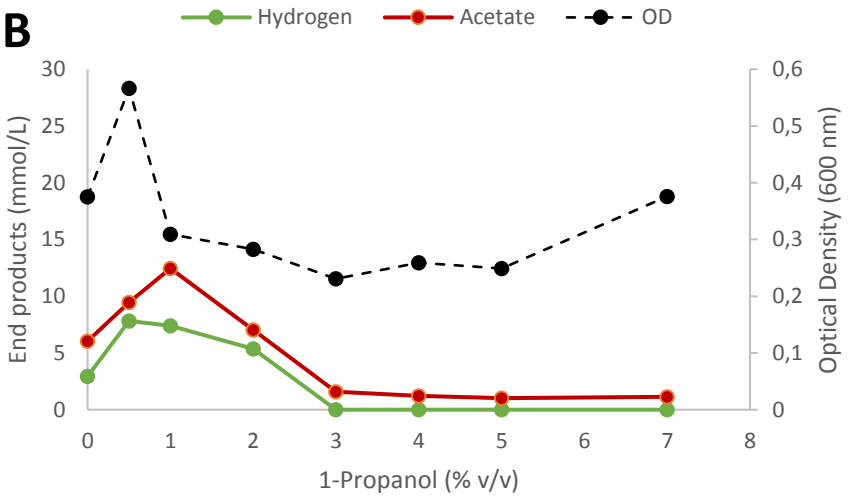
Figure 3. Impact of liquid-gas phase ratios on end product formation after 5 days from cultures of *T. pseudoethanolicus* containing (A) glucose, (B) glucose + 1-propionate, (C) glucose + 1-butyrate, (D) glucose + 3-methyl-1-butyrate, (E) glucose + 2-methyl-1-butyrate. Five different L-G phase ratios were used (0.05, 0.34, 0.98, 2.08, and 5.40). Standard deviation are shown as error bars.

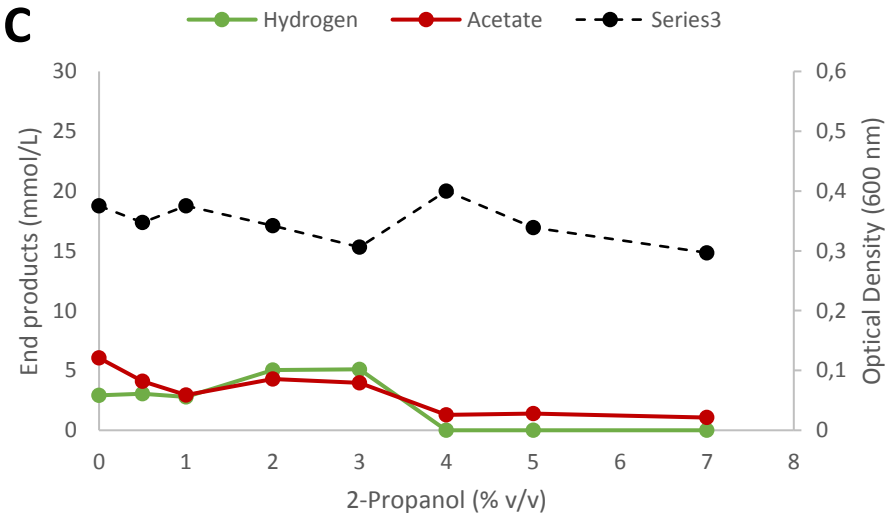
3.4. Inhibitory impact of the alcohols on end product formation from glucose

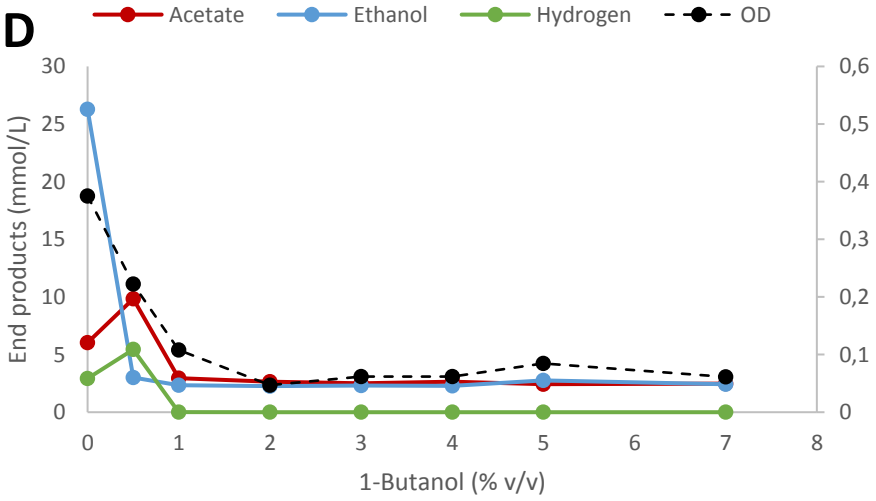
Understanding the impact of the conversion of SCFAs to their corresponding alcohols is necessary to determine at which alcohol concentration become toxic to cells. It is well known that alcohols in general inhibit bacteria by disrupting the cell membrane when in high enough concentrations (Bajapi & Iannotti, 1988; Huffer, Clark, Ning, Blanch, & Clark, 2011). The severity of inhibition by alcohols is more apparent with higher order alcohols such as butanol as compared with ethanol. For example, most *T. pseudoethanolicus* can tolerate ethanol up to 2.5% but only 1% v/v 1-butanol (Huffer et al., 2011) although some strains of *T. ethanolicus* can tolerate up to 10% v/v of ethanol (Rani, Swamy, & Seenayya, 1997). In order to investigate the inhibitory effects of aliphatic alcohols, *T. pseudoethanolicus* was cultivated on glucose (20 mM) in the presence of exogenously added alcohols (C2 to C6 alcohols) for 5 days as shown in Figure 4A-H.

In case of ethanol, 1-propanol and 2-propanol, ethanol is not shown in Figures 4A-C, either because it was exogenously added or because both propanol types co-elute with ethanol on the GC. The strain tolerated ethanol up to 4% concentration, and in fact hydrogen and acetate production was considerable higher at concentrations between 0.5 to 3% as compared with control (no added ethanol) (Figure 4A). At 4% and higher ethanol concentration end product formation was gradually lower until at 7% when a complete inhibition occurred. Similarly, higher hydrogen and acetate concentrations were observed on 1-propanol when added between 0.5 to 2.0% concentration although a complete inhibition of glucose for both alcohols were observed at 3% and higher concentrations (Figure 4B). The addition of 2-propanol resulted in a complete inhibition at 4% concentration and somewhat higher hydrogen and acetate production was observed at 2.0 to 3.0% concentration as compared with cultivation on glucose without acid addition (Figure 4C). It is known that 1-butanol strongly inhibits most bacteria at concentrations around 1% (Ezeji, Milne, Price, & Blaschek, 2010), and this was reflected in the results with *T. pseudoethanolicus* in present study. The strain produced higher amounts of hydrogen and acetate at 0.5% 1-butanol concentrations but was inactive at higher concentrations (Figure 4D). The remaining alcohols tested (2-methyl-1-propanol, 1-pentanol, 2-methyl-1-butanol, 1-hexanol) all inhibited the strain at concentration of 0.5% (v/v), except for 2-methyl-1-butanol (1.0% v/v) (Figure 4E-H).


A
Hydrogen
Acetate
OD
End products (mmol/L)
Optical Density (600 nm)
Ethanol (% v/v)


B
Hydrogen
Acetate
OD
End products (mmol/L)
Optical Density (600 nm)
1-Propanol (% v/v)


C
Hydrogen
Acetate
Series3
End products (mmol/L)
Optical Density (600 nm)
2-Propanol (% v/v)


D
Acetate
Ethanol
Hydrogen
OD
End products (mmol/L)
1-Butanol (% v/v)

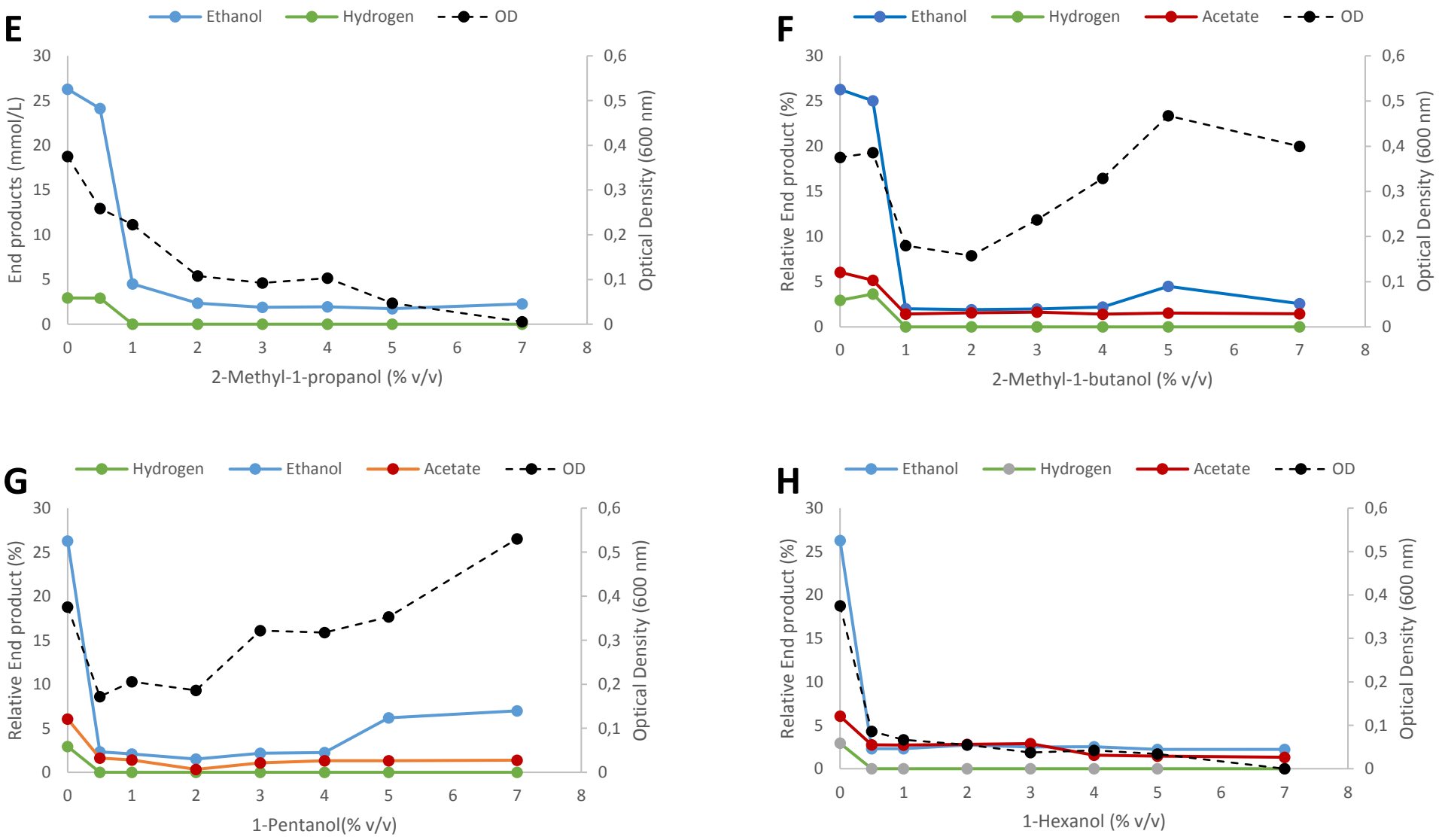


Figure 4. Impact of alcohol addition on end product formation of glucose using (A) ethanol (B) 1-propanol (C) 2-propanol (D) 1-butanol (E) 2-methyl-1-propanol (F) 2-methyl-1-butanol (G) 1-pentanol (H) 1-hexanol.

264

3.5. Effect of the ratio of glucose and fatty acid on alcohol formation

An effort to increase the amount of carboxylic acid converted to its corresponding alcohol, the use of additional reducing potential by increasing the initial concentration of glucose was investigated on three model carboxylic acids, 1-propionate, 2-methyl-1-propionate, and 1-butyrate. Figure 5 A-C) shows the increase in the conversion of SCFA to alcohol from 0 to 40 mM glucose which corresponds to a molar ratio of 0.5 to 2 of glucose to SCFA.

Without addition of any glucose 3.41 mM of 1-propanol were produced from 1-propionate, presumable by using electrons produced from oxidation of substrates present in the yeast extract (Figure 5A). Increased glucose concentrations increased the amount of the alcohol from the acid, reaching a maximum at 40 mM glucose with 74.1% conversion. Clearly, there is a substrate inhibition that occurs between 20 and 30 mM of glucose as reflected in the decrease in the amount of ethanol produced per mole of glucose degraded with similar results being obtained with increasing initial glucose concentrations without the addition of exogenously added SCFA (data not shown). It is well known that many thermophiles are strongly inhibited at moderate (20 – 30 mM) initial substrate concentrations (Scully and Orlygsson, 2015). It is unlikely that this decrease of glucose degradation is caused by the addition of the alcohol since 20 mM of 1-propanol is only 0.12% and as stated above the strain tolerates up to 3% 1-propanol concentration. Similar results were also obtained from cultivation of the strain in the presence of 1-butanol and 2-methyl-1-propanol; maximum conversion were observed at the highest concentration of glucose applied, or 50.7 and 53.4%, respectively (Figure 5B-C). Also, similar trends in less glucose degradation as with the culture amended with 1-propanol were observed.

To achieve higher conversions, another culture mode such as fed-batch or continuous culture maybe more appropriate. Furthermore, the use of a biphasic system to remove the produced SCOHs from the fermentation broth may increase titres. This approach has proven successful with butanologenic *Clostridia* using ionic liquids (Domańska & Królikowski, 2012), *in situ* vacuum distillation (Mariano, et al. 2012), and fed-batch supplemented with a non-ionic surfactant such as Tween 80 (Qin, Duns, Pan, & Xin, 2018; Xin et al., 2018).

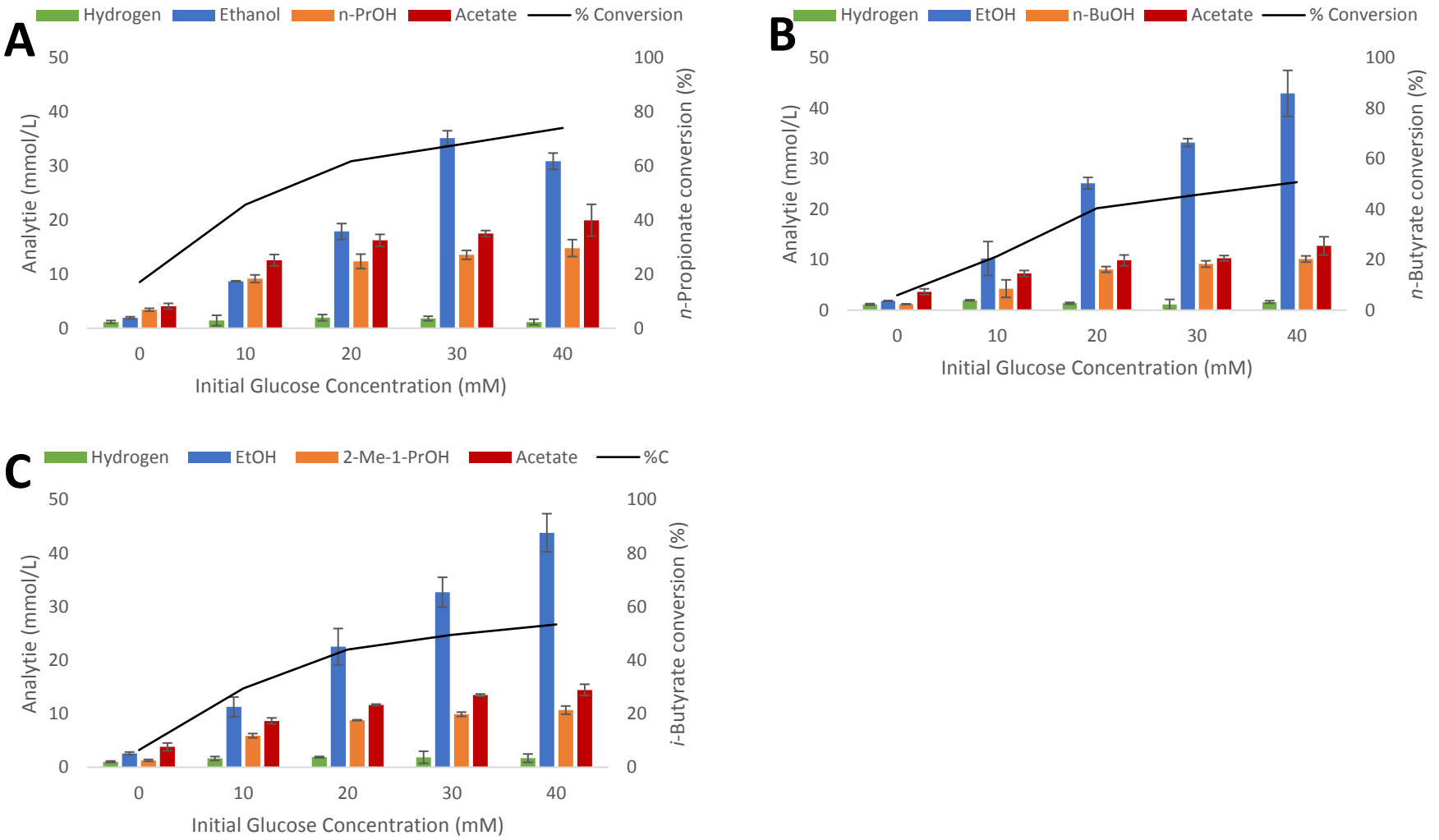


Figure 5. Impact of glucose concentration on end product formation by *T. pseudoethanolicus* in the presence of (A) 1-propionate (B) 1-butyrate (C) 2-methyl-1-propionate bioconversion. Additionally, the percent of glucose is shown (%C).

3.6. ^{13}C NMR studies

Isotopically labelled studies of the conversion of ^{13}C1 acetate, ^{13}C1 1-propionate, and ^{13}C1 1-butyrate have been previously reported by *T. pseudoethanolicus* (Scully et al. 2019). Here we demonstrate that ^{13}C1 3-methyl-1-butyrate is also converted to its corresponding alcohol in the presence of glucose (Figure 6). The appearance of a new peak (δ 60.3 ppm) can be attributed to the formation of 3-methyl-1-butanol. This is similar to the production of 3-methyl-1-butanol from ^{13}C2-labeled leucine recently reported (Scully and Orlygsson, 2019a; Scully and Orlygsson, 2019b; **Paper IV and Paper V, respectively**). Using ^{13}C1 butyrate as a model, the fermentation kinetics were monitored over 96 hours of fermentation as shown in Figure 7. ^{13}C1 1-butyrate (183.5 ppm) is rapidly converted to the ^{13}C1 1-butanol (60.3 ppm) achieving maximum conversion between 16 and 24 hours (Figure 7) which is similar to the kinetic experiment performed earlier (Figure 2B).

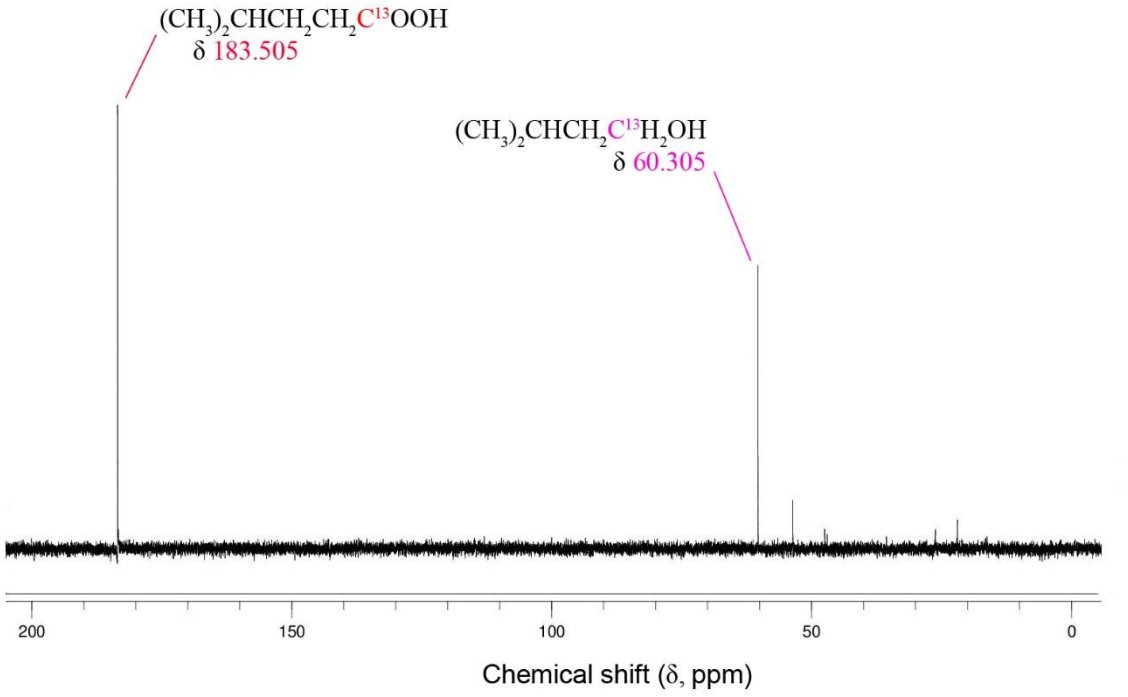


Figure 6 – Conversion of ^{13}C1 3-Methyl-1-butyrate to 3-methyl-1-butanol by *T. pseudoethanolicus* using glucose as the source of reducing potential

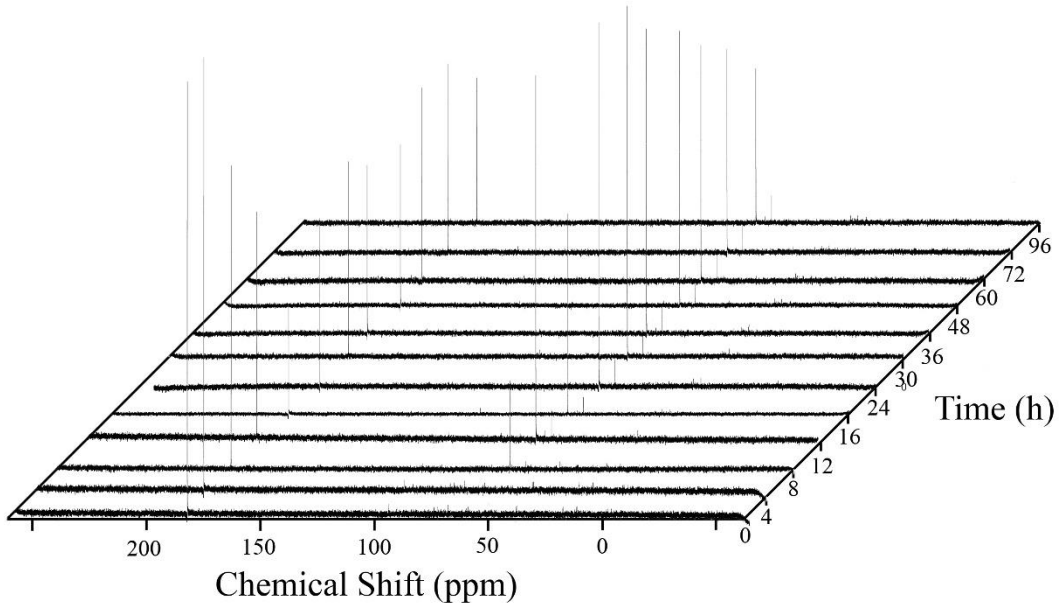


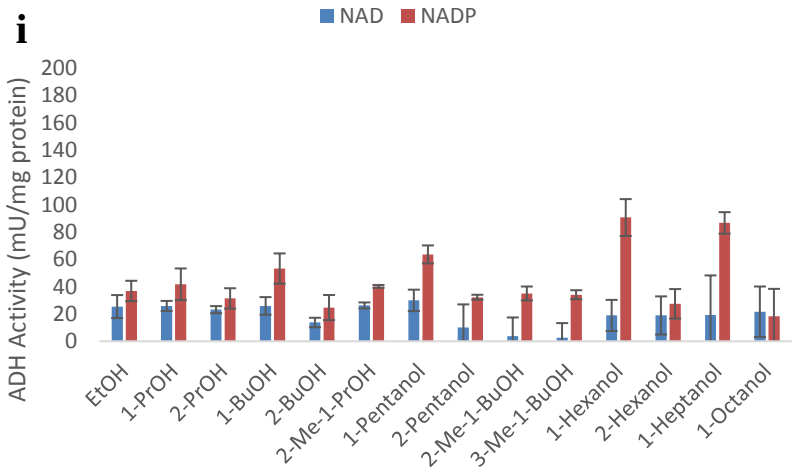
Figure 7 – Time course of ^{13}C1 1-butyrate conversation 1-butanol by *T. pseudoethanolicus* using glucose as the source of reducing potential.

3.7. Enzymatic assays

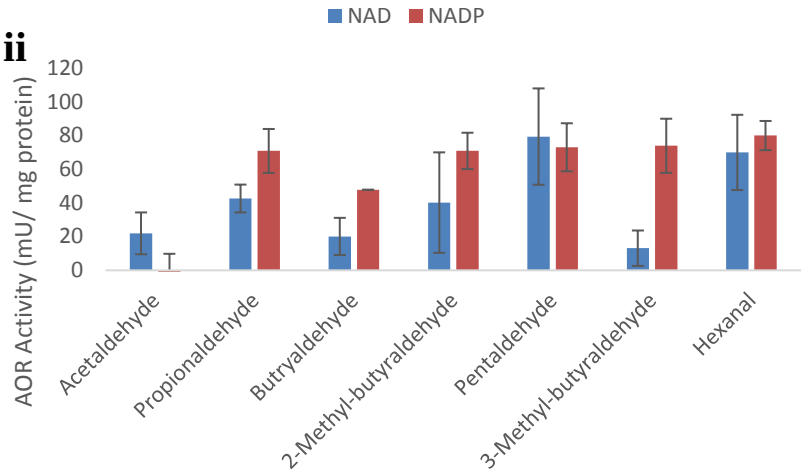
T. pseudoethanolicus was cultivated on glucose with and without the addition of C5 fatty acids for 24 hours at which time the activities of alcohol dehydrogenase (ADH) and aldehyde oxidoreductase (AOR) were determined with both NAD^+ and $NADP^+$ as cofactors as summarized in Figure 8. Generally, the ADH and AOR activities were higher in glucose-grown cultures containing exogenously added fatty acids. The activity of ADHs towards longer alcohols was higher with the addition of 1-pentanoate and 3-methyl-1-butyrate as compared with glucose only. Many *Thermoanaerobacter* strains, including *T. pseudoethanolicus*, have multiple ADHs, including both primary and secondary-specific ADHs which differ in their cofactor specificity (Bryant, Wiegel, & Ljungdahl, 1992; Lamed & Zeikus, 1981; Zhou et al., 2017); both primary specific and secondary specific ADHs are active under the examined conditions. This may suggest that higher ADH expression can be achieved by adding SCFAs to the fermentation medium. Interestingly, lower AOR activities on C2-C4 aldehydes are observed using NAD^+ as a cofactor in the C5-alcohol grown cells as compared with the glucose controls; for example, AOR activities towards propionaldehyde are 42.6, 67.9, and 71.4 mU/mg protein for cells grown on glucose, glucose and 3-methyl-1-butyrate, and glucose and 1-pentanoate, respectively. Interestingly, the ADHs present seem to be able to oxidize 1-heptanol and 1-octanol although the corresponding AOR activity was not examined. It should be noted that C7 and C8 alcohols are poorly soluble in aqueous systems so non-aqueous or biphasic systems may be necessary to achieve reduction of the corresponding C7 and C8 carboxylic acids.

A

i

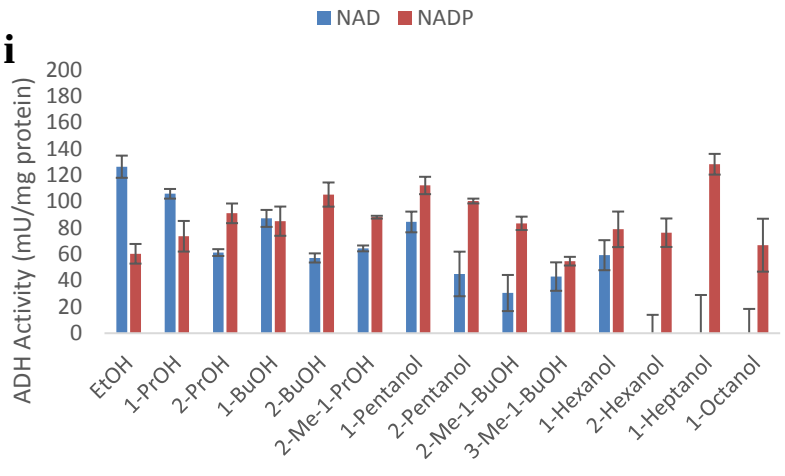

NAD
NADP
ADH Activity (mU/mg protein)
200
180
160
140
120
100
80
60
40
20
0
EtOH
1-PrOH
2-PrOH
1-BuOH
2-BuOH
2-Me-1-PrOH
1-Pentanol
2-Pentanol
2-Me-1-BuOH
3-Me-1-BuOH
1-Hexanol
2-Hexanol
1-Heptanol
1-Octanol

ii

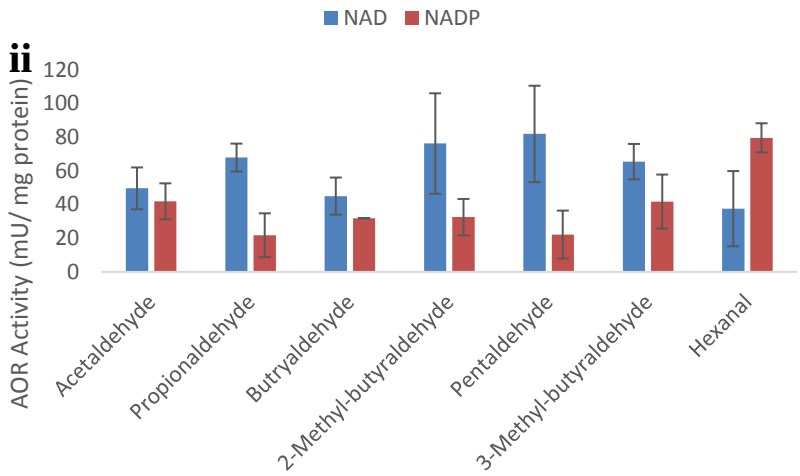

NAD
NADP
AOR Activity (mU/ mg protein)
120
100
80
60
40
20
0
Acetaldehyde
Propionaldehyde
Butryaldehyde
2-Methyl-butyraldehyde
Pentaldehyde
3-Methyl-butyraldehyde
Hexanal

B

i


NAD
NADP
ADH Activity (mU/mg protein)
200
180
160
140
120
100
80
60
40
20
0
EtOH
1-PrOH
2-PrOH
1-BuOH
2-BuOH
2-Me-1-PrOH
1-Pentanol
2-Pentanol
2-Me-1-BuOH
3-Me-1-BuOH
1-Hexanol
2-Hexanol
1-Heptanol
1-Octanol

ii


NAD
NADP
AOR Activity (mU/ mg protein)
120
100
80
60
40
20
0
Acetaldehyde
Propionaldehyde
Butryaldehyde
2-Methyl-butyraldehyde
Pentaldehyde
3-Methyl-butyraldehyde
Hexanal

C

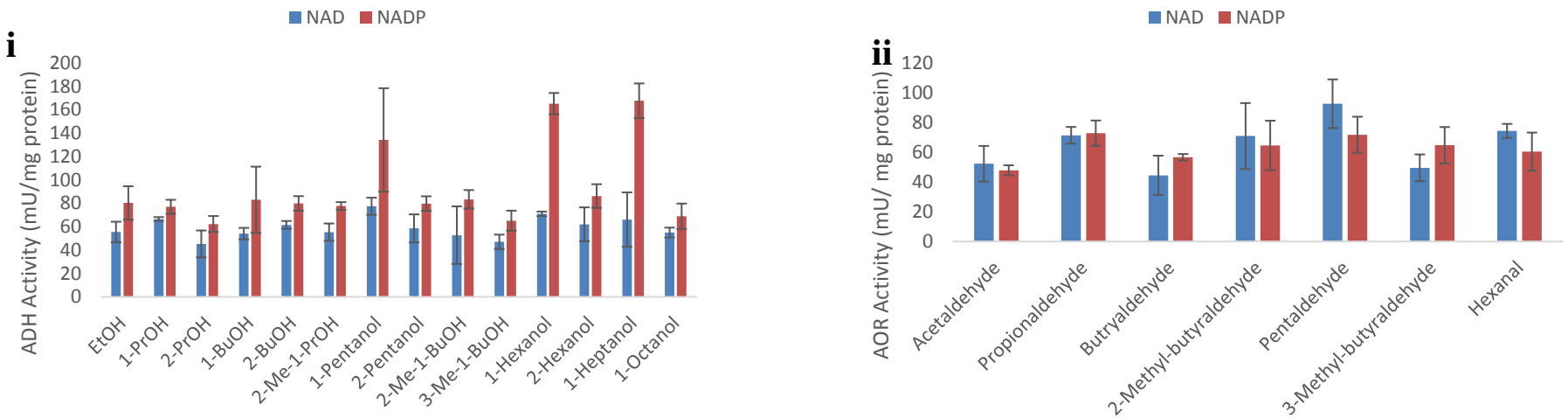


Figure 8 – Enzyme activities of *T. pseudoethanolicus* cells grown on 20 mM glucose (A), 20 mM 3-methyl-1-butryate + 20 mM glucose (B), 20 mM pentanoate + 20 mM glucose (C). Panel (i) specific ADH activity, (ii) specific aldehyde oxidoreductase activity. Values represent the average of triplicates with standard deviation presented as error bars.

Conclusions

Thermoanaerobacter pseudoethanolicus rapidly converts C2-C6 short-chain fatty acids to their corresponding alcohols in the presence of glucose. Culture conditions such as liquid-gas phase ratio have little impact on the conversion of the short-chain fatty acids to alcohols although providing additional reducing potential (glucose) increases the amount converted. C4 and higher alcohols are particularly inhibitory to *T. pseudoethanolicus* suggesting that other culture modes may be necessary to improve the amount of fatty acids converted. The use of *T. pseudoethanolicus* for the reduction of carboxylic acids present a potentially useful route to the production of alcohols from inexpensive carboxylic acids.

Acknowledgements

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Conflict of Interest

The authors declare that they are no conflicts of interest.

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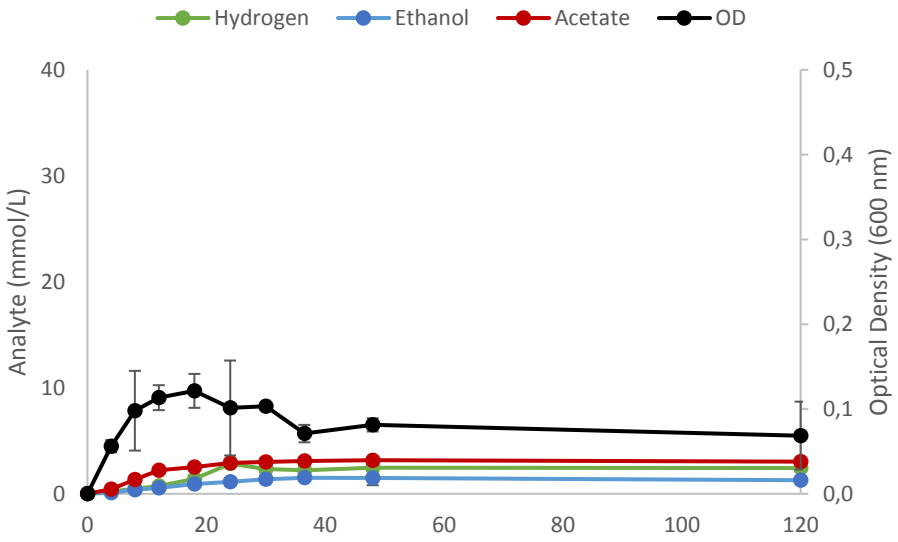
Supplemental Figure 1 - Time-course studies of yeast extract (control) fermentation by *T. pseudoethanolicus*. Values represent the average of triplicate fermentations with standard deviation presented as error bars.



Supplemental Table 1 Impact of liquid-gas phase ratios on end product formation after 5 days from cultures of *T. pseudoethanolicus*

Substrate (20 mM) + carboxylic acid (20 mM)	L-G ratio	**Analyte** (mmol/L) Hydrogen	Ethanol	Alcohol	Acetate	Carboxylic acid	Carboxylic acid conversion (%)	Optical Density (600 nm)	Carbon balance (%)
Glucose	0.09	0.96±0.19	25.84±3.26	ND	14.65±3.81	ND	ND	0.26±0.02	101.2
	0.34	2.04±0.35	25.25±1.14	ND	7.19±0.32	ND	ND	0.28±0.01	81.1
	1	3.72±0.50	30.43±2.26	ND	6.94±2.25	ND	ND	0.24±0.02	93.4
	2.12	6.03±0.27	27.39±2.26	ND	4.21±0.09	ND	ND	0.22±0.00	79.0
	5.26	4.65±0.42	32.31±3.46	ND	3.62±0.40	ND	ND	0.22±0.01	89.8
Glucose +1-propionate	0.09	1.05±0.15	13.52±0.98	11.18±0.64 (1-PrOH)	22.43±1.04	10.45±0.82	55.9	0.18±0.02	96.0
	0.34	0.33±0.27	9.10±1.73	8 .96±1.06 (1-PrOH)	15.07±0.57	12.24±0.03	44.8	0.06±0.01	84.9
	1	0.75±0.11	10.06±0.30	9.57±0.34 (1-PrOH)	14.62±0.50	12.00±0.19	47.9	0.07±0.02	77.1
	2.12	1.12±0.09	11.17±0.23	10.38±0.30 (1-PrOH)	15.34±0.34	11.58±0.39	51.9	0.08±0.02	92.2
	5.26	1.95±0.19	10.96±0.39	10.48±0.25 (1-PrOH)	16.23±1.08	12.49±0.23	52.4	0.12±0.05	94.2
Glucose +1-butyrate	0.09	0.59±0.08	23.28±0.36	11.22±0.29 (1-BuOH)	19.40±0.68	13.45±1.18	56.1	0.25±0.04	89.8
	0.34	0.96±0.05	24.71±0.97	11.14±0.46 (1-BuOH)	17.50±0.43	10.68±0.91	55.7	0.29±0.04	106.8
	1	0.89±0.80	20.63±0.24	9.96±0.15 (1-BuOH)	13.99±0.19	12.26±0.44	49.8	0.23±0.02	94.7
	2.12	2.00±0.13	22.34±0.37	10.71±0.24 (1-BuOH)	14.91±0.44	11.01±0.09	53.4	0.20±0.02	98.3
	5.26	1.85±0.63	23.37±1.67	10.71±1.07 (1-BuOH)	17.55±0.73	12.25±0.69	53.4	0.25±0.03	106.5
Glucose + 2-Me-1-butyrate	0.09	0.75±0.08	24.43±0.45	6.85±0.32 (2-Me-1-BuOH)	18.02±0.83	13.39±1.54	34.3	0.25±0.01	104.4
	0.34	0.76±0.24	27.00±1.85	8.15±0.29 (2-Me-1-BuOH)	15.11±0.43	13.36±0.32	40.8	0.20±0.01	106.0
	1	1.35±0.06	24.19±0.62	7.22±0.24 (2-Me-1-BuOH)	11.91±0.30	14.77±0.51	36.1	0.18±0.00	98.6
	2.12	2.04±0.05	24.70±0.72	7.24±0.29 (2-Me-1-BuOH)	12.03±0.77	15.09±0.23	36.2	0.22±0.02	98.4
	5.26	1.69±0.49	24.63±1.17	7.28±0.69 (2-Me-1-BuOH)	11.92±2.66	16.13±1.10	36.4	0.20±0.01	99.9
Glucose + 3-Me-1-butyrate	0.09	0.64±0.05	19.18±1.70	4.07±0.29 (3-Me-1-BuOH)	14.10±1.29	16.42±0.69	20.4	0.26±0.09	89.6
	0.34	1.09±0.05	29.08±1.88	5.89±0.37 (3-Me-1-BuOH)	12.55±0.63	16.17±0.48	29.5	0.22±0.02	106.1
	1	1.41±0.06	25.62±0.70	5.15±0.11 (3-Me-1-BuOH)	9.83±0.13	17.76±0.92	25.8	0.18±0.02	97.3
	2.12	1.92±0.26	25.78±3.80	4.92±0.54 (3-Me-1-BuOH)	9.14±1.04	18.09±1.08	24.9	0.19±0.01	96.6
	5.26	2.62±0.30	25.72±1.37	5.62±0.03 (3-Me-1-BuOH)	9.81±0.43	16.93±0.03	28.1	0.22±0.00	96.8

Supplemental Table 3 - Impact of glucose concentration on end product formation by *T. pseudoethanolicus* in the presence of (A) 1-propionate (B) 1-butyrate (C) 2-methyl-1-propionate bioconversion. Additionally, the percent of glucose consumed is shown (%C). Standard deviation is presented as error bars.

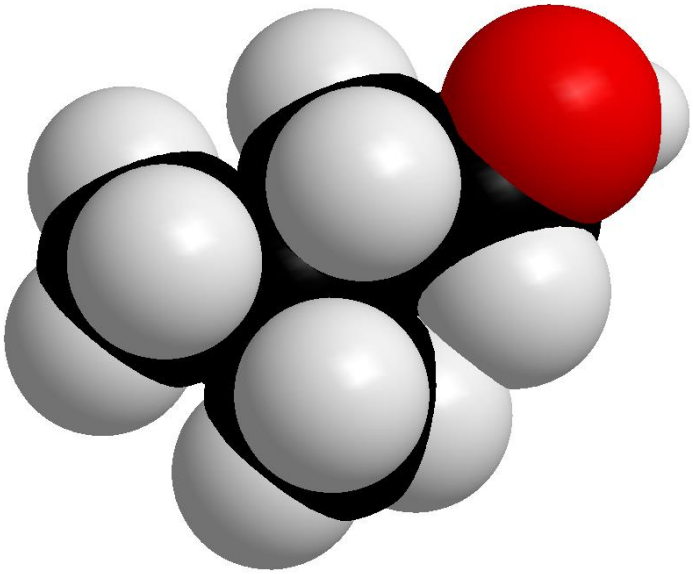
		Analyte (mmol/L)							
Substrate + carboxylic acid (20 mM)	Glucose (mM)	Hydrogen	Ethanol	Alcohol	Acetate	Carboxylic acid	Carboxylic acid conversion (%)	Optical Density (600 nm)	Carbon balance (%)
Glucose	0	0.13±0.04	1.08±0.14	ND	3.34±0.20	ND	ND	0.23±0.01	ND
	10	1.07±0.21	12.44±0.27	ND	4.24±0.26	ND	ND	0.30±0.01	83.4
	20	2.26±0.43	32.48±1.38	ND	7.03±1.13	ND	ND	0.28±0.02	98.8
	30	4.02±0.27	49.23±1.72	ND	15.46±0.63	ND	ND	0.39±0.03	107.8
	40	5.26±0.14	48.63±0.64	ND	25.33±0.78	ND	ND	1.13±0.24	92.5
Glucose +1-propionate	0	1.16±0.26	1.94±0.19	3.41±0.27 (1-PrOH)	4.07±0.53	17.37±0.85	17.1	0.37±0.10	103.9
	10	1.43±0.97	8.67±0.10	9.15±0.71 (1-PrOH)	12.58±1.05	10.59±1.31	45.8	0.45±0.12	102.5
	20	1.95±0.57	17.88±1.47	12.36±1.34 (1-PrOH)	16.23±1.12	7.86±0.75	61.8	0.34±0.01	90.6
	30	1.80±0.42	35.14±1.37	13.56±0.83 (1-PrOH)	17.49±0.57	6.57±1.59	67.8	0.46±0.05	91.0
	40	1.15±0.52	30.87±1.51	14.81±1.57 (1-PrOH)	19.96±2.95	5.25±0.34	74.1	1.14±0.17	88.6
Glucose +2-methyl-1-propionate	0	1.03±0.14	2.59±0.25	1.29±0.18 (2-Me-1-PrOH)	3.81±0.72	19.16±1.80	6.4	0.31±0.05	102.3
	10	1.64±0.37	11.29±1.83	5.90±0.40 (2-Me-1-PrOH)	8.66±0.56	14.70±1.70	29.5	0.51±0.08	101.4
	20	1.89±0.13	22.52±3.41	8.79±0.09 (2-Me-1-PrOH)	11.65±0.14	10.35±1.09	44.0	0.45±0.08	88.9
	30	1.86±1.13	32.71±2.78	9.90±0.40 (2-Me-1-PrOH)	13.48±0.22	9.14±0.86	49.5	0.71±0.09	81.5
	40	1.70±0.79	43.81±3.57	10.67±0.77 (2-Me-1-PrOH)	14.44±1.07	6.54±1.57	53.4	1.17±0.16	94.3
Glucose +1-butyrate	0	1.14±0.15	1.83±0.07	1.18±0.05 (1-BuOH)	3.64±0.58	15.78±2.17	5.9	0.31±0.08	84.8
	10	1.95±0.10	10.23±3.36	4.26±1.75 (1-BuOH)	7.25±0.61	13.96±1.32	21.3	0.30±0.06	89.3
	20	1.36±0.17	25.16±1.13	8.07±0.56 (1-BuOH)	9.86±1.07	12.23±1.64	40.4	0.47±0.12	92.2
	30	1.12±0.00	33.20±0.78	9.14±0.64 (1-BuOH)	10.27±0.55	10.31±0.42	45.7	0.60±0.16	78.7
	40	1.61±0.25	42.94±4.56	10.14±0.61 (1-BuOH)	12.73±1.81	9.08±0.33	50.7	0.90±0.04	93.6

Supplemental Table 4 - Time-course studies of fermentation of 20 mM glucose, 20 mM 1-butyrate + 20 mM glucose, and 20 mM 3-methyl-1-butyrate + 20 mM glucose by *T. pseudoethanolicus*. Values represent the average of triplicate fermentations with standard deviation presented as error bars.

Substrate	Time (h)	**Analyte** (mmol/L) Hydrogen	Ethanol	Alcohol	Acetate	Carboxylic acid	Glucose (remaining)	Carboxylic acid conversion (%)	Glu consumed(%)	Optical Density (600 nm)	Carbon balance (%)
Yeast extract (control)	0	0.00±0.00	0.00±0.00	ND	0.00±0.00	ND	ND	ND	ND	0.00±0.00	ND
	4	0.24±0.07	0.12±0.20	ND	0.45±0.07	ND	ND	ND	ND	0.06±0.01	ND
	8	0.48±0.13	0.38±0.03	ND	1.34±0.03	ND	ND	ND	ND	0.10±0.05	ND
	12	0.75±0.20	0.57±0.13	ND	2.21±0.25	ND	ND	ND	ND	0.11±0.01	ND
	18	1.41±0.29	0.91±0.18	ND	2.51±0.18	ND	ND	ND	ND	0.12±0.02	ND
	24	2.84±0.25	1.13±0.34	ND	2.92±0.01	ND	ND	ND	ND	0.10±0.06	ND
	30	2.34±0.37	1.38±0.39	ND	2.99±0.17	ND	ND	ND	ND	0.10±0.00	ND
	36,5	2.22±0.48	1.51±0.30	ND	3.09±0.24	ND	ND	ND	ND	0.07±0.01	ND
	48	2.47±0.31	1.48±0.67	ND	3.17±0.19	ND	ND	ND	ND	0.08±0.01	ND
	120	2.43±0.15	1.28±0.24	ND	3.04±0.16	ND	ND	ND	ND	0.07±0.04	ND
Glucose (20 mM)	0	0.00±0.00	0.00±0.00	ND	0.00±0.00	ND	20.00±0.00	ND	0.0	0.00±0.00	100.0
	4	0.00±0.00	1.06±0.31	ND	1.50±0.20	ND	20.00±0.00	ND	0.0	0.05±0.00	103.8
	8	0.22±0.02	1.52±0.52	ND	2.68±0.90	ND	18.30±1.10	ND	8.5	0.09±0.01	102.0
	12	0.74±0.20	8.88±1.65	ND	6.66±1.06	ND	14.70±1.47	ND	26.5	0.22±0.02	112.4
	18	1.24±0.20	13.51±0.52	ND	7.21±1.34	ND	9.10±1.53	ND	54.5	0.34±0.02	97.4
	24	1.55±0.38	29.55±0.49	ND	7.68±0.62	ND	2.40±0.48	ND	88.0	0.31±0.06	105.1
	30	2.08±0.42	31.30±3.52	ND	5.73±1.28	ND	2.30±0.57	ND	88.5	0.30±0.02	104.1
	36,5	1.76±0.36	29.80±0.58	ND	5.71±1.55	ND	0.70±0.18	ND	96.5	0.30±0.01	92.3
	48	2.62±0.47	35.04±2.13	ND	6.88±1.74	ND	0.00±0.00	ND	100	0.25±0.01	104.7
	120	2.46±0.18	30.22±0.54	ND	6.49±0.67	ND	0.00±0.00	ND	100	0.12±0.05	91.8
Glucose (20 mM) + 1-butryate (20 mM)	0	0.00±0.00	0.00±0.00	0.00±0.00 (1-BuOH)	0.00±0.00	20.00±0.00	20.00±0.00	0.0	0.0	0.00±0.00	100.0
	4	0.00±0.00	0.36±0.24	0.00±0.00 (1-BuOH)	1.00±0.28	19.68±0.25	20.00±0.00	0.0	0.0	0.06±0.01	101.7
	8	0.17±0.12	1.45±0.15	0.40±0.08 (1-BuOH)	2.50±0.16	18.61±1.15	17.98±0.53	2.0	10.1	0.11±0.02	98.2
	12	0.64±0.05	12.50±1.66	3.47±0.72 (1-BuOH)	6.36±0.99	14.45±2.25	13.56±0.91	17.4	32.2	0.37±0.06	106.5
	18	0.82±0.17	18.15±1.24	4.23±0.23 (1-BuOH)	9.47±0.37	12.34±1.01	9.54±1.22	21.2	52.3	0.42±0.02	105.5
	24	1.11±0.10	21.88±4.80	8.73±2.03 (1-BuOH)	13.85±0.74	8.69±1.96	3.21±0.32	43.7	84.0	0.25±0.01	99.3
	30	1.13±0.30	24.82±1.78	10.11±0.72 (1-BuOH)	14.89±0.66	9.79±1.33	2.47±0.19	50.6	87.7	0.17±0.01	107.6
	36,5	1.29±0.08	27.27±0.30	10.68±1.41 (1-BuOH)	16.15±2.87	9.83±1.80	0.60±0.23	53.4	97.0	0.19±0.05	108.6
	48	1.44±0.09	27.34±3.65	10.43±1.53 (1-BuOH)	15.16±1.37	6.50±1.60	0.00±0.00	52.2	100.0	0.15±0.00	99.1
	120	1.25±0.41	26.22±1.18	11.19±0.23 (1-BuOH)	15.87±0.93	6.43±1.10	0.00±0.00	56.0	100.0	0.13±0.01	99.5

Supplemental Table 4 (continued)- Time-course studies of fermentation of 20 mM glucose, 20 mM 1-butyrate + 20 mM glucose, and 20 mM 3-methyl-1-butyrate + 20 mM glucose by *T. pseudoethanolicus*. Values represent the average of triplicate fermentations with standard deviation presented as error bars.

Substrate	Time (h)	**Analyte** (mmol/L) Hydrogen	Ethanol	Alcohol	Acetate	Carboxylic acid	Glucose (remaining)	Carboxylic acid conversion (%)	Glu consumed(%)	Optical Density (600 nm)	Carbon balance (%)
Glu (20 mM) +	0	0.00±0.00	0.00±0.00	0.00±0.00 (3-Me-1-BuOH)	0.00±0.00	20.00±0.00	20.00±0.00	0.0	0.0	0.00±0.00	100.0
3-me-1-butryate (20 mM)	4	0.00±0.00	0.46±0.07	0.00±0.00 (3-Me-1-BuOH)	1.29±0.13	20.42±1.05	20.00±0.00	0.0	0.0	0.05±0.00	103.6
	8	0.32±0.04	1.64±0.37	0.00±0.00 (3-Me-1-BuOH)	2.49±0.11	19.68±2.33	18.30±1.10	0.0	8.5	0.09±0.00	100.7
	12	0.43±0.04	8.94±1.72	0.00±0.00 (3-Me-1-BuOH)	4.94±0.64	18.52±2.88	14.70±1.47	0.0	26.5	0.35±0.04	103.0
	18	0.84±0.21	15.13±2.16	1.13±0.07 (3-Me-1-BuOH)	8.52±0.54	14.22±1.34	9.10±1.53	5.7	54.5	0.38±0.03	95.3
	24	1.73±0.16	26.55±0.87	2.26±0.14 (3-Me-1-BuOH)	12.22±1.10	12.99±1.25	2.40±0.48	11.3	88.0	0.43±0.03	98.0
	30	1.87±0.16	26.93±2.15	2.24±0.13 (3-Me-1-BuOH)	12.72±0.41	13.18±1.65	2.30±0.57	11.3	88.5	0.36±0.04	102.6
	36,5	1.59±0.17	31.13±0.93	2.29±0.10 (3-Me-1-BuOH)	13.26±0.64	13.66±1.18	0.70±0.18	11.5	96.5	0.26±0.08	102.9
	48	2.15±0.16	27.66±1.65	2.31±0.10 (3-Me-1-BuOH)	13.05±0.14	13.82±1.36	0.00±0.00	11.6	100.0	0.24±0.03	94.7
	120	1.64±0.40	27.87±0.72	6.16±0.27 (3-Me-1-BuOH)	13.44±0.80	13.12±0.72	0.00±0.00	30.8	100.0	0.20±0.00	101.0



And now, the end is near;
And so I face the final curtain.
My friend, I'll say it clear,
I'll state my case, of which I'm certain.

I've lived a life that's full.
I've traveled each and every highway;
And more, much more than this,
I did it my way.

Regrets, I've had a few;
But then again, too few to mention.
I did what I had to do
And saw it through without exemption.

I planned each charted course;
Each careful step along the byway,
And more, much more than this,
I did it my way.

Yes, there were times, I'm sure you knew
When I bit off more than I could chew.
But through it all, when there was doubt,
I ate it up and spit it out.
I faced it all and I stood tall;
And did it my way.

I've loved, I've laughed and cried.
I've had my fill; my share of losing.
And now, as tears subside,
I find it all so amusing.

To think I did all that;
And may I say - not in a shy way,
"Oh no, oh no not me,
I did it my way".

For what is a man, what has he got?
If not himself, then he has naught.
To say the things he truly feels;
And not the words of one who kneels.
The record shows I took the blows -

And did it my way!

Yes, it was my way.