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Insights into Protein and Amino Acid Metabolism of *Thermoanaerobacter mathranii*

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Abstract: Few investigations have been carried out into the members of the genus *Thermoanerobacter* for protein and amino acid metabolism compared to carbohydrates, mostly due to the intense interest in bioethanol and biohydrogen in recent decades. The present study investigates the biotechnological potential of *Thermoanaerobacter mathranii* (DSM 11426) in terms of its ability to produce high-energy alcohols from amino and fatty acids. End product formation from glucose (in the presence and absence of butyrate) as well as from selected proteins and amino acids were analyzed. *T. mathranii* did not degrade any of the proteins tested to a large extent but degraded several amino acids, namely serine and the branched-chain amino acids (leucine, isoleucine, valine) when cultivated in the presence of thiosulfate. The main end products from the branched-chain amino acids were a mixture of their corresponding branched-chain fatty acids and alcohols, with the strain producing a concentration of the corresponding branched-chain alcohol between 1.0 and 1.7 mM and 8.2–10.9 mM of the corresponding fatty acid. ^{13}C2-labeled leucine revealed that the strains degraded the amino acid in the presence of thiosulfate, producing 3-methyl-1-butyrate, which was then used as an electron acceptor which led to the accumulation of 3-methyl-1-butanol. The strain is highly ethanologenic, producing more than 1.2 mol of ethanol per mol of glucose degraded. The strain was able to reduce volatile fatty acids during glucose fermentation to their corresponding alcohol, further suggesting this strain may be of greater biotechnological value beyond bioethanol production.

Keywords: *Thermoanaerobacter*; protein; amino acid; fatty acid reduction

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1. Introduction

The production of biofuels and other biomolecules from renewable biomass has been an area of intense investigation over the past 40 years. The main attention given towards biofuel production has been on the utilization of carbohydrates. The urge to use complex biomass for the production of second-generation ethanol has led to research on the use of thermophilic anaerobic bacteria, mainly because of their broad substrate range facilitating the degradation of the wide variety of sugars present in such biomass. These proteinaceous materials are often a substantial portion of biomass but have not been extensively investigated as a source of biofuels and other bio-manufactured products.

Among thermophilic anaerobic bacteria, *Thermoanaerobacter* species have been intensively investigated for their ability to produce ethanol from complex biomass because of their broad substrate spectrum. All species within *Thermoanaerobacter* are obligatory anaerobes that ferment various carbohydrates into ethanol, acetate, lactate, hydrogen, and carbon dioxide [1,2]. These bacteria originate from various habitats like hot springs, hydrothermal vents, and oil fields [3–10]. Most of the species within the genus can catabolize the hexoses and pentoses present in lignocellulosic and macroalgae biomasses as well as many of the various disaccharides commonly encountered, notably cellobiose. Paired with the ability to utilize starch and often xylan, *Thermoanaerobacter* species are excellent

candidates for the production of biofuels such as hydrogen and ethanol from complex biomass. As an example, many *Thermoanaerobacter* species are highly ethanologenic, with *T. ethanolicus*, *T. pseudethanolicus*, *Thermoanaerobacter* strain J1, and *Thermoanaerobacter* strain AK15 demonstrating ethanol yields above 1.5 mol of ethanol from 1 mole of glucose [11–15]. Additionally, many of these strains also show high yields of ethanol from hydrolysates from various lignocellulosic biomasses [16–20]. Thus, our knowledge of carbohydrate metabolism leading to the production of second-generation ethanol has increased considerably in the past two decades. However, our understanding of the role of protein and amino acid metabolism among thermophilic anaerobes has not been fully explored, despite its importance in various ecosystems as a part of nutrient cycling [21–23].

Most of our early understanding of protein degradation under anaerobic conditions was derived from mesophilic anaerobes, particularly proteolytic pathogens such as *Clostridum botulinium* [24–28], *Clostridium perfringens* [29], and *Clostridium tetanmorphum* [30], among others [31–37]. By comparison, studies on thermophilic anaerobes have been limited to several genera like *Caloramator* and *Thermoanerobacter* species, of which several strains are known to be proteolytic to some extent although the specific usage patterns of strains are often limited due to relatively few proteins and proteogenic amino acids being tested [38–40]. The importance of the need of electron acceptors for protein and amino acid degradation has been known for some time due to the unfavorable thermodynamics involved and is important knowledge for understanding the role of thermophilic bacteria in hot environments [41–43]. As an example, it is known that the acetogen *Thermoanaerobacter kivui* uses alanine in the presence of thiosulfate [44], although the use of other amino acids was not reported. *Coprothermobacter* (formerly *Thermobacteroides*) *proteolyticus*, as the name suggests, can utilize gelatin in the presence of a methanogen resulting in fermentation products such as branched-chain fatty acids (BCFAs) associated with the degradation of branched-chain amino acids (BCAAs) [45].

While several studies by our group have previously focused upon the BCAA catabolism of *Thermoanaerobacter* strains, namely *Thermoanaerobacter* strain AK85 [46], *T. pseudethanolicus* [47], and *T. brockii* [48], there has been one comprehensive investigation of all twenty proteogenic amino acids using *Thermoanaerobacter* strain AK90 [49], which is not deposited in a publicly accessible culture collection. This study has shown that species within the genus *Thermoanaerobacter* can degrade the BCAA in the presence of hydrogen-scavenging organisms, like hydrogenotrophic methanogens, as well as by using thiosulfate in the medium to scavenge the electrons produced in the oxidative deamination of these amino acids. Interestingly, co-cultivating *Thermoanaerobacter* strains with a hydrogenotrophic methanogen during growth on BCAAs resulted in the production of their corresponding BCFAs only. However, by adding thiosulfate to the medium these bacteria produce a mixture of both their corresponding BCFA and branched-chain alcohols (BCOHs). The production of an alcohol thus seems to be dependent on the electron-scavenging system used by these bacteria.

In the early 1990s, *Thermoanaerobacter* strain A3, which would go on to be described as *Thermoanaerobacter mathranii*, was isolated from an alkaline hot spring (70 °C, pH 8.5) in Hveragerði (SW Iceland) through the enrichment of sediment material in anaerobic media containing 0.2% *w*/*v* xylan [50]. The strain produced ethanol as a dominant end product from xylose with other end products being carbon dioxide, hydrogen, and lactate. Strain A3 was formally described as a new species, *Thermoanaerobacter mathranii*, several years later, during which time one of the authors of the original paper describing this strain, Indra Mathrani, had passed away due to food poisoning, resulting in the strain bearing his namesake in his honor [4]. The strain grows between 50 and 75 °C with optimum growth at 70 °C and grows best at near neutral pH. Like other *Thermoanaerobacter* species, *T. mathranii* is a mixed acid producer although ethanol is the dominant end product from glucose. Notably, *T. mathranii* subsp. *Alimentarius* is not as sensitive to ethanol as other thermophilic anaerobes, being able to tolerate 4% *v*/*v* without prior adaptation, making it a promising candidate for bioethanol production [51]. The strain has been intensively investigated for

its ethanol production purpose, both the wild type (Ahring et al., 1999) as well as several genetically modified strains that have originated from the A3 type strain [16,17]. Thus, *T. mathranii*, much like *T. pseudethanolicus*, is a strong candidate for bioethanol production from lignocellulosic biomass hydrolysates as it can degrade the constituent hexoses and pentoses comprising lignocellulosic biomass. As proteins their component amino acids are an integral part of biomass, and understanding their metabolism is of importance and potentially of biotechnological value. Unfortunately, the protein and amino acid metabolism, much like the end products of non-glucose carbohydrates, is seldom reported during the characterization of novel strains and has rarely garnered attention. To the best of our knowledge, the protein and amino acid metabolism of *T. mathranii* have not been investigated in any detail. Fortunately, *T. mathranii*, under the NCBI Bioproject PRJNA33329, was sequenced and annotated by the DOE Joint Genome Institute (JGI) and uploaded to NCBI. JGI performed the genomic annotation with the Prokaryotic Genomic Annotation Pipeline (PGAP) program. The genome assembly is further available on NCBI with the reference genome accession number ASM9296v1.

The present investigation is directed towards gaining insight into the protein and amino acid metabolism of *Thermoanaerobacter mathranii*. Of particular interest is the strain's potential to generate the corresponding BCOHs like the previously reported *Thermoanaerobacter* strains, as well as a better understanding of the potential reductive route from the fatty acids produced by BCAA catabolism to their corresponding primary alcohol.

2. Materials and Methods

2.1. Culture Medium and Preparation

All reagents were obtained from Sigma Aldrich unless otherwise noted. Keratin was locally obtained and used without further preparation. *Thermoanaerobacter mathranii* (DSM 11426) was acquired from DSMZ and was cultivated in Basal Mineral (BM) medium prepared as previously described [52]; the 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: $FeCl_2{\cdot}4H_2O$ 2.0g, 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. The medium was prepared by adding the buffer to distilled water containing resazurin and boiling the mixture for 10 min and cooling under nitrogen flushing. The mixture was then transferred to serum bottles using the Hungate technique [53,54] and autoclaved (121 °C) for 60 min. All other components of the medium were added separately through filtered (0.45 µm) sterilized solutions. All experiments were conducted at 65 °C and at a pH of 7.0 with a liquid–gas (L-G) ratio of 1:1 unless otherwise noted. In all cases, experiments were performed in triplicate.

2.2. API ZYM Test

API ZYM strips (BioMérieux, Lyon, France) were used as directed by the manufacturer's instructions with minor adaptations to higher incubation temperatures. Overnight cultures of *T. mathranii* cultivated on glucose (20 mM) were used for the assay. After inoculation of the strain (~50 µL) into the API ZYM strip wells, strips were incubated for 2 h at 65 °C in a sealed humidified bag by placing the inoculated strips on moist paper towels. Then, 50 µL of ZYM A reagent (25 g tris(hydroxymethyl)aminomethane, 10 g sodium dodecylsulfate, and 11 mL of concentrated HCl in 100 mL of dH_2O) were added followed by 50 µL of freshly prepared ZYM B reagent (0.12 g of Fast Blue BB in 40 mL of methanol and 60 mL of dimethylsulfoxide) and the mixture was developed for 20 min under a bright fluorescent light. All test strips were performed in duplicate.

2.3. Substrate Utilization Spectrum

The ability of the strain to utilize amino acids was tested at 20 mM concentration in the absence and presence of thiosulfate (20 mM). Protein degradation (casein, collagen, gelatine, keratin) was tested at 0.2% (*w*/*v*) concentration. Cultures were incubated for a period of 5 days at which time the end products were analyzed. Experiments were carried out in 25 mL serum bottles with a liquid–gas phase ratio of 1.0.

To investigate the electron flow during glucose fermentation, in the presence and absence of butyrate as an external electron acceptor, the strain was cultivated in BM medium on glucose (20 mM) only and on glucose (20 mM) with butyrate (20 mM). The experiment was performed in 57 mL serum bottle with a liquid phase ratio of 1.0.

2.4. NMR Experiments

Thermoanaerobacter mathranii was cultivated (14 days) on 20 mM ^{13}C2 leucine with and without the addition of thiosulfate (40 mM) as well as on leucine without thiosulfate but with ^{13}C1 3-methyl-1-butyrate. The methodology used has been described elsewhere [46,55].

2.5. Genome Search

To analyze the genome of *T. mathranii* subsp. *mathranii* A3, the reference genome assembly and PGAP annotation files were downloaded from NCBI's genome database. The Refseq annotation file was searched manually to identify relevant genes corresponding to the BCAA fermentation, carboxylic acid reduction, and ethanol fermentation pathways. The Expsasy Enzyme nomenclature database was applied to search for alternative naming conventions of enzymes and determine the EC number. The protein sequences were cross referenced with the Uniprot database by BLAST to confirm the enzymatic activity of the sequence in question. It was through this method that relevant enzymes were detected within the *T. mathranii* genome. Protein alignments were conducted with Uniprot align software.

2.6. Analytical Methods

Hydrogen was analyzed with a Perkin Elmer Auto System XL gas chromatograph according to [56] (Waltham, MA, USA). Alcohols and volatile fatty acids were quantified by gas chromatography using a Perkin Elmer Clarus 580 gas chromatograph as previously described [56]. Optical density was determined by measuring absorbance at 600 nm with a Perkin Elmer Lambda-25 UV-Vis spectrophotometer in a cuvette with a pathlength of 1 cm. Hydrogen sulfide was analyzed as described by Cline (1969) [57].

3. Results and Discussion

3.1. Enzymatic Activities (API ZYM)

Unlike other genera commonly described in the literature, *Thermoanaerobacter* strains have not undergone many of the routine chemotaxonomic tests using commercially available screening kits like many other mesophilic bacteria have. To obtain an overview of the common carbohydrate and amino acid-related enzyme activities produced by *T. mathranii*, various strains were evaluated; the use of the API ZYM strip proved to be facile although attempts to use the strips at lower temperatures did not produce good results supporting the thermophilic nature of the enzymes. *T. mathranii* was found to be positive for esterase, trypsin, acid phosphatase, and naphthol-AS-BI-phosphohydrolase (Supplementary Table S1).

Being highly saccharolytic, it is a not surprising that the strain was found to be negative for the enzymes typically involved in carbohydrate catabolism, such as α- and β-galactosidase and α- and β-glucosidase (Supplementary Table S1). There are several possible reasons for this observation. The API ZYM test strips use substrate analogs that contain a sterically bulky naphthyl which may not be compatible with the active site of the enzymes in question. Furthermore, as the *T. mathranii* culture used for this assay was

cultivated on glucose, it could be the case that the basal levels of expression for other import systems are weakly expressed; repeating such an assay after cultivating the organism on other carbohydrates could result in the genes responsible for the catabolism of these substrates being upregulated and thus expressed at levels that would allow their activity to be more readily detectable in this format. *T. mathranii* tests positive for the presence of an esterase that is active on four carbon substrates but seems to lack activity on the C8 and C14 esters. While the explicit physiological function of esterases in *Thermoanaerobacter* is not known, it has been speculated that it may serve to help with the deacylation of hemicellulose and other polymeric carbohydrates. Esterases have broad biotechnological potential and cold-active esterases have demonstrated a broad catalytic promiscuity giving them a wide range of applications in organic synthesis. To the authors' knowledge, few if any thermostable esterases have been exploited, making this a potentially interesting avenue for future work. The presence of two phosphatases, acid phosphatase and naphthol-AS-BI-phosphohydrolase, indicates that *T. mathranii* is capable of scavenging phosphate from its environment from both inorganic and organic sources. *T. mathranii* tested positive for trypsin, a common serine protease, which suggests that this strain may have some role in protein hydrolysis in its environment even if the strain itself does not seem to be highly proteolytic (see later). The trypsin produced by *T. mathranii* has not been previously described but warrants further investigation. The biotechnological potential of trypsin is well-established with this enzyme having found applications.

3.2. Degradation of Amino Acids

Much less attention has been paid to the capacity of thermophiles to degrade amino acids. Most of the information on amino acid catabolism among Clostridia has been gained from well-known proteolytic members of *Clostridium sporogenes* [36], *Clostridium botulinum* [31], and *Clostridium sticklandii* [32–34]. The degradation of amino acids is a complex process involving several oxidation and reduction steps and some amino acids can be degraded via multiple routes often with specific conditions being necessary. Generally, the most common pathways employ a two-step mechanism involving a preliminary oxidative deamination of the amino acid, yielding a corresponding α-keto acid, which is then oxidatively decarboxylated to give a one-carbon shorter carboxylic acid [21]. However, under anaerobic conditions, this route is only possible for amino acids with high oxidation stages, such as serine and threonine [43]. The reduced amino acids, such as the BCAAs (leucine, isoleucine, and valine) as well as alanine, are usually not degraded as single substrates under anaerobic conditions unless an electron-scavenging element is added to the medium. In the 1990s, several investigations showed that these reduced amino acids were degraded when the amino acid-degrading bacteria could dispose of the electrons produced during the oxidation of these amino acids to an external electron accepter (either by co-cultivating the amino acid degrading bacterium with a hydrogenotrophic methanogen or through the use thiosulfate as a chemical electron acceptor). For instance, Fardeau et al. (1997) demonstrated that *Thermoanaerobacter brockii* degraded the BCAAs only in the presence of thiosulfate, producing a one-carbon shorter branched-chain carboxylic acid [41]. Later investigations revealed that the BCAA were not only degraded to their corresponding fatty acid, but to a mixture of their corresponding BCFAs and BCOHs by *Thermoanaerobacter brockii*, and the *Caldanaerobacter subterraneaus* subsp. *yonseiensis*, in the presence of thiosulfate [48].

In the present study, *Thermoanaerobacter mathranii* degraded serine and the three BCAAs (Figure 1), but only in the presence of thiosulfate. Aside from *T. brockii*, some work has been carried out on other *Thermoanaerobacter* strains which reveals broad differences in the ratio of the alcoholic to carboxylic acid products. Table 1 shows selected data available in the literature concerning leucine metabolism whereas the Supplementary Materials show similar data for all three BCAAs. Compared to other *Thermoanaerobacter* strains, *T. mathranii* uses relatively few of the 20 proteogenic amino acids. For comparison, *Thermoanaerobacter*

strain AK90 degraded a greater variety of amino acids, and in addition to serine, six other amino acids were degraded in the presence of thiosulfate [49].

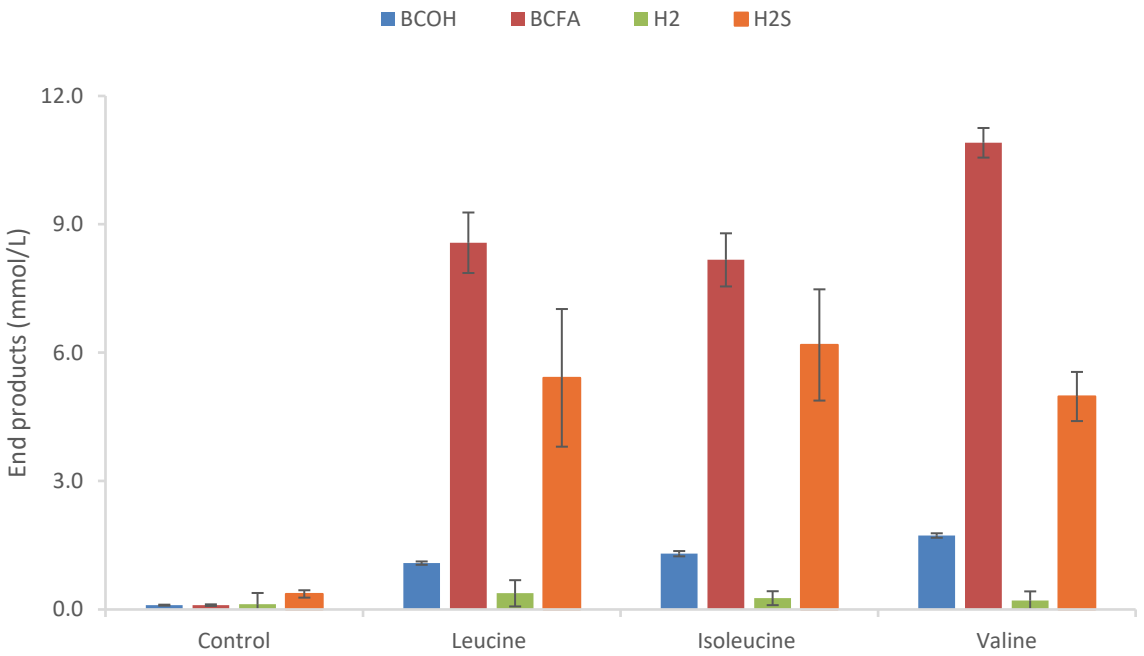


Figure 1. End product formation from BCAA fermentation by *T. mathranii* with and without thiosulfate (40 mM) after five-day cultivation (65 °C, pH 7.0). In this instance, the BCOH and BCFA reported corresponds to those produced from each specific BCAA; thus, leucine yields 3-methyl-1-butanol and 3-methyl-1-buryate, isoleucine produced 2-methyl-1-butanol and 2-methyl-1-butyrate, and valine yields 2-methyl-1-propanol and 2-methyl-1-propionate. Values represent the average of triplicate fermentations with standard deviation shown as error bars. Control contains yeast extract only.

Table 1. Comparison of end products formed from branched-chain amino acid fermentation by selected *Thermoanaerobacter* strains.

Strain	Substrate	RCOOH (mM)	ROH (mM)	ROH/RCOOH Ratio	Reference
T. mathranii (DSM 11426)	Leucine + S_2O_3	8.6	2.8	0.33	This study
T. pseudethanolicus (DSM 2355)	Leucine + S_2O_3	10.3	1.2	0.12	(Scully and Orlygsson, 2020) [47]
Thermoanaerobacter brockii (DSM 1457)	Leucine + S_2O_3	13.6	1.5	0.11	(Scully and Orlygsson, 2014) [48]
T. ethanolicus (DSM 2246)	Leucine + S_2O_3	5.1	0.6	0.12	(Scully et al., 2015) [49]
Thermoanaerobacter strain AK68	Leucine + S_2O_3	13.0	2.5	0.15	(Scully et al., 2015) [49]
Thermoanaerobacter strain AK85	Leucine + S_2O_3	10.6	1.4	0.13	(Scully and Orlygsson, 2019) [46]
Thermoanaerobacter strain AK90	Leucine + S_2O_3	9.7	3.5	0.36	(Scully and Orlygsson, 2015) [49]
Thermoanaerobacter strain AK152	Leucine + S_2O_3	8.8	2.3	0.26	(Scully et al., 2015) [49]

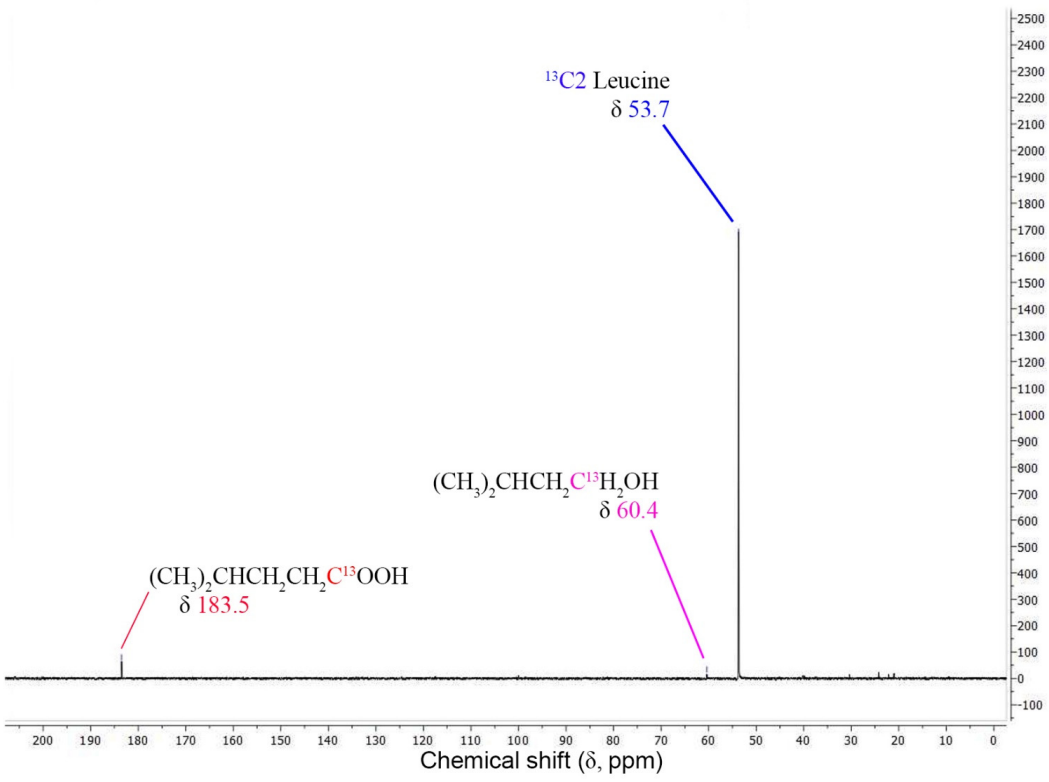
Similarly to other *Thermoanaerobacter* strains, *T. mathranii* produced a mixture of BCFAs and BCOHs from the BCAAs and the concentration of the acid was always considerably higher than the alcohol in all cases (Figure 1).

Leucine was thus degraded to a mixture of 3-methyl-1-butyrate and 3-methyl-1-butanol, isoleucine to 2-methyl-1-butyrate and 2-methyl-1-butanol, and valine to 2-methyl-1-propionate and 2-methyl-1-propanol. The highest amount of BCFA and BCOH accounted for valine degradation resulting in 1.73 and 10.90 mM of the 2-methyl-1-propanol and 2-methyl-1-propionate, respectively. *T. mathranii* generally has higher ROH-to-RCOOH ratios with ratios of 0.16 for valine, 0.33 for leucine, and 0.38 for isoleucine than other strains

examined thus far, including other members of *Thermoanaerobacter* (Table 1; Supplemental Table S2). Compared to other *Thermoanaerobacter* and *Caldanaerobacter* strains degrading BCAAs described in the literature to date, *T. mathranii* produces higher concentrations of BCOHs, such a 2-methyl-1-propanol from valine. For comparison, *Thermoanaerobacter* strains typically have ROH/RCOOH ratios between 0.05 and 0.22 for valine, 0.11 and 0.36 for leucine, and 0.09 and 0.31 for isoleucine when thiosulfate is used as the terminal electron acceptor (Table 1; Supplemental Table S2).

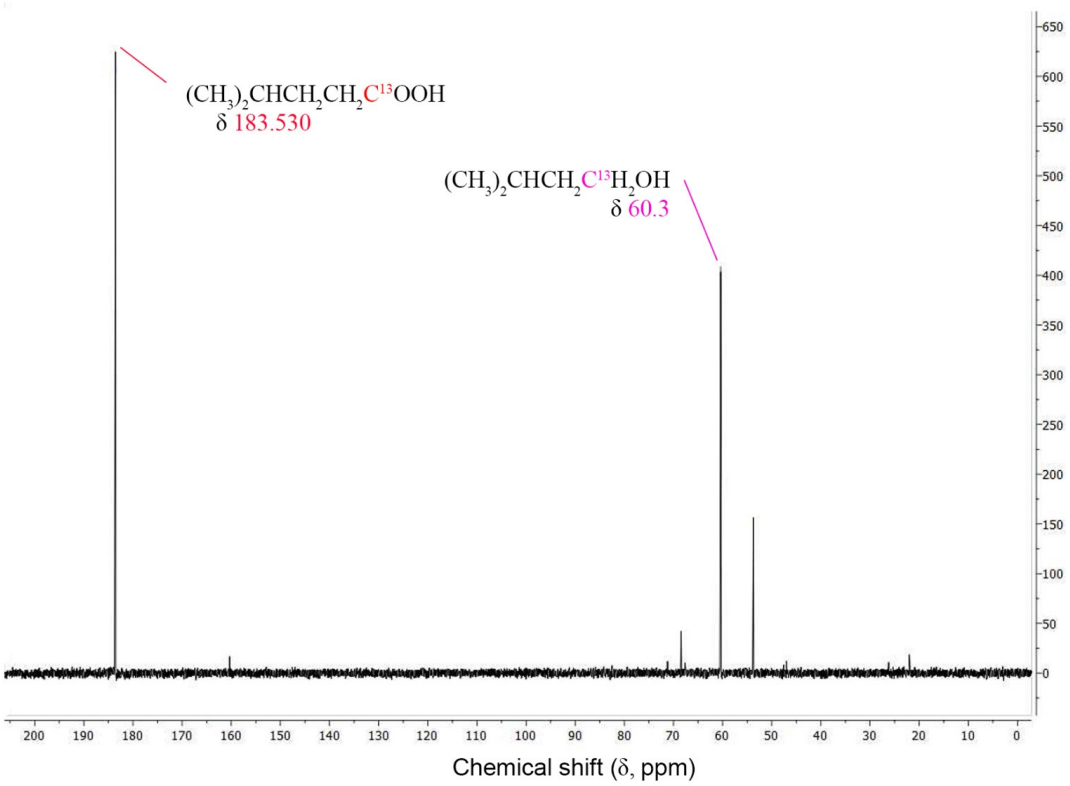
Later studies on other strains within the genera *Thermoanaerobacter* and *Caldanaerobacter* showed that this ability to produce a mixture of alcohols and acids from BCAA was common among both genera [49]. Investigations to understand in more detail the reaction pathway these bacteria use to produce both acid and alcohol have been carried out, with *Thermoanaerobacter* strain AK85 showing that the partial pressure of hydrogen was indeed of great importance for the ratio of the end products formed [46]. To demonstrate the conversion of leucine to a mixture of its corresponding fatty acid and alcohol *T. mathranii* was cultivated on ^{13}C2-labeled leucine, without and with thiosulfate. When the strain was cultivated without thiosulfate, no labeled end products were formed (Figure 2a). Conversely, when thiosulfate was added, both C2 3-methyl-1-butyrate and C2 3-methyl-1-butanol were produced (Figure 2b).

To demonstrate whether the 3-methyl-1-butyrate can be converted to its corresponding alcohol, the strain was finally cultivated on leucine with ^{13}C1-labeled 3-methyl-1-butytate in the presence of thiosulfate. This resulted in the formation of 3-methyl-1-butanol showing that the fatty acid can indeed act as an electron acceptor instead of thiosulfate and produce the corresponding alcohol (Figure 2c). Earlier studies with *Thermoanaerobacter* strain AK85 and *Thermoanaerobacter pseudethanolicus* have also shown that by using ^{13}C1 leucine as a substrate BCAAs are first converted to BCFAs which in turn can be used as an electron donor and converted to the corresponding alcohol [46].

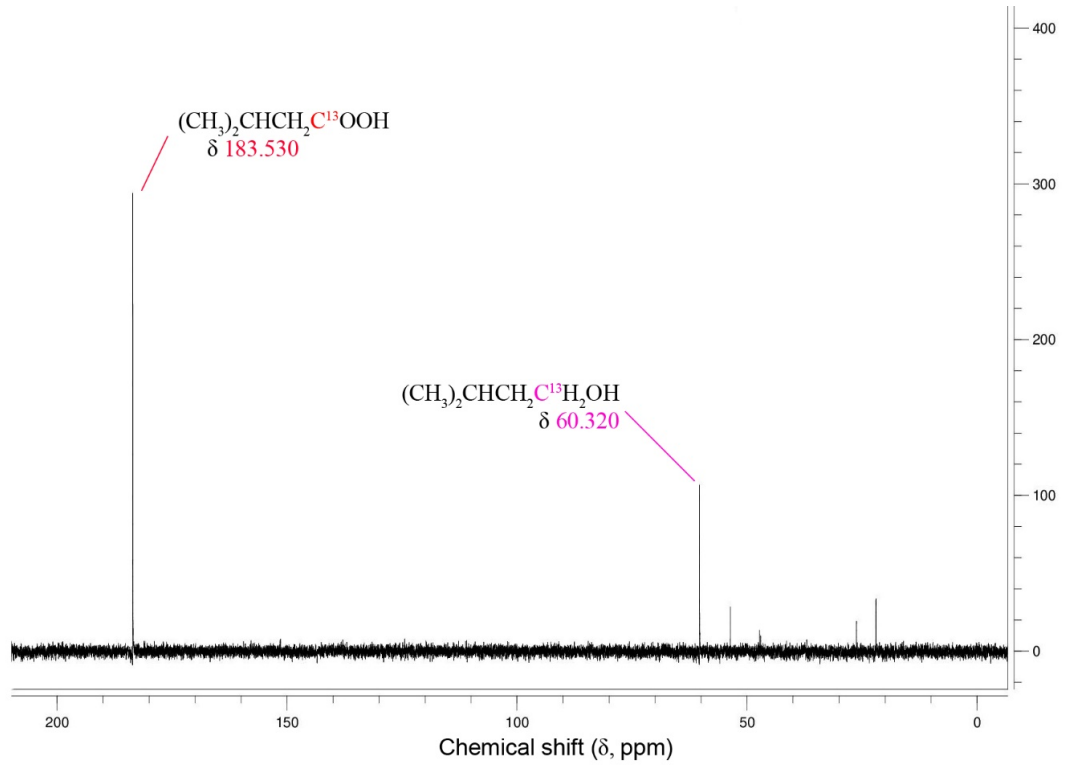


(a)

Figure 2. *Cont.*



(**b**)



(**c**)

Figure 2. ^{13}C NMR−spectrogram for the fermentation of ^{13}C2 leucine by *T. mathranii* in the absence (**a**) and presence of thiosulfate (**b**) and ^{13}C1 3−methyl−1−butyrate in the presence of unlabeled leucine and thiosulfate (**c**). Fermentations were performed at 65 °C and pH 7.0 over 14 days. Blue, red, and magenta peaks correspond to the ^{13}C−labeled carbon of the leucine, 3-methyl-butyrate, and 3-methyl-butanol, respectively.

3.3. Degradation of Proteins

Protein degradation by thermophilic anaerobic bacteria has received much less attention compared to carbohydrates. Strictly anaerobic mesophiles capable of degrading protein have received more attention, such as *Clostridium botulinum* [28], *Clostridium difficile* [58], and *Clostridium perfringens* [29]. Studies on thermophilic anaerobes have been limited to several genera like *Caloramator* and *Thermoanaerobacter*. The importance of electron acceptors for protein and amino acid degradation has been known for some time now and is important knowledge for understanding the role of thermophilic bacteria in hot environments [42].

T. mathranii was tested for growth on four types of proteins, casein, collagen, gelatin, and keratin at concentrations of 0.2% *w*/*v* in the presence or absence of 20 mM of thiosulfate (Figure 3). The strain only produced acetate and ethanol slightly above control values for casein, collagen, and gelatin as compared to yeast extract controls, while cultivation on keratin resulted in end products similar to control values. However, the addition of peptone and yeast extract enhanced both the growth of and formation of end products by the strain.

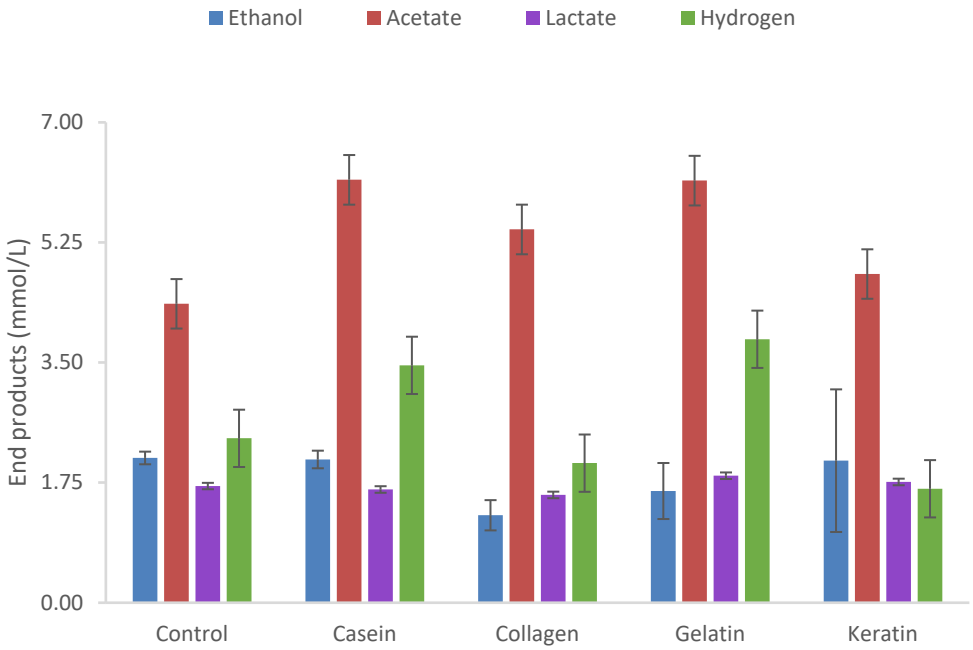


Figure 3. Fermentation of casein, collagen, gelatine, and keratin (0.2% *w*/*v*) by *T. mathranii* for five days' cultivation (65 °C, pH 7.0). Values represent the average of triplicate fermentations with standard deviation shown as error bars. Control contains yeast extract only.

Interestingly, BCFAs and BCOHs were not above control values; although, when the fermentation products were analyzed after five days, higher concentrations could have been observed if additional time for peptide hydrolysis was provided for complete degradation.

3.4. Conversion of Fatty Acids to Alcohols

Being able to reduce BCFAs to their corresponding alcohol during the degradation of the BCAAs, as shown above, is of interest and has led to an investigation of this phenomenon. As is common among thermophilic anaerobes, *Thermoanaerobacter* and *Caldanaerobacter* dispose of the electrons produced during glucose (and other carbohydrates) oxidations to pyruvate to produce ethanol or lactate. Recent investigations have, however, demonstrated that some *Thermoanaerobacter* strains can also reduce other electron acceptors like fatty acids which are converted to their corresponding alcohols [46,59–61]. This was tested for *Thermoanaerobacter mathranii* by cultivating the strain on glucose only and on

glucose in the presence of butyrate. When the strain was cultivated on glucose only it led to a mixture of ethanol, acetate, and lactate according to Equation (1):

$$1\ \text{Glucose} \rightarrow 1.23\ \text{Ethanol} + 0.26\ \text{Acetate} + 0.31\ \text{Lactate} + 1.49\ CO_2 + 0.51\ H_2 \quad (1)$$

When the strain was cultivated on glucose with the addition of 20 mM of butyrate, the reaction stoichiometry changed according to Equation (2).

$$1\ \text{Glucose} + 1\ \text{Butyrate} \rightarrow 0.85\ \text{Ethanol} + 0.65\ \text{Acetate} + 0.13\ \text{Lactate} + 0.47\ \text{Butyrate} + 1.50\ CO_2 + 0.07\ H_2 + 0.37\ \text{Butanol} \quad (2)$$

Thus, as expected, the strain produces less ethanol and more acetate in the presence of butyrate as an electron acceptor and the fatty acid is partially converted to its corresponding alcohol, in this case butanol. It is suggested that the reducing potential normally used during glucose fermentation that would be disposed of via ethanol production or the reduction of pyruvate to lactate is instead diverted to the reduction of butyrate.

3.5. Genome and Pathway of Amino Acid Metabolism

T. mathranii's genome was used to investigate the possible enzymes present that are responsible for the amino acid metabolism shown to be active. The strain only degraded serine and the BCAA in the present study. Serine was weakly degraded to a mixture of acetate and ethanol when used as the sole substrate but mainly to acetate when thiosulfate was added to the medium. The strain was shown to be positive for genes responsible for serine-*O*-acetyltransferase and serine hydrolase. The BCAAs (leucine, isoleucine, valine) were only degraded when the strain was cultivated in the presence of thiosulfate. Genomic studies indeed show the presence of BCAA-specific ABC transporter permeases as well as BCAA transaminases. A proposed scheme for BCAA utilization and the production of BCFA and BCOH is shown in Figure 4. Supplementary Table S3 comprises the enzymes found to be present in the genome of *T. mathranii*.

Other genome studies show the activity of several dehydrogenases, such as histidinol-, homoserine-, and L-lactate dehydrogenases as well as several genes responsible for glutamate and histidine metabolism. These two amino acids were, however, not utilized by *T. mathranii* in the present study. The presence of enzymes involved in carboxylic acid reduction, like acetaldehyde decarboxylase, alcohol dehydrogenase, acetate kinase, acetyl phosphotransferase, pyruvate ferredoxin oxidoreductase, pyruvate formate lyase, and aldehyde reductase (Table 2, Supplementary Table S3), were investigated using *Thermoanaerobacter psudethanolicus* as a reference. Both strains were positive for most of the enzyme pathways investigated. However, there are several distinct differences between the species. *T. mathranii* exhibited copies of all the enzymes searched for excluding aldehyde ferredoxin oxidoreductase and thiosulfate reductase, which was not found in either strain. *T. pseudethanolicus* is positive for the aldehyde ferredoxin oxidoreductase but not BCAA ABC transporter permease or amino acid permease (Table 2). Concerning the utilization of thiosulfate as an electron acceptor, both strains are positive for thiosulfate transporter family proteins and an unannotated thiosulfide reductase family protein is suspected for the utilization of thiosulfate as an electron scavenger with the reduced product being hydrogen sulfide, as is a well-known phenomenon of most *Thermoanerobacter* strains [5,6,8,62].

Further, proteins from *T. mathranii* and *T. psuedethanolicus* involved in thiosulfate and BCAA metabolism were aligned against each other. This was primarily carried out to investigate the reasoning behind *T. mathranii's* elevated BCOH synthesis when compared to *T. pseudethanolicus* as the reference (Table 1 and Supplementary Table S2). The ARS subunits A, B, and C all displayed greater than 93% sequence homology. The YeeE/YedE thiosulfate transporters displayed 84% and 89% homology. Therefore, thiosulfate utilization is likely equivalent between the strains although this warrants continued investigation.

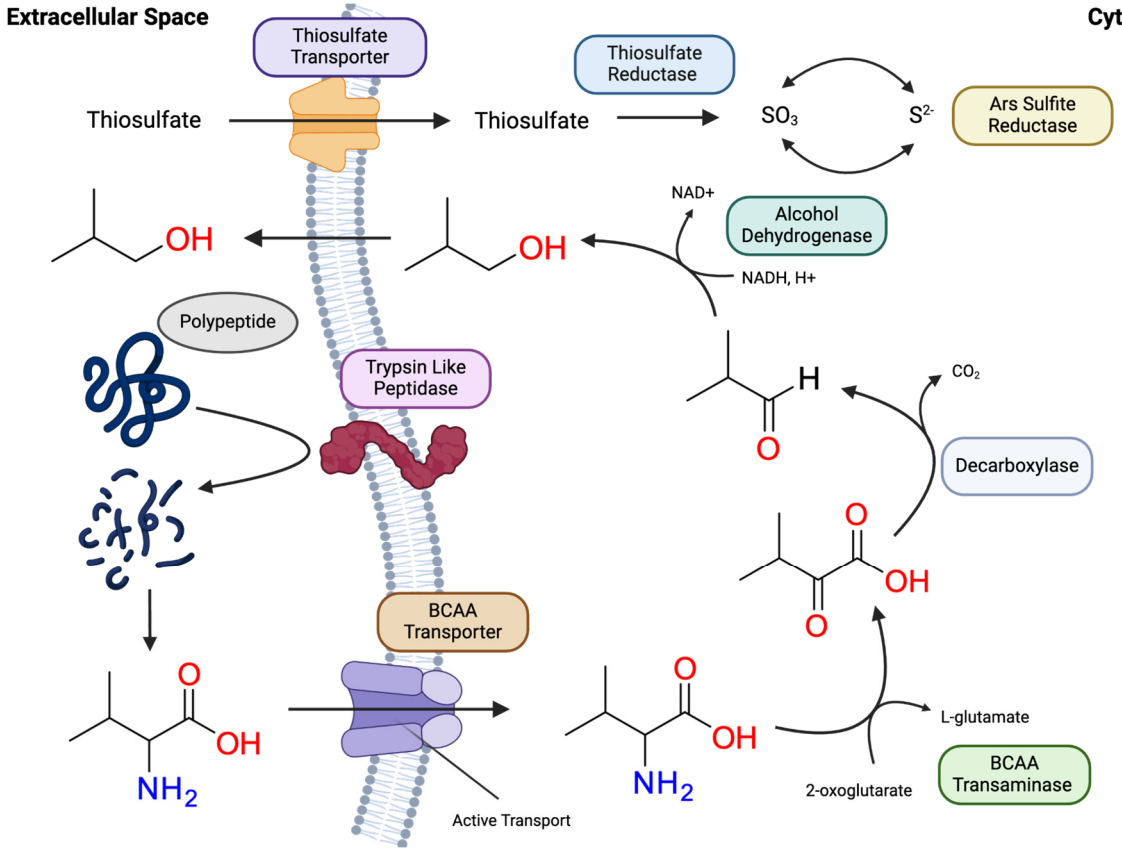


Figure 4. Details of the proposed pathway for branched-chain amino acid fermentation within *T. mathranii* in the presence of thiosulfate as a reducing agent. It should be noted that this mechanism is being proposed for all BCAAs including valine, leucine, and isoleucine. Within the figure, exogenous polypeptides from the environment are degraded by the trypsin-like peptidase, thus releasing BCAAs. As displayed, BCAAs such as valine are imported into the cell with a BCAA ABC transporter. Valine subsequently undergoes transamination and decarboxylation, resulting in compounds C and B, respectively. *T. mathranii* exhibits the BCAA transaminase listed in Table 2 and Supplementary Table S3, but the enzyme involved decarboxylation step is illusive. Compound B may be reduced by one of the several potential alcohol dehydrogenases (listed in Supplementary Table S3) allowing the alcohol compound to passively diffuse through the membrane. Alternatively, the aldehyde itself can passively diffuse through the membrane. For the reducing potential, thiosulfate is imported via a specific thiosulfate transporter. Again, no obvious thiosulfate reductase was found in the annotation. However, Ars sulfite reductase is present for cycling SO_3 and S^{2-} concentrations. Therefore, the presence of the thiosulfate reductase is implied. 2−methyl−1−propanol is A; 2−methyl−propanal is B; and 2−methyl−1−propionate is C; Dashed lines represent passive diffusion. This figure was created with biorender.com.

The remaining relevant proteins exhibited similar amino acid sequences, as seen in Supplementary Table S3. The BCOH production and genomic analysis data suggest that the *T. mathranii's* synthesis of BCOHs from the corresponding BCAAs are a result of the presence of the BCAA-specific amino acid transporters and amino acid permeases. Continued investigation is required to examine the transcriptome and genetic knockouts to determine the exact cause of the unregulated BCOH production.

T. mathranii was tested for the utilization of four proteins: casein, collagen, gelatine, and keratin. The strain only produced small amounts of the end products from these proteins but was found to be positive for several protein- and peptide-degrading enzymes. For example, the strain was positive for trypsin-like peptidase domain-containing protease,

ATP-dependent Cip protease ATP binding subunit CipX, and ATP-dependent protease ATPase subunit HsIU.

Table 2. Comparison of selected genes present in *T. mathranii* and *T. pseudethanolicus*. It is important to note the lack of Aldehyde ferredoxin oxidoreductase (AOR) within *T. mathranii*. Indicating that the pyruvate fermentation mechanism must operate distinctly. Further, *T. pseudethanolicus* does not exhibit a BCAA transporter permease, while *T. mathranii* does. This is indicative that *T. mathranii* is able to utilize exogenous branched-chain amino acids more readily than *T. pseudethanolicus*. Excluding the AOR, amino acid permeases, and BCAA transporters, both strains exhibit a similar genetic profile. Y and N indicate where the gene was found in the annotation for yes and no, respectively. NA indicates that the gene was not found in any of the annotations. * Indicates the gene has multiple subunits. [ ] are the total number of copies found within each respective strain.

Gene	Pathway(s)	*T. mathranii*	*T. pseudethanolicus*
Bifunctional aldehyde alcohol dehydrogenase	BCAA fermentation RCOOH reduction Ethanol fermentation	Y	Y
Aldehyde ferredoxin oxidoreductase	RCOOH reduction Ethanol fermentation	N	Y
Alcohol dehydrogenase	BCAA fermentation RCOOH reduction Ethanol fermentation	Y [4]	Y [4]
BCAA transaminase	BCAA fermentation	Y	Y
BCAA ABC transporter permease	BCAA fermentation	Y	N
Amino Acid Permease	Amino Acid importation	Y [2]	N
Amino Acid ABC transporter Substrate Binding Protein	Amino Acid importation	Y	Y
Oxaloacetate decarboxylase subunit alpha	BCAA fermentation	Y	Y
Butyrate kinase	RCOOH reduction	Y [2]	Y [2]
Butyryl phosphotransferase	RCOOH reduction	Y	Y
Pyruvate–ferredoxin oxidoreductase	Pyruvate fermentation	Y [2]	Y [2]
Acetate kinase	Pyruvate fermentation	Y	Y
Acetyl phosphotransferase	Pyruvate fermentation	Y	Y
YeeE/YedE thiosulfate transporter family protein	Thiosulfate utilization	Y [2]	Y [3]
Thiosulfate reductase	Thiosulfate utilization	NA	NA
Ars sulfite reductase *	Thiosulfate utilization	Y	Y
Trypsin-like peptidase	Protease	Y [2]	Y [2]

* Indicates the gene has multiple subunits and [ ] are the total number of copies found within each respective strain.

4. Conclusions

Thermoanaeroacter mathranii is a well-established bioethanol-producing organism although its protein and amino acid metabolism have received little attention until now. Despite possessing a trypsin-like activity, *T. mathranii* does not degrade the proteins investigated to a significant extent. However, *T. mathranii*, like several other members of the genus, is capable of producing 3-methyl-1-butanol, 2-methyl-1-butanol, and 2-methyl-1-propanol from the corresponding BCAAs of leucine, isoleucine, and valine, respectively. Investigations of the genome highlight many of the genes necessary for a pathway for their utilization, including several proteases, although two enzymes in the proposed pathway have yet to be identified, specifically for the decarboxylation step and the inferred thiosulfate reductase; the presence of these enzymes is implied by the corresponding activity of the strain. These findings warrant further investigations into the annotated genome of *T. mathranii* to identify the exact enzymes responsible for the two aforementioned enzymatic reactions. Additionally, the presence of esterases, which are of biotechnological importance, particularly for organic chemists, paired with the *T. mathranii´*s established history of ethanol production and its ability to utilize several of the proteogenic amino acids makes it a promising candidate for the utilization of complex biomass. The ability of *T. mathranii* to

produce alcohols other than ethanol is a potential use that further strengthens the case that this strain is of potential importance for bioprocessing for the generation of more complex alcohols as products

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation10110576/s1, Supplemental Table S1. API ZYM assay results for *Thermoanaerobacter mathranii* as compared to *T. pseudethanolicus*; Supplemental Table S2. Comparison of end products formed from branched-chain amino acid fermentation by *Caldanaerobacter* and *Thermoanaerobacter* strains; Supplemental Table S3. *Thermoanaerobacter manthranii* genes.

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