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Article

Protein and Amino Acid Metabolism of *Thermoanaerobacter mathranii*

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Abstract: The members within the genus *Thermoanerobacter* have been much less investigated for protein and amino acid metabolism as compared with carbohydrates. The present study investigates the biotechnological potential of *Thermoanaerobacter mathranii* (DSM 11426) concerning its ability to produce high energy alcohols from amino and fatty acids. End-product formation was analyzed from glucose in the presence and absence of butyric acid as well as from selected proteins, and all amino acids. *T. mathranii* did not degrade any of the proteins tested to a large extent but degrade 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. 13C2-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 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

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 on 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 a wide variety of sugars present in such biomass. The proteinaceous materials are often a substantial portion of biomass, but has 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 obligate anaerobes; that ferment various carbohydrates to 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 ethanol from one mole of glucose [11–15]. Additionally,

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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 is not explored fully, 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 is derived from mesophilic anaerobes, particularly proteolytic pathogens such as *Clostridum botulinium* [24–28], *Clostridium perfringens* [29], *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 to understand 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 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 have 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. These studies have shown that species within the genus *Thermoanaerobacter* can degrade the BCAA in the presence of a 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 BCAA resulted in the production of their corresponding BCFA 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 the alcohol seems thus to be dependent on the electron scavenging system 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, Indra Mathrani, of the original paper describing this strain 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 an optimum for 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]. To our best knowledge, protein and amino acid metabolism of *T. mathranii* has not been investigated in any detail earlier. *T. mathranii* under NCBI Bioproject PRJNA33329, was sequenced and annotated by the DOE Joint Genome Institute (JGI) and uploaded to NCBI. JGI performed the genomic annotation with Prokaryotic Genomic Annotation Pipeline (PGAP) program. The genome assembly is further available on NCBI with reference genome accession number ASM9296v1.

 doi:10.20944/preprints202406.0551.v1

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 BCOH like previously reported *Thermoanaerobacter* strains as well as better understanding a 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 from milled 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, $MgCl_2$ x $6H_2O$ 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: $FeCl_2$ x 4 H_2O 2.0g, EDTA 0.5 g, $CuCl_2$ 0.03 g, H_3BO_3, $ZnCl_2$, $MnCl_2$ x 4 H_2O, $(NH_4)Mo_7O_{24}$, $AlCl_3$, $CoCl_2$ x 6 H_2O, $NiCl_2$, and 0.05 g, Na_2S x 9 H_2O 0.3 g, and 1 mL of concentrated HCl. The medium was prepared by adding the buffer to distilled water containing resazurin and boiled 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 filter (0.45 μm) sterilized 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 in triplicate.

2.2. Substrate Utilization Spectrum

The ability of the strain to utilize amino acids were tested at 20 mM concentration in 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 end products were analyzed. Experiments were done in 25 mL serum bottles with 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.

2.3. 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].

2.4. 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 by manual identification for relevant genes corresponding to the BCAA fermentation, carboxylic acid reduction, and ethanol fermentation pathways. The Expsasy Enzyme nomenclature database was applied in searching for alternative naming conventions of enzymes and determining 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 is through this method that relevant enzymes were detected within the *T. mathranii* genome.

 doi:10.20944/preprints202406.0551.v1

2.5. Analytical Methods

Hydrogen was analyzed by Perkin Elmer Auto System XL gas chromatograph according to [56]. 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 by 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 Activity

Unlike other genera commonly described in the literature, *Thermoanaerobacter* strains have not undergone some of the routine chemotaxonomic tests using commercially available screening kits as have many other mesophilic bacteria. In order to get an overview of the common carbohydrate and amino acid-related enzyme activities present among *T. mathranii* 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 napthol-AS-BI-phosophohydrolase (Supplementary Table S1).

Being highly saccharolytic, it is a little surprising that the strain was found negative on the enzymes typically involved in carbohydrate catabolism, such as α- and β-galactosidase, α- and β-glucosidase (Supplementary Table S1).

T. mathranii tests positive for the presence of an esterase that is active on four carbon substrates but seems to lack activity on C8 or 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 deacylation of hemicellulose and other polymeric carbohydrates. Esterases have broad biotechnological potential and cold-active esterases have demonstrated 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 napthol-AS-BI-phosophohydrolase, 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 to use 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 one carbon shorter fatty acids (McInerney, 1988). 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 BCAA (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 the electrons produced during oxidation of these amino acids to external electron accepter (either by co-cultivating the amino acid degrading bacterium with

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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 BCAA only in the presence of thiosulfate, producing one carbon shorter BCFA [41]. Later investigations revealed that the BCAA were not only degraded to their corresponding fatty acid, but to a mixture of their corresponding BCFA and BCOH by *Thermoanaerobacter brockii*, and *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. Apart from *T. brockii* some work has been done 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 literature concerning leucine metabolism whereas Supplementary shows similar data for all three BCAAs BCAA. Compared to other *Thermoanaerobacter* strains, *T. mathranii* uses relatively few of the 20 proteogenic amino acids. For comparison, *Thermoanaerobacter* strain AK90 showed a greater variety of amino acids degraded, in addition to serine, six other amino acids were degraded in the presence of thiosulfate.

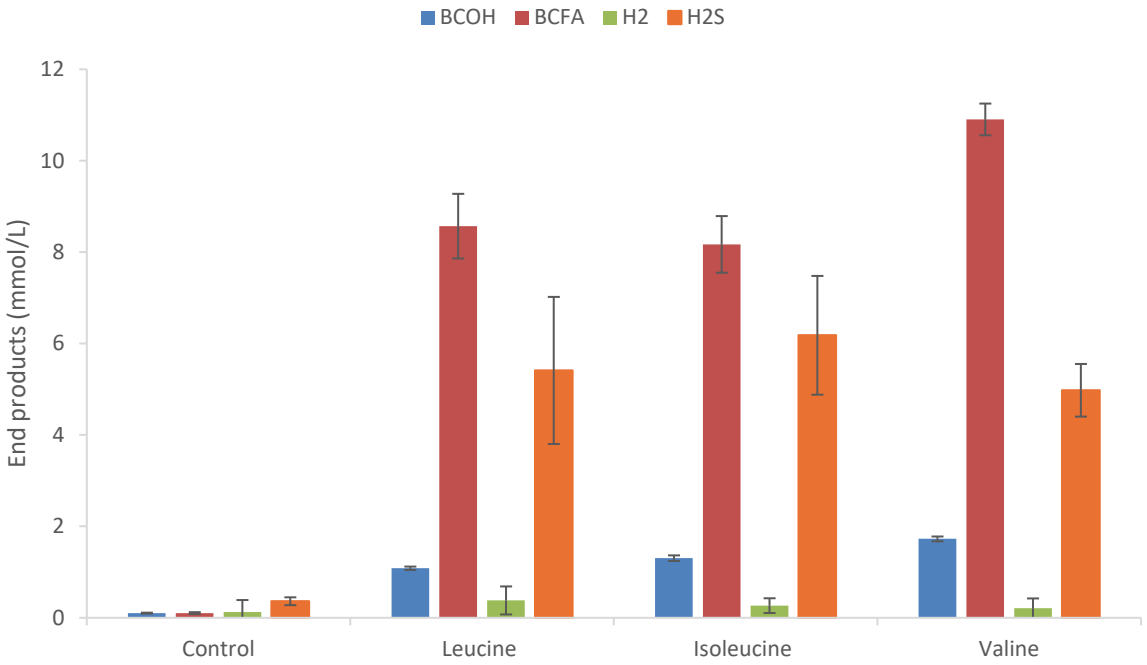

Figure 1. End-product formation from BCAA fermentation by *T. mathranii* with and without thiosulfate (40 mM) after five-day cultivation (65°C, pH 7.0). Values represent the average of triplicate fermentations with standard deviation shown as error bars. Control contains yeast extract only.

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 & Orlygsson, 2020)
Thermoanaerobacter brockii (DSM 1457)	Leucine + S_2O_3	13.6	1.5	0.11	(Scully & Orlygsson, 2014)
T. ethanolicus (DSM 2246)	Leucine + S_2O_3	5.1	0.6	0.12	(Scully et al., 2015)
Thermoanaerobacter strain AK68	Leucine + S_2O_3	13.0	2.5	0.15	(Scully et al., 2015)

 doi:10.20944/preprints202406.0551.v1

Thermoanaerobacter strain AK85	Leucine + S_2O_3	10.6	1.4	0.13	(Scully & Orlygsson, 2019)
Thermoanaerobacter strain AK90	Leucine + S_2O_3	9.7	3.5	0.36	(Scully & Orlygsson, 2015)
Thermoanaerobacter strain AK152	Leucine + S_2O_3	8.8	2.3	0.26	(Scully et al., 2015)

Similar to other *Thermoanaerobacter* strains, *T. mathranii* produced a mixture of BCFAs and BCOHs from the BCAAs and the concentration of the acid was always considerable 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 is 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 while 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 to 0.22 for valine, 0.11 to 0.36 for leucine, and 0.09 to 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 the acid and the alcohol have been done with *Thermoanaerobacter* strain AK85 showed that indeed the partial pressure of hydrogen was of great importance for the ratio of 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).

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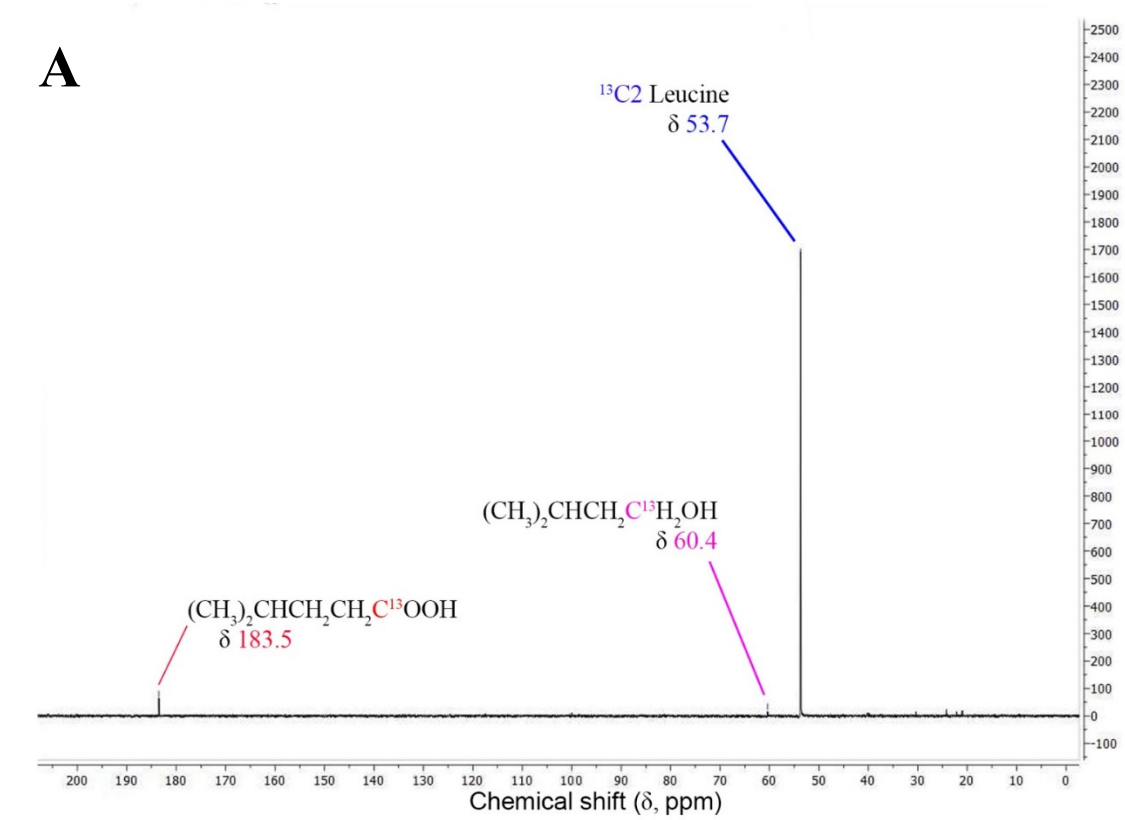

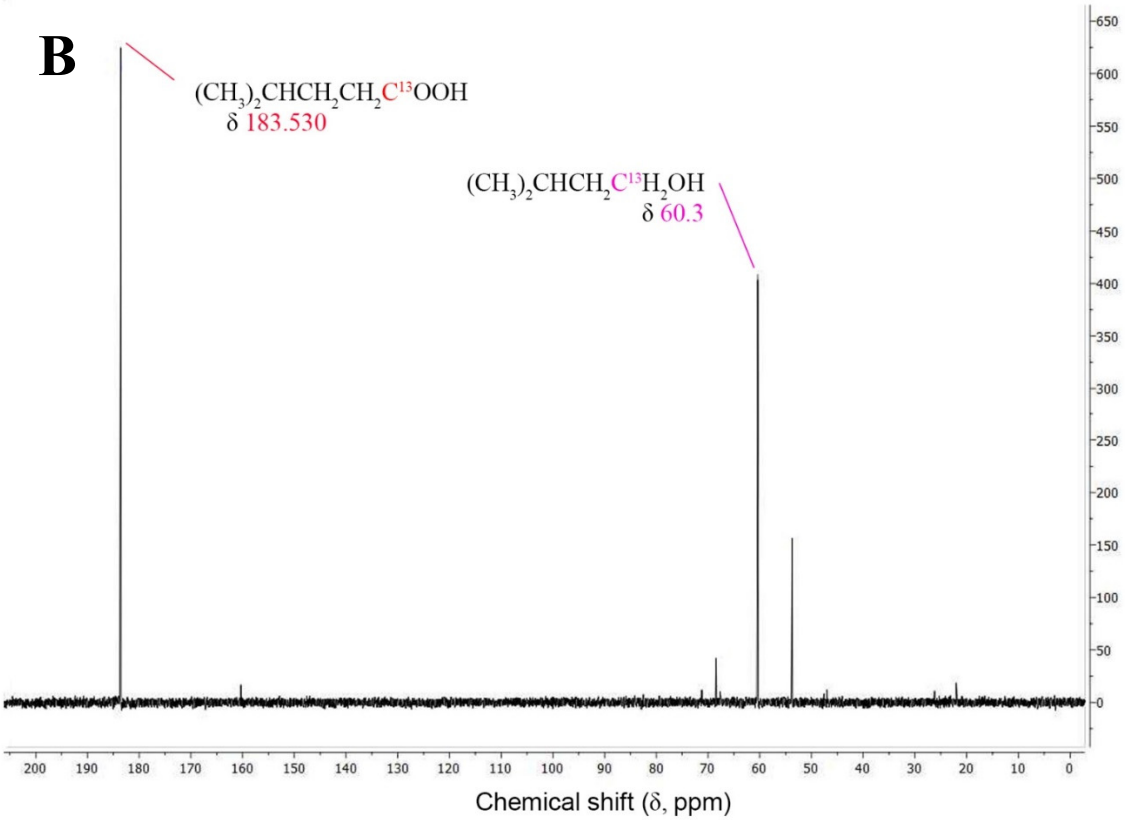

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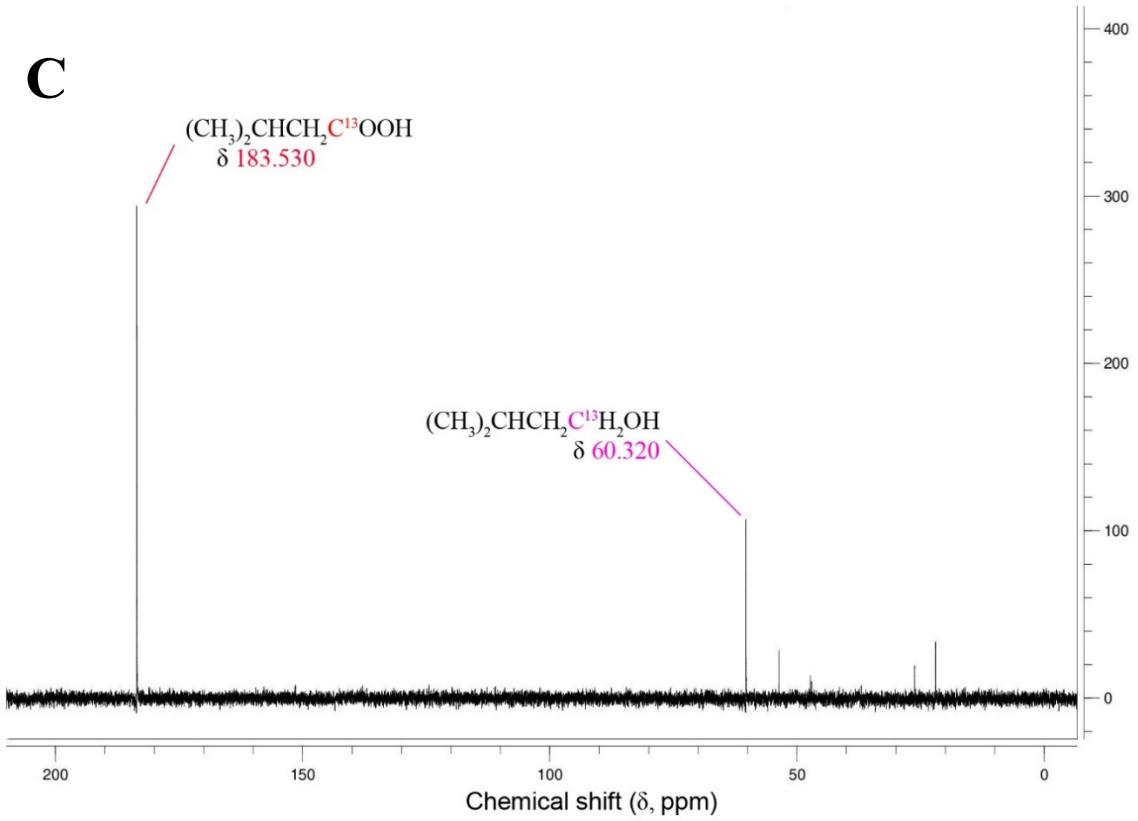

Figure 2. ^{13}C NMR-spectrogram for the fermentation of ^{13}C2 leucine by *T. mathranii* in the absence (a) and presence of thiosulfate (b) and ^{13}C1 3-methyl-1-butyrate in the presence of unlabeled leucine and thiosulfate (c). Fermentations were performed at 65°C at pH 7.0 over 14 days. Blue, red,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

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 that BCAAs are first converted to the BCFAs which in turn is used as an electron donor and is converted to its corresponding alcohol [58].

3.3. Degradation of Proteins

Protein degradation by thermophilic anaerobic bacteria has received much less attention as compared to carbohydrates. Strictly anaerobic mesophiles capable of degrading protein have more attention such as *Clostridium botulinum* [28], *Clostridium difficile* [59],*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 to understand 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, gelatin as compared to yeast extract controls while cultivation on keratin resulted in end products similar to control values. However, addition of peptone and yeast extract enhanced both growth and end product formation by the strain (results not shown).

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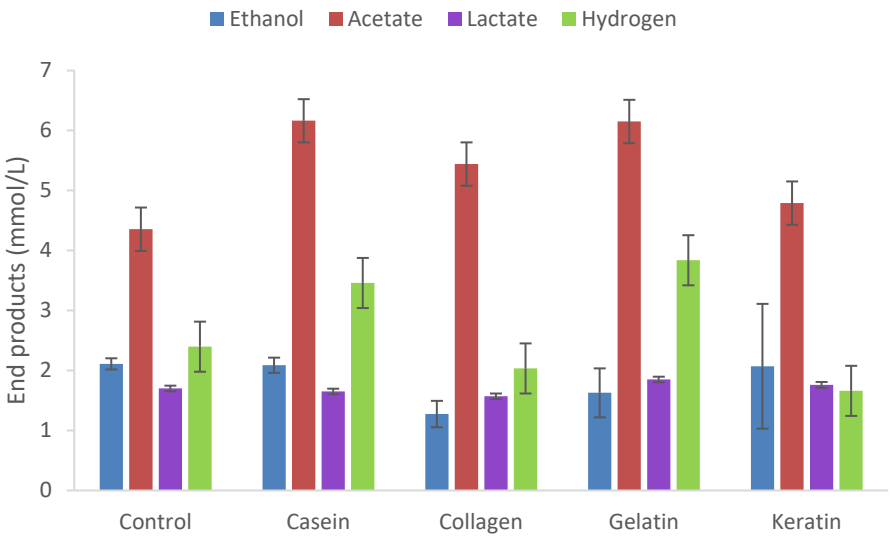

Figure 3. Fermentation of casein, collagen, gelatine and keratin (0.2% w/v) by *T. mathranii* for five days cultivation (65°C, pH 7.0). Values represent the average of triplicate fermentations with standard deviation shown as error bars. Control contains yeast extract only.

Interestingly, BCFAs and BCOHs were not above control values although as fermentation products were analyzed after five days, higher titers could have been higher if additional time for peptide hydrolysis is needed 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 investigation of the general ability of this phenomenon. As common among thermophilic anaerobes, *Thermoanaerobacter* and *Caldanaerobacter* dispose of their 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 [46,60–62]. 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):

$$1\ \text{Glucose} \rightarrow 1.23\ \text{Ethanol} + 0.26\ \text{Acetate} + 0.31\ \text{Lactate} + 1.49\ CO_2 + 0.51\ H2 \quad (1)$$

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

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

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, butanol. Instead of reducing pyruvate to only ethanol and lactate only, the reducing power is also used to reduce butyrate to its corresponding alcohol, butanol.

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 a

 doi:10.20944/preprints202406.0551.v1

sole substrate but mainly to acetate when thiosulfate was added to the medium. The strain was shown positive for genes responsible for serine-*O*-acetyltransferase and serine hydrolyase. 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 present in the genome of *T. mathranii.*

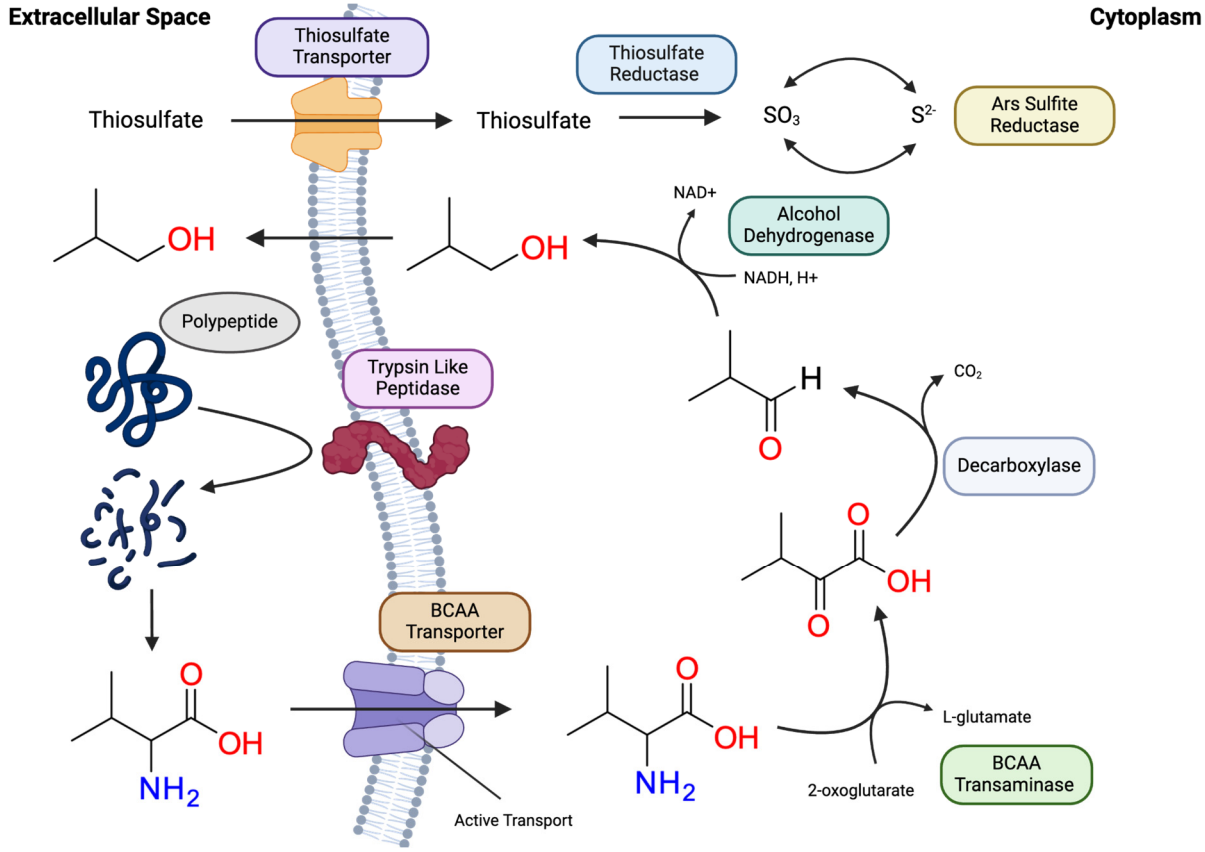

Figure 4. Details of the proposed pathway for branched chain amino acid fermentation within *T. mathranii* in the presence of thiosulfate as a reducing agent. It should be noted, that this mechanism is being proposed for all BCAAs including: valine, leucine, and isoleucine. Within the figure, exogenous 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. While *T. mathranii* exhibits a BCAA transaminase listed in Table 2 and Supplementary Table S3; the enzyme involved decarboxylation step is illusive. Compound B may be reduced by one of the several potential alcohol dehydrogenases (listed in Supp. Table S2) allowing the alcohol compound 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; 2-methyl-1-propionate is C; Dashed lines represent passive diffusion.

Other genome studies show 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. Presence of enzymes involved in carboxylic acid reduction were investigated like acetaldehyde decarboxylase, alcohol dehydrogenase, acetate kinase, acetyl phosphotransferase, pyruvate

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ferredoxin oxidoreductase, pyruvate formate lyase and aldehyde reductase (Table 2, Supplementary Table S3) using *Thermoanaerobacter psudethanolcus* as a reference. Both strains were positive for most of the enzyme pathways investigated with the only major difference between the species is that *T. pseudethanolicus* is positive for aldehyde ferredoxin oxidoreductase but *T. mathranii* not, and *T. mathranii* is positive for BCAA ABC transporter permease but *T. pseudethanolcus* not (Table 2). Concerning the utilization of thiosulfate as an electron acceptor the strain is positive for thiosulfate transporter family protein and thiosulfide reductase family protein as expected being able to utilize thiosulfate as an electron scavenger with the reduced product being hydrogen sulfide as is a well known phenomenon of most *Thermoanerobacter* strains [5,6,8,63].

Table 2. Comparison of selected genes present in *T. mathranii* and *T. pseudethanolicus*. Of important note is 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 differences in AOR and BCAA transporters, both strains exhibit a similar genetic profile. Y and N indicates 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 fermenation RCOOH reduction Ethanol fermentation	Y	Y
Aldehyde ferredoxin oxidoreductase	RCOOH reduction Ethanol fermentation	**N**	Y
Alcohol dehydrogenase	BCAA fermenation RCOOH reduction Ethanol fermentation	Y [4]	Y [4]
BCAA transaminase	BCAA fermentation	Y	Y
BCAA ABC transporter permease	BCAA fermentation	Y	**N**
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 fermenation	Y [2]	Y [2]
Acetate kinase	Pyruvate fermenation	Y	Y
Acetyl phosphotransferase	Pyruvate fermenation	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, [ ] are the total number of copies found within each respective.

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The strain was tested for the utilization of four proteins; casein, collagen, gelatine and keratin. The strain did only produce small amounts of 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.

4. Conclusions

Here we confirm the activity of BCAA fermentation via ^{13}C NMR and propose a mechanism for the synthesis of BCOHs in *T. mathranii.* The significance of this pathway has multiple implications across the genus. First, not all *Thermoanaerobacter* species within the genus exhibit BCAA transporters that are able to take advantage of this mechanism. *T. pseudethanolicus,* a highly studied organism, does not have BCAA transporters in their corresponding genomes. Thus, their ability to ferment branched chain alcohols are significantly reduced.

As shown, *T. mathranii* is able to serve as a platform for the bio-manufacturing of BCOHs for producing 3-methyl-1-butanol, 2-methyl-1-butanol, and 2-methyl-1-propanol from the corresponding BCAAs of leucine, isoleucine, and valine respectively. While two enzymes in the proposed pathway have yet to be identified for the decarboxylation step and the inferred thiosulfate reductase specifically; the presence of these enzymes are implied by the corresponding activity of the strain. These findings warrants further investigation in the annotated genome of *T. mathranii* to identify the exact enzymes responsible for the two aforementioned enzymatic reactions.

The data herein additionally showcases a potential for proteolytic activity within the strain. Not only does *T. mathranii* have a trypsin like peptidase (Table 2, Supplementary Table S2), the strain also expresses a specific BCAA transporter. By comparison, *T. pseudethanolicus* exhibits a similar genomic profile but, do not have an annotated BCAA transporter. In particular *T. pseudethanolicus* is able to perform a similar fermentation reaction using BCAA.

The presence of a BCAA transporter and trypsin like peptide can potentially indicate that *T. mathranii's* role in the microbiome may be involved in the nitrogen cycling and alcohol formation. Further, *T. mathranii* was tested positive for trypsin activity. This hints that not only the strain is naturally proteolytic but may be involved in the nitrogen cycle. While the exact relationships between *T. mathranii* and other thermophilic species are speculated. it is undeniable that this strain has displayed some level of proteolytic activity and should facilitate more research into the proteolytic capacity of the strain.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org, Supplemental Table S1. API ZYM assay results for *Thermoanaerobacter mathrani*i 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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