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Posted Date: 14 December 2023

doi: 10.20944/preprints202312.1105.v1

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Article

# Biotechnological Prospects of *Thermoanaerobacter* AK15; End-Product Formation from Sugars, Amino Acids, Lignocellulosic and Macroalgae Hydrolysates

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**Abstract:** The present study is on the biotechnological potential of *Thermoanaerobacter* strain AK15 concerning its ability to produce ethanol and valuable alcohols from carbohydrates and amino acids as well as investigations on their enzyme capability. The strain AK15 was found to be positive for alkaline phosphate, C4 esterase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, and  $\alpha$ -glucosidase. Strain AK15 is highly ethanologenic, producing a maximum of 1.57 mol ethanol per mol of glucose degraded at high liquid-gas phase ratios. The strain degrades most of the sugars tested as well as starch and pretreated hydrolysates of biopolymers (Whatman paper, newspaper, Timothy grass, Rhubarb leaves, and several macroalgae species) to ethanol mainly. The strains degraded serine and threonine when used as a single substrate, producing mainly acetate and ethanol as end products, and the branched-chain amino acids (leucine, isoleucine, valine) when cultivated in the presence of thiosulfate. The main end products from branched-chain amino acids were a mixture of their corresponding branched-chain fatty acids and alcohols. Finally, the strain was also shown to use butyrate as an electron sink during glucose degradation resulting in the reduced product butanol in addition to end products produced from glucose. Thus, strain AK15 is a promising candidate for ethanol and fine chemical production.

**Keywords:** *Thermoanaerobacter* carbohydrate; protein; sugar; amino acid; end products

## 1. Introduction

Production of both biofuels and fine chemicals from renewable sources is in more demand than ever before. The urge to replace fossil fuels with renewable alternatives is becoming more and more urgent because of the global climate changes on Earth as well as the geopolitical uncertainties associated with major oil exporting nations. As such, the United Nations Sustainable Development goals place a great emphasis on climate action and sustainable development.

While there are several viable options for biofuels that can meet the needs of a liquid energy carrier to replace gasoline, bioethanol, and other alcohols are promising due to their ease of production, low toxicity, direct compatibility of blended fuels with many existing combustion engines, and high volatility [1]. Furthermore, bioethanol production has enjoyed a long history but its production as a biofuel at industrial scales has been primarily limited to using biomass containing starch and sucrose as a substrate which poses several problems, namely the environmental impact and negligible energy yield from cultivating these crops and increasing the price of food [2,3]. To this end, there has been a lot of interest in using other biomasses that do not compete with food utilization such as lignocellulosic and seaweed biomass. Lignocellulosic biomass is composed of cellulose, hemicellulose, and lignin [4] while seaweeds have a much more diverse composition of carbohydrates which often includes cellulose but can also include  $\beta$ -glucans such as laminarin, uronic-acid containing carbohydrates such as carrageenan, alginate, agarose, mannitol, and sulfated polysaccharides [5,6]. As with terrestrial biomass, the exact composition varies as a function of the type of seaweed in addition to other variables related to growth conditions. Both lignocellulosic and

algal biomass present a challenge to the production of bioethanol given the greater diversity of carbohydrate building blocks present making finding an organism for bioprocessing more challenging.

The production of bioethanol from simple biomass has also led to the debate on the utilization of food and feed for fuel production. Therefore, utilization of more complex biomass is a better alternative, but its use is more complex because of the complex structure and the pretreatment needed for their degradation to hexoses and pentoses. Additionally, the most common microorganism used for bioethanol production has a limited capacity of degrading the wide variety of sugars present in lignocellulosic biomass. This has led to a search for more feasible microorganisms with a broader substrate range than yeasts. To this end, thermophilic anaerobes have been promising candidates for this purpose and many studies in the past twenty years have focused on ethanol production from complex biomass [2,3]. Production of fine chemicals, like 1,2-propanediol, 1,3-propanediol, and branched-chain alcohols have historically been produced from non-renewable sources, like gasoline and gases [7]. More environmental routes to such chemicals are thus more feasible and have led to the investigation of the production of various fine chemicals [8]. Several thermophilic bacteria have been used for the production of various fine chemicals, e.g. *Thermoanaerobacterium* and *Caldicellulosiruptor* for 1,2 propanediol [9,10], *Caloramator* for 1,3 propanediol [11], and *Thermanaerobacter* and *Caldanaerobacter* for production of branched-chain alcohols [12].

All species within *Thermoanaerobacter* are obligate anaerobes; ferment various carbohydrates to ethanol, acetate, lactate, hydrogen, and carbon dioxide [13] and originate from various habitats like hot springs, hydrothermal vents, and oil fields [14–17]. Species within the genus *Thermoanaerobacter* have received considerable attention due to their biotechnological potential, having a very broad substrate spectrum, especially among the sugars present in lignocellulosic biomass, and high ethanol yields from lignocellulosic biomasses [18–20]. The most common species that have been investigated for their ethanol production are *Thermoanaerobacter ethanolicus* and *Thermoanaerobacter pseudethanolicus* [21,22]. Recent investigations have also shown the ability of some species within the genus to produce valuable high-carbon alcohols from specific amino acids [12,23] as well as being able to reduce fatty acids to their corresponding alcohol in during growth on both sugars and amino acids [24,25]. It is of interest that the end products formed during fermentation can be manipulated by controlling environmental factors. Thus, a very good ethanol producing strain under specific conditions can be cultivated under conditions that may lead to that the main end product of the strain being acetate and hydrogen. This has been shown for *Thermoanaerobacter* strain AK5 where the ethanol and production varied from being 0.7 mol ethanol/mol glucose to 1.7 mol ethanol/mol glucose, simply by cultivating the strain at different liquid to gas phase ratios [26]. Similarly, acetate produced changed from 0.14 to 0.74 mol acetate/mol glucose. This change was even more dramatic when the strain was cultivated in the presence of thiosulfate or in the presence of a hydrogenotrophic methanogen. Under these conditions, ethanol became only a minor end product but acetate, by far, is the major volatile end product. Due to the broad substrate range of *Thermoanaerobacter*, they have often been linked to biofuel production from complex biomass (lignocellulose). Several reports show that *Thermoanaerobacter* species produce between 3 to 6 mM ethanol g<sup>-1</sup> dw of various biomass types, such as grass, hemp, barley straw, corn straw, and so forth ([3] and references therein).

The present investigation is on both the carbohydrate and amino acid metabolism of *Thermoanaerobacter* strain AK15 as well as their potential to utilize complex biomass and macroalgae hydrolysates. *Thermoanaerobacter* strain AK15 was isolated from an alkaline hot spring (pH 8.6, Temperature 60°C) in Viti in the Krafla area in NE-Iceland as previously described by Scully and co-workers [12]. The strain is, like other *Thermoanaerobacter* species, strictly anaerobic and ferments a wide variety of sugars, both pentoses, hexoses, and disaccharides. The end products are ethanol, lactate, acetate, CO<sub>2</sub>, and H<sub>2</sub> [13].

## 2. Results

### 2.1. Phylogeny

Thermoanaerobacter strain AK15 is most closely related to *Thermoanaerobacter uzonensis* with 98.6 % similarity [12].

### 2.2. API ZYM

The use of API ZYM strips has been commonly employed for the characterization of mesophilic microorganisms. With modification, the use of the API ZYM strip proved to be facile at elevated temperatures (65°C) although attempts to use the strips at lower temperatures did not produce good results supporting the thermophilic nature of the enzymes. It was found, however, that the use of a humidified bag was essential to prevent the strips from drying out during incubation. To the author's knowledge, this is the first use of these strips for the characterization of some of the basic enzyme chemistries present among thermophilic anaerobic bacteria.

*Thermoanaerobacter* strain AK15 was found to be positive for alkaline phosphate, C4 esterase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, and  $\alpha$ -glucosidase (Table 1). *T. uzonensis* was also tested with API ZYM since it is closely related to strain AK15. The strain was also positive on C4 esterase, acid phosphatase, and naphthol-AS-BI-phosphohydrolase as strain AK15 but tested negative on alkaline phosphatase and  $\alpha$ -glucosidase. However, *T. uzonensis* was positive for C14 lipase,  $\beta$ -glucosidase, and N-Acetyl- $\beta$ -glucosaminidase but strain AK15 was negative for these three enzymes (Table 1). Being saccharolytic, it is surprising that strain AK15 was found to be negative for the presence of most of the enzymes involved in the carbohydrate utilization present on the API ZYM strips (e.g.  $\alpha$ - and  $\beta$ -galactosidase,  $\beta$ -glucosidase) although the strain does test positive for  $\alpha$ -glucosidase. The strain tested positive for the presence of esterase that is active on four carbon substrates but seems to lack activity on C8 and C14 esters. While the explicit physiological function of esterases in *Thermoanaerobacter* strains is not known, it has been speculated that they may serve to help with deacylation of hemicellulose and other polymeric substrates. Esterases have a broad biotechnological potential and cold-active esterases have demonstrated broad catalytic promiscuity giving them a wide range of applications in organic synthesis. The author's 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 the strain is capable of scavenging phosphate from its environment from both inorganic and organic sources.

**Table 1.** API ZYM data for strain AK15 and *Thermoanaerobacter uzonensis*.

	Strain AK15	<i>T. uzonensis</i>
Alkaline phosphatase	+	-
Esterase (C4)	+	+
Lipase esterase (C8)	-	+
Lipase (C14)	-	+
Leu arylamidase	-	-
Val arylamidase	-	-
Cys arylamidase	-	-
Trypsin	-	-
$\alpha$ -Chymotrypsin	-	-
Acid phosphatase	+	+
Naphthol-AS-BI-phosphohydrolase	+	+
$\alpha$ -Galactosidase	-	-
$\beta$ -Galactosidase	-	-
$\beta$ -Glucuronidase	-	-
$\alpha$ -Glucosidase	-	-
$\beta$ -Glucosidase	-	+

N-Acetyl- $\beta$ -glucosaminidase	-	+
$\alpha$ -Mannosidase	-	-
$\alpha$ -Fucosidase	-	-

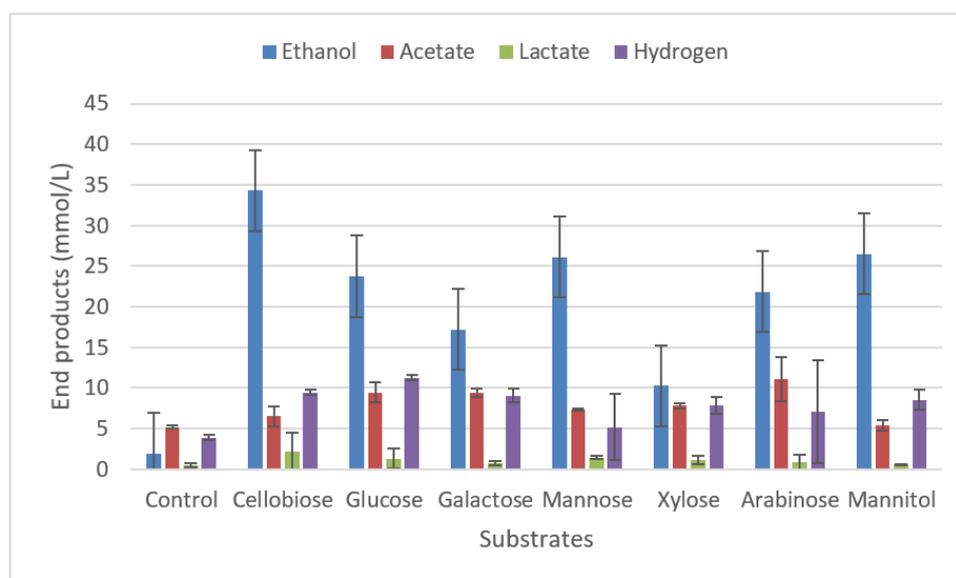
### 2.3. Substrate spectra

*Thermoanaerobacter* AK15 was cultivated on the main carbohydrates present in lignocellulosic and algal biomass, including hexoses (glucose, mannose, galactose), pentoses (arabinose, xylose), disaccharides (cellobiose), as well as several deoxysugars (L-fucose and L-rhamnose), and mannitol which is found in seaweeds. Additionally, strain AK15 was tested for growth on polymeric carbohydrate substances, all 20 of the proteogenic amino acids with and without the inclusion of thiosulfate (20 mM), and various proteins with and without thiosulfate (20 mM).

#### 2.3.1. Degradation of carbohydrates

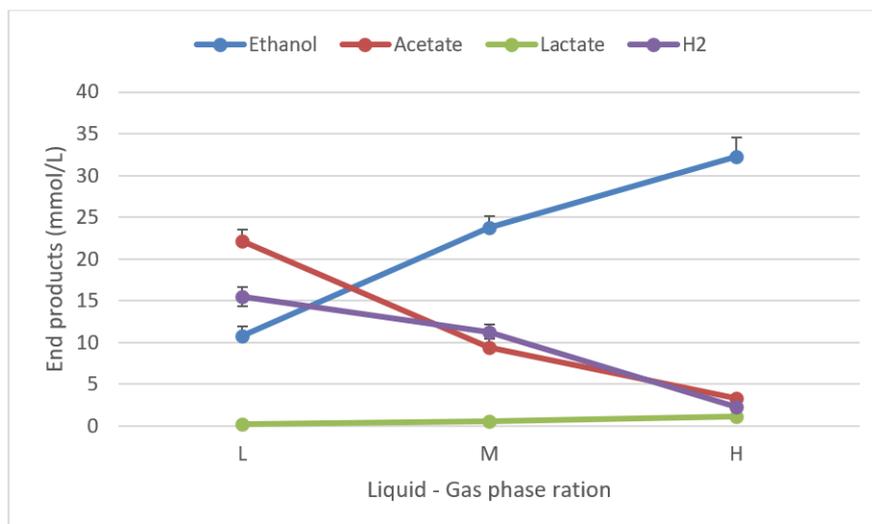
*Thermoanaerobacter* AK15 degraded cellobiose and all tested monosaccharides except for the two deoxysugars, L-fucose and L-rhamnose (Figure 1 (deoxysugars now shown)). Some thermophiles have been shown to degrade deoxysugars like *Caldicellulosiruptor* species of which more than half of the type strains within the genus degraded L-rhamnose to 1,2-propanediol and three strains degraded L-fucose to 1,2-propanediol [10]. Other thermophilic anaerobes known to produce 1,2-propanediol are *Clostridium* [27,28].

The strain degraded mannitol to ethanol mainly, as has also been shown for *T. uzonensis*, the closest relative to strain AK15 [29]. Mannitol utilization was not tested in the original characterization study of Wagner and coworkers describing *T. uzonensis* [30].



**Figure 1.** End product formation from various substrates. From left to right, acetate, ethanol, lactate, and hydrogen. Standard deviations are presented as error bars.

The original data on ethanol production from glucose for *T. uzonensis* showed 1.15 mol glucose per mol glucose degraded [30] while *Thermoanaerobacter* strain AK15 produces 1.1 ethanol mol glucose<sup>-1</sup> (Figure 1). Other well-known species of producing high amounts of ethanol from sugars within the genus are i.e. *T. ethanolicus* and *T. pseudethanolicus* [21,22]. The other end products are mainly acetate with traces of lactate; this was also the case for other carbohydrates tested. It is a well-known phenomenon that the ratio of end products is highly influenced by culture conditions, particularly hydrogen accumulation. Therefore, a simple experiment was performed on the strain by cultivating it in three different liquid–gas phase ratios: 0.09, 1.00, and 0.87. As shown in Figure 2, the end product profile changes dramatically during growth under different pH<sub>2</sub> for the strain.

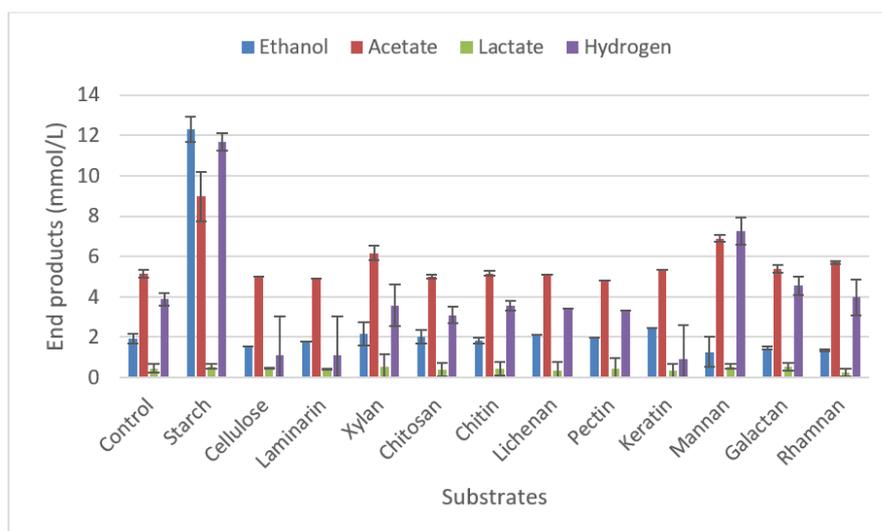


**Figure 2.** End product formation from glucose (20 mM) at different liquid – gas phase ratios. Standard deviations are presented as error bars.

At high pH<sub>2</sub>, the main end product is ethanol, or 33.3 mM which corresponds to 1.57 mol/mol glucose degraded (controls subtracted) but drops to 0.44 mM at the lowest pH<sub>2</sub>. Similarly, acetate and hydrogen decrease at high pH<sub>2</sub> conditions. This highlights the importance of the culture conditions on end-product formation and has been reported for various thermophilic bacteria in recent studies [20,26]. Thus, the strain is highly ethanologenic and can be manipulated to convert more than 75% of the glucose to ethanol. Also, being versatile concerning the utilization of a wide variety of carbohydrates, strain AK15 may be considered as a good candidate for bioethanol production from lignocellulosic biomass. To investigate this further the strains were cultivated on several lignocellulosic and algal biomass pretreated with dilute acid and cellulases.

### 2.3.2. Degradation of polymeric carbohydrate substrates.

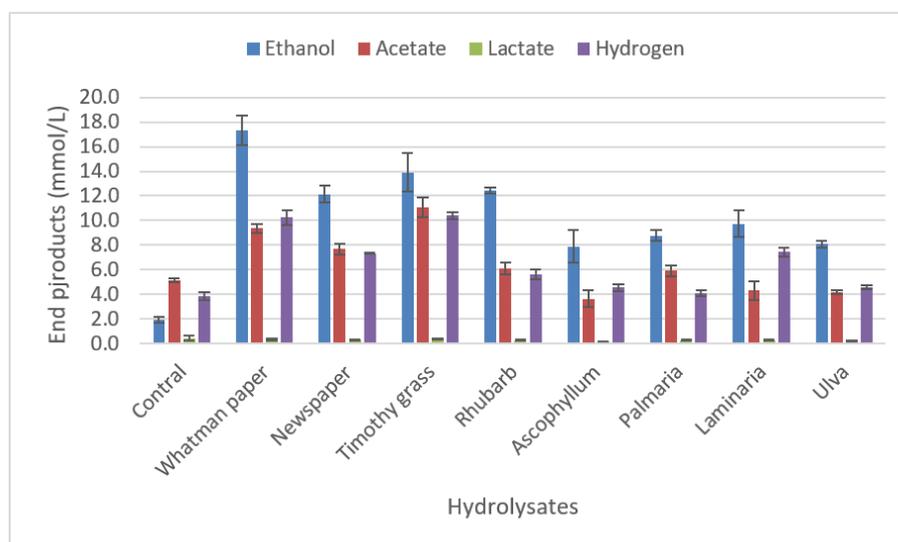
The ability of microorganisms to be good candidates for bioprocessing of lignocellulosic or algal biomass should include the capability of degrading a wide range of glycosidic bonds. The ability of strain AK15 to degrade  $\beta$ -1,3-O glycosidic bonds was evaluated using lichenan and laminarin. The ability of strains to utilize the hemicellulose fraction was evaluated using xylan, mannan ( $\beta$ -O-(1-4) linked mannose), galactan, and rhamnan. The strain did not utilize any of these substrates. Of the polymeric carbohydrates tested, strain AK15 only showed end product formation from starch (Figure 3). *T. uzonensis* is however reported as starch negative [30] although many of members of the genus degrade starch as the sole carbon source. The main end product from starch was ethanol (12.3 mM). No growth was observed when the strain was cultivated on cellulose, as previously reported for *Thermoanaerobacter* species [13] nor did the strain degrade laminarin, chitosan, lichenan, keratin, mannan, galactan, or rhamnan. There are only several species within the genus that are known to degrade keratin with the best known being “*Thermaanaerobacter keratinophilus*” [31], although this strain is likely falls within the modern genus of *Caldanaerobacter*.



**Figure 3.** End product formation from various polymeric substrates. Standard deviations are presented as error bars.

### 2.3.3. Degradation of biomass hydrolysates

The strain was tested on several complex biomass types, both lignocellulosic biomass (grass, newspaper, rhubarb leaves) and macroalgae types (*Ascophyllum nodosum*, *Palmaria palmata*, *Laminaria digitata*, *Ulva lactuca*) as shown in Figure 4. Whatman paper was used as a reference in same concentration as for the biomass samples.



**Figure 4.** End product formation from hydrolysates made from lignocellulosic and macroalgae biomasses. Concentration of hydrolysates was 2.5% (v/v). Standard deviations are presented as error bars.

As for the end product formation from individual sugars, the main end product from the biomass hydrolysates used was ethanol. Glucose degradation under the same conditions resulted in the formation of 23.8 mM of ethanol, or 1.15 mol/mol glucose degraded. Theoretical yields of glucose from 2.5% w/v Whatman paper that is completely hydrolyzed is 15.4 mM. The amount of ethanol produced from Whatman paper hydrolysates is 15.4 mM, or 1.0 mol ethanol/mol glucose which corresponds to 5.6 mM/g glucose, assuming all glucose is released during hydrolysis (control ethanol production subtracted). Not surprisingly, ethanol yields were lower in lignocellulosic biomasses

(newspaper, grass, rhubarb) because its more complex nature. Yields on newspaper, grass, and rhubarb were 3.7, 4.4, and 3.9 mM g<sup>-1</sup> dw). The highest yields of ethanol from complex biomass by thermophilic bacteria is by *Thermoanaerobacter* strain BG1L1 on wheat straw and corn stover, or 8.5-9.2 mM ethanol g<sup>-1</sup> dw [32,33]. Yields from grass are relatively good compared with other similar species. *Thermoanaerobacter* AK5 produces 4.1 mM g<sup>-1</sup> dw grass [26], *Thermoanaerobacterium* strain AK17 produces 5.5 mM g<sup>-1</sup> dw [34], and 4.1 mM from strain J1 [20]. In all cases the grass hydrolysates were pretreated with acid, as well as with enzymatic pretreatment as performed in present study. Thus, *Thermoanaerobacter* strain AK15 seems to be suitable for bioethanol production from complex biomass with good ethanol yields.

Macroalgae is another type of biomass that may be of future use as a potential biomass for bioethanol production. In present study, four types of macroalgae were used, two brown algae (*Ascophyllum nodosum*, *Laminaria digitata*), one red algae (*Palmaria palmata*), and one green algae (*Ulva lactuca*). Ethanol yields for these macroalgae were in most cases lower compared with lignocellulosic biomass. The highest yields were from *Laminaria digitata*, or 2.8 mM g<sup>-1</sup> dw which corresponds to 25% of theoretical yields. Most data on ethanol production from various macroalgae species in literature originate from yeast fermentation of hydrolysates produced by a wide variety of pretreatment methods. Examples of comparatively high ethanol yields from green macroalgae are 0.47 g g<sup>-1</sup> of *Ulva lactuca* pretreated with cellulase isolated from *Cladosporium sphaeraspermum* (pH 4, 25°C, 42h) by using *Saccharomyces cerevisiae* [35]. Similarly, high yields have been obtained from red and brown macroalgae species (

[36], and references therein). Recent investigation of the fermentation of *Laminaria digitata* hydrolysates, pretreated with enzymatic hydrolysis showed 0.30 gg<sup>-1</sup> of consumed substrate (59% of theoretical yields) [37].

#### 2.3.4. Degradation of amino acids.

There is a huge amount of data concerning sugar metabolism of thermophilic bacteria in general, with most focus on ethanol production from complex biomass. Much less attention has been on 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* [38], *Clostridium botulinum* [39] and *Clostridium sticklandii* [40]. The degradation of amino acids is a complex process involving several oxidation and reduction steps, and only possible under specific conditions and unlike carbohydrates, many amino acids are not degraded as single substrates because of the thermodynamics involved in the reactions [41]. There are various pathways to degrade the 20 amino acids present in proteins, some of which are highly reduced and are not degraded unless the electrons produced in the oxidative step are scavenged. The most common way to degrade amino acids is to use a two-step mechanism, first an oxidative deamination of the amino acid, yielding a corresponding keto acid, which is oxidatively decarboxylated to give one carbon shorter fatty acids [41]. This is possible under anaerobic conditions only for amino acids with high oxidation stages [41]. The so-called reduced amino acids, e.g. the branched-chain amino acids (leucine, isoleucine, valine) and alanine. In the 1990s, several investigations showed that the reduced amino acids could only be degraded when the amino acid-degrading bacteria could dispose of the electrons produced during the oxidation of these amino acids to an external electron acceptor. This could be done either by co-cultivating the amino acid degrading bacterium with a hydrogenotrophic bacterium, i.e. methanogens, or by using thiosulfate as a chemical electron acceptor. This was showed by *Thermoanaerobacter brockii* during growth on the branched-chain amino acids [42] where leucine, isoleucine, and valine were degraded to isovalerate, 2-methylbutyrate, and isobutyrate, in the presence of thiosulfate, respectively. Later investigations revealed that the branched-chain amino acids were not only degraded to their corresponding fatty acid but to a mixture of their corresponding fatty acid and alcohol by *Thermoanaerobacter brockii*, and *Caldanaerobacter subterraneus* subsp. *yonseiensis*, when thiosulfate was added into the culture medium [23]. In present investigation, serine and threonine were the only amino acids that were degraded without the addition of thiosulfate, mainly to ethanol and acetate. In the presence of thiosulfate, these two amino acids were

also degraded with a change in the proportion of the end product formation. In the case of serine degradation, acetate became the main end product and ethanol minor product. Similarly, acetate became the main end product in the presence of thiosulfate during threonine degradation, but ethanol increased as well. Equations 1A and 1B show the stoichiometry of serine degradation without and with thiosulfate, and equations 2A and 2B for threonine, respectively.

Serine 20 mM  $\rightarrow$  6.91 mM Ethanol + 11.33 mM Acetate (Equation 1A)

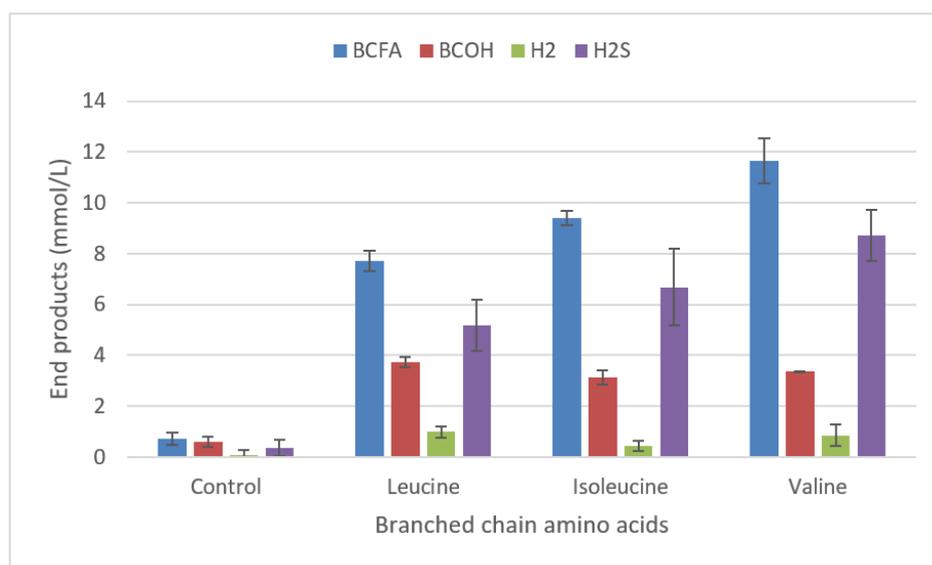
Serine 20 mM + S<sub>2</sub>O<sub>3</sub>  $\rightarrow$  1.21 mM Ethanol + 21.41 mM Acetate (Equation 1B)

Threonine 20 mM  $\rightarrow$  3.42 mM Ethanol + 6.15 mM Acetate (Equation 2A)

Threonine 20 mM + S<sub>2</sub>O<sub>3</sub>  $\rightarrow$  10.68 mM Ethanol + 16.70 mM Acetate (Equation 2A)

Similarly, *Thermoanaerobacter brockii* shifts its end product formation during growth on serine in the presence of electron acceptor, pushing end product towards more oxidized end product like acetate and less reduced end products, like ethanol, were produced [23,42]. Addition of thiosulfate in the study of *Thermoanaerobacter* AK90 showed a more variety of amino acids degraded, in addition to serine, six other amino acids were degraded in the presence of thiosulfate [24].

In present study, the thiosulfate addition resulted in the degradation of the branched-chain amino acids (leucine, isoleucine, and valine) (Figure 6). As previously reported for other *Thermoanaerobacter* strains [23,24,43], *Thermoanaerobacter* strain AK15 produced a mixture of branched-chain fatty acids and branched-chain alcohols from these amino acids and the concentration of the acid was always higher than the alcohol.



**Figure 5.** End product formation from leucine, isoleucine, and valine (all individually at 20 mM concentrations) in the presence of thiosulfate (40 mM). For simplicity formation of acetate, ethanol and lactate is not shown but was in similar concentrations as in control bottles. Standard deviations are presented as error bars.

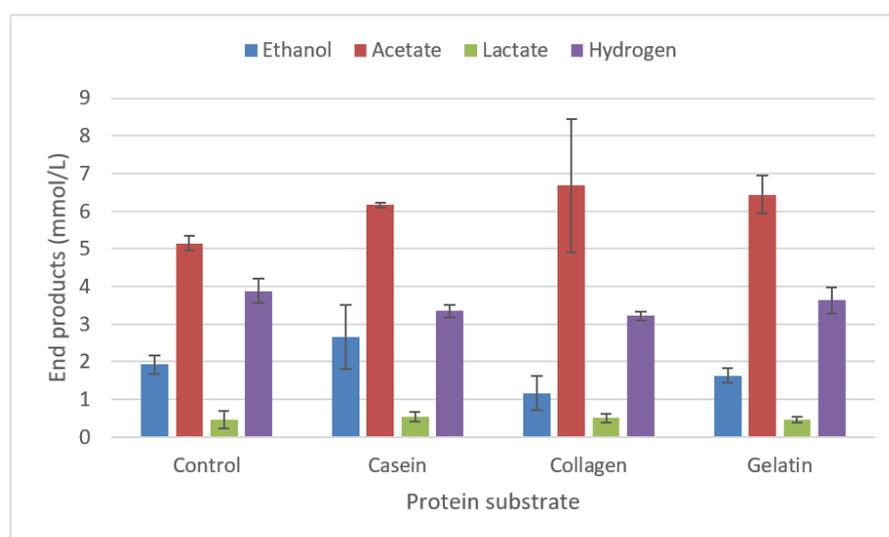
Leucine was thus degraded to 3-methylbutyrate and 3-methylbutanol, isoleucine to 2-methylbutyrate and 2-methylbutanol, and valine to 2-methylpropionate and 2-methylpropanol. Branched chain amino acids have been reported to be degraded to branched-chain fatty acids and alcohols under anaerobic conditions, mostly by *Lactobacillus* and yeasts that use the Ehrlich pathway. Usually, the concentration of these compounds is of importance for a flavor of foods and beverages [44]. Early studies of thermophilic anaerobic bacteria were performed in several investigations by on *Thermoanaerobacter brockii*. This bacterium was described to degrade the branched-chain amino acids to their corresponding branched-chain fatty acids, but only when thiosulfate was added to the culture medium, acting as a hydrogen scavenger. By adding thiosulfate

to the medium, the thermodynamics of the oxidation step of the deamination was lowered and which resulted in the corresponding keto acids which was further decarboxylated to their, one-carbon shorter, branched-chain fatty acid [42]. Recent work in our laboratory has since then shown that indeed the main bottleneck for the degradation of these reduced amino acids is the first energy demanding deamination step. Original investigations also showed that both *Thermoanaerobacter brockii* and *Caldanaerobacter subterraneus* subsp. *yonseiensis* degraded the branched-chain amino acids to only their corresponding BCFA's when co-cultivated in the presence of a hydrogenotrophic methanogen, but to a mixture of their BCFA and BCOH in the presence of thiosulfate [23]. Thus, it seems to be of important how effective the electron acceptor is that determining the product distribution during the degradation of BCAAs. Later studies on other strains within the genera of *Thermoanaerobacter* and *Caldanaerobacter* showed that this ability to produce a mixture of alcohols and acids from BCAA was common among both genera [12]. 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 which is closely related to *Thermoanaerobacter uzonensis*. This study showed that indeed the partial pressure of hydrogen was of great importance for the ratio of end products formed [43]. Finally, it was demonstrated by NMR studies that these bacteria first produce the BCFA which in turn is converted to their corresponding BCOH, both for *Thermoanaerobacter* strain AK85 and *Thermoanaerobacter pseudethanolicus* [43,45].

### 2.3.5. Degradation of proteins

Protein degradation by thermophilic anaerobic bacteria has received much less attention as compared with carbohydrates. Mesophilic anaerobic protein degrading bacteria have however got much more attention, mainly because of the high number of pathogens that are proteolytic. 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 [46].

The strain was tested for growth on three types of proteins, casein, collagen, and gelatin (Figure 6). A slight increase was observed in acetate from casein and collagen as compared to controls. However, the addition of peptone and yeast extract enhanced both growth and end-product formation by the strain (results not shown).



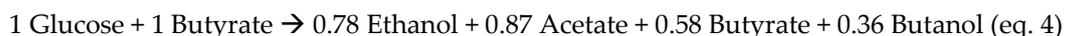
**Figure 6.** End product formation from casein, collagen and gelatin. Concentration is 0.2% (w/v). Standard deviations are presented as error bars.

### 2.3.6. Conversion of fatty acids to alcohols

Recent investigations have shown that bacteria within the genera of *Thermoanaerobacter* and *Caldanaerobacter* can dispose their electrons produced during glucose (and other sugars) oxidations not only to pyruvate to produce ethanol or lactate but may also use other electron acceptors like fatty acids which are converted to their corresponding alcohols [47–49]. This was tested for strain AK15 by cultivating the strain on glucose only and on glucose in the presence of butyrate. The strain degraded glucose to a mixture of ethanol, acetate, and lactate according to equation 3:



When the strain was cultivated on glucose with the addition of 20 mM of butyrate the reaction stoichiometry changed according to equation 4.



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. No lactate and hydrogen were observed as end products at end of fermentation in the presence of butyrate. This was also investigated for other volatile fatty acids, like propionate, branched chain fatty acids and pentanol, with similar results of the conversion of the fatty acid to their corresponding alcohol (results not shown). *Thermoanaerobacter pseudethanolicus* has recently been shown to convert fatty acids to alcohols, during sugar degradation [25,48] as well as *Thermoanaerobacter thermohydrosulfuricus*, strain AK152 [49]. Production of high carbon alcohols from complex biomass by adding cheap volatile fatty acids to the fermentation broth of the hydrolysates is indeed a new way of biofuel production that may be of great importance in the near future.

## 4. Materials and Methods

### 3.1. Chemicals

All chemicals were obtained from Sigma-Aldrich unless otherwise noted. L-fucose and fucoidan were obtained from Dextra (Reading, UK). Rhamnogalacturonan (from soy), mannan (from ivory nut), and galactan (from lupin) were obtained from Megazyme. Keratin was locally obtained from milled and used without further preparation.

### 3.2. Culture medium and preparation

*Thermoanaerobacter* strain AK15 was cultivated in Basal Mineral (BM) medium prepared as previously described [50]; the medium consisted of (per liter): NaH<sub>2</sub>PO<sub>4</sub> 2.34 g, Na<sub>2</sub>HPO<sub>4</sub> 3.33 g, NH<sub>4</sub>Cl 2.2 g, NaCl 3.0 g, CaCl<sub>2</sub> 8.8 g, MgCl<sub>2</sub> × 6H<sub>2</sub>O 0.8 g, yeast extract 2.0 g, resazurin 1 mg, trace element solution 1 mL, vitamin solution (DSM141) 1 mL, and NaHCO<sub>3</sub> 0.8 g. The trace element solution consisted of the following on a per liter basis: FeCl<sub>2</sub> × 4H<sub>2</sub>O 2.0g, EDTA 0.5 g, CuCl<sub>2</sub> 0.03 g, H<sub>3</sub>BO<sub>3</sub>, ZnCl<sub>2</sub>, MnCl<sub>2</sub> × 4H<sub>2</sub>O, (NH<sub>4</sub>)Mo<sub>7</sub>O<sub>24</sub>, AlCl<sub>3</sub>, CoCl<sub>2</sub> × 6H<sub>2</sub>O, NiCl<sub>2</sub>, and 0.05 g, Na<sub>2</sub>S × 9H<sub>2</sub>O 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 [51] 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.

### 3.3. Bacterial strain

*Thermoanaerobacter* strain AK15 was isolated from an alkaline hot spring, Viti (pH 8.6; temperature 60°C) in NE-Iceland according to Scully and coworkers [12]. The strain has been

sequenced for 16S rRNA and is most closely related to *Thermoanaerobacter uzonensis* [12]. The strain was persevered 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 65°C. All inoculation stocks of the strain were taken from frozen (-20°C) culture with rigorously degassed 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 1:1). Cultures were grown for five days and screened for end-product formation. In one set of experiments, *Thermoanaerobacter uzonensis* (DSM 18761) was used. The strain was obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ).

### 3.4. API ZYM test

API ZYM strips were performed according to the manufacturer's directions except that after inoculation of the strain to the API ZYM wells, it was incubated for 2 hours at 65°C in a humidified bag in duplicate.

### 3.5. Substrate utilization spectrum

The ability of strains to utilize selected hexoses (D-glucose, D-mannose, D-galactose), pentoses (D-xylose, D-arabinose, D-ribose), methypentoses (L-fucose and L-rhamnose), sugar alcohols (mannitol), and cellobiose was evaluated at a concentration of 20 mM except for cellobiose (10 mM). Amino acids were tested at 20 mM concentration in the absence and presence of thiosulfate. Polymeric substrates (starch, cellulose, laminarin, xylan, chitosan, chitin, casein, keratin, collagen, lichenan, pectin, and keratin), were evaluated at a concentration of 0.2% (w/v) except laminarin (0.1% (w/v)). Cultures were incubated for a period of 5 days at which time end products were analysed. Experiments were done in 25 mL serum bottles with liquid-gas phase of 1.0 ratio.

Samples of cellulose and complex biomass was obtained from Whatman paper, printed newspaper, grass (*Phleum pratense*), rhubarb (*Rheum rhabarbarum*), *Ascophyllum nodosum*, *Palmaria palmata*, *Laminaria digitata*, and *Ulva lactuca*. The biomass was pretreated as previously described with 0.1% H<sub>2</sub>SO<sub>4</sub> and enzymes (CelluclastR and Novozyme 188) as previously described [34], rendering hydrolysates that were diluted to a concentration of 2.5% (w/v).

### 3.6. Influence of liquid-gas phase ratio

The influence of partial hydrogen pressure ( $\rho_{H_2}$ ) on end product formation was investigated with different ratios of liquid and gas phases when grown on 20 mM glucose. The liquid phase varied from 5.0, 29.45, and 50 mL in serum bottles with a total volume of 57 mL; thus, the L-G volume ratio varied from 0.09, 1.00, and 5.62, respectively. A control experiment at each L-G consisted of BM medium containing yeast extract (0.2% w/v) only. After 5 days of incubation, end products were quantified by GC.

### 3.7. Analytical methods.

Hydrogen was analyzed by Perkin Elmer Auto System XL gas chromatograph according to Orlygsson and Baldursson [52]. Alcohols and volatile fatty acids were measured by gas chromatography using a Perkin-Elmer Clarus 580 gas chromatograph as previously described [52]. Lactate was quantified colorimetrically according to the method of Taylor [53] with modification according to Scully and Orlygsson [49]. Optical density was determined by measuring absorbance at 600 nm by a Shimadzu UV-1800 UV-Vis spectrophotometer in a quartz cuvette with a pathlength of 1 cm. Hydrogen sulfide was analysed as described by Cline [54].

## 4. Conclusions

The data presented shows that the thermophilic anaerobic bacterium *Thermoanaerobacter* strain AK15 is a potentially good organism for biotechnological applications. The bacterium is highly ethanologenic, producing more than 1.57 mol ethanol per mol glucose degraded. It also shows good

ethanol yields from complex biomass hydrolysates but not from most of the untreated biopolymers and proteins applied. The strain is also interesting concerning producing fine chemicals like branched-chain alcohols from branched-chain amino acids and converting volatile fatty acids to high-carbon fuel molecules. Finally, the strain possesses many enzymes of importance for bioprocessing purposes.

**Author Contributions:** Conceptualization, J.O and S.M.S.; Methodology, J.O and S.M.S.; Software, J.O and S.M.S.; Validation, J.O.; Formal analysis, J.O and S.M.S.; Investigation, S.M.S.; Resources, J.O.; Data curation, J.O and S.M.S.; Writing—original draft preparation, J.O.; Writing—review and editing, J.O and S.M.S.; Visualization, J.O. and S.M.S.; Methodology, J.O and S.M.S.; Supervision, J.O.; Project administration, J.O. Both authors have read and agreed to the published version of the manuscript.

**Funding:** This research received no external funding.

**Institutional Review Board Statement:** Not applicable.

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** Not applicable.

**Acknowledgments:** Not applicable.

**Conflicts of Interest:** The authors declare no conflict of interest.

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