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Influence of Inhibitory Compounds on Biofuel Production from Oxalate-Rich Rhubarb Leaf Hydrolysates Using *Thermoanaerobacter thermohydrosulfuricus* Strain AK91

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Abstract: The present investigation is on bioethanol and biohydrogen production from oxalate-rich rhubarb leaves which are an underutilized residue of rhubarb cultivation. Rhubarb leaves can be the feedstock for bioethanol and biohydrogen production using thermophilic, anaerobic bacteria. The fermentation of second-generation biomass to biofuels by *Thermoanaerobacter* has already been reported as well as their high ethanol and hydrogen yields although rhubarb biomass has not been examined for this purpose. *Thermoanaerobacter thermohydrosulfuricus* strain AK91 was characterized (temperature and pH optima, substrate utilization spectrum) which demonstrates that the strain can utilize most carbohydrates found in lignocellulosic biomass. Additionally, the influence of specific culture conditions, namely the partial pressure of hydrogen and initial glucose concentration, were investigated in batch culture and reveals that the strain is inhibited. Additionally, batch experiments containing common inhibitory compounds, namely carboxylic acids and aldehydes, some of which are present in high concentrations in rhubarb. Strain AK91 is not affected by alkanolic carboxylic acids and oxalate up to at least 100 mM although the strain was inhibited by 40 mM of malate. Interestingly, strain AK91 demonstrated the ability to reduce alkanolic carboxylic acids to their primary alcohols; more detailed studies with propionate as a model compound demonstrated that AK91's growth is not severely impacted by high propionate loadings although 1-propanol titers did not exceed 8.5 mM. Additionally, ethanol and hydrogen production from grass and rhubarb leaf hydrolysates was investigated in batch culture for which AK91 produced 7.0 and 6.3 mM g⁻¹, respectively.

Keywords: second generation ethanol production; lignocellulosic biomass; rhubarb; *Thermoanaerobacter*



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1. Introduction

There has been an increased emphasis on developing alternatives to grain-based first generation biofuels such as lignocellulosic and macro algal biomass, neither of which directly compete with food and feed production, using fermentative microbes such as yeasts [1–3] as well as ethanol-producing bacteria [4–6]. The selection of crops as a raw material for bioprocessing is highly dependent upon local growing conditions as well as crop traits and composition. Recent work has demonstrated that the use of perennial crops grown on marginal lands that are not otherwise suitable for cultivation can bypass the conflict between the environmental impacts associated with land usage while minimizing carbon footprint [7]. While first generation biomass includes biomass that contains a large fraction of easily fermentable sugars, second-generation biomass uses complex lignocellulosic biomass such as organic agricultural waste (e.g., stems, straw, leaves, husks), industry waste (e.g., woodchips, skins, pulp), and non-food crops (e.g., grass) as a raw material [6]. A potential second generation biomass widely cultivated in Iceland is the oxalate- and malate-rich leaves of perennial plant Rhubarb (*Rheum rhabarbarum*) which are discarded

after the petiole is utilized for its nutritional value although the presence of potentially inhibitory compounds such as oxalate and malate poses a challenge for bioprocessing.

The main components of lignocellulose are lignin, hemicellulose, and cellulose, all of which are tightly bound together necessitating pre-treatment prior to enzymatic deconstruction and fermentation. Pre-treatment regiments routinely consist of a combination of physical, chemical, physio-chemical, or enzymatic methods to liberate fermentable carbohydrates but employ elevated temperatures and acidic conditions [8]. The pre-treatment process is often accompanied by the generation of inhibitory compounds that later often negatively affect the fermentation process by hindering the growth of microorganisms; alternately, inhibitory compounds generated during biomass pre-treatment can be removed with an extra detoxification step. Thus, the goals of pre-treatment are to minimize the formation of unwanted compounds and maximize sugar extraction. Inhibitory furans, such as 2-furfuraldehyde (2-FF) and 5-hydroxymethyl-2-furfuraldehyde (5-HMF), which are formed from five and six carbon monosaccharides, respectively, under conditions commonly found in acidic pre-treatment [8]. 2-Furfuraldehyde has been shown to strongly inhibit alcohol dehydrogenases in yeast while 5-HMF has been found to be inhibitory for some important metabolic enzymes [9]. Additionally, the hemicellulose fraction is often partially acylated and these acyl groups can undergo hydrolysis liberating organic acids under common pre-treatment conditions; carboxylic acids that are fully protonated can cross the cell membrane and cause inhibition by lowering the intracellular pH. The lignin fraction is composed of a random heteropolymeric material consisting of aromatic residues, namely hydroxyphenyl, guaiacyl, and syringyl monomers such as *p*-coumaryl, coniferyl, and sinapyl alcohols, respectively [8]. While lignin is highly resistant to degradation, the partial hydrolysis of the lignin can liberate these toxic aromatic alcohols, aldehydes, and carboxylic acids which can negatively impact microbial growth and alter fermentation performance.

Rhubarb is a perennial species which produces long fleshy edible stalks and large leaves that are poisonous due to the presence of many compounds including, oxalic acid, and maleic acid thus making it a potentially challenging raw material for bioprocessing. While rhubarb stalks are rich in sugars, namely sucrose, and are regarded as a food source, the leaves contain several organic acids making them unfit for human and animal consumption. Oxalic acid is a strong dicarboxylic acid that can be corrosive with a pK_a values of 1.25 and 4.14 and a high solubility in water (143 g/L at 25 °C); it has notable toxicity with an approximate LD_{50} of 0.6 g/kg (human) [10]. The typical value of oxalic acid in rhubarb is about 0.5% *w/w* but may be minimized by cooking the leaves [10,11].

Due to their relative abundance, ease of cultivation, and low cost, rhubarb leaves are a potential renewable feedstock for biofuel production that does not compete with food production. In Iceland, rhubarb has been harvested in Eyjafjörður (N-Iceland) and in Árnessýsla (S-Iceland) for its sugar-rich stem used in the food industry although harvested quantities are not available. Additionally, Iceland's annual import of rhubarb is 50–60 tons which is used to supplement locally grown crops in order to meet market demand although rhubarb producers in Eyjafjörður are aiming to increase production and ultimately export rhubarb.

While a wide range of bioprocessing organisms have been considered for the fermentation of lignocellulosic biomass to bioethanol, namely yeasts [1,3] and highly ethanologic bacteria such as *Zymomonas*, a commonly encountered drawback of these microorganisms is their limited ability to ferment components of lignocellulosic biomass. In this regard, thermophilic bioprocessing organisms within the genus of *Thermoanaerobacter* have demonstrated a diverse applicability to the conversion of cellulosic biomass into biofuels [4,6,12]. *Thermoanaerobacter* strains degrade a wide variety of substrates; hexoses, pentoses, methylpentoses, disaccharides, and tolerate various extremes of temperature, pH, and can grow in the presence of inhibitory compounds [13]. Conversely, like many thermophilic anaerobes, many strain studies so far demonstrate low tolerances for initial substrate concentration [14–16] but this may be overcome using other fermentation modes.

The purpose of this work was to investigate the production of bioethanol and biohydrogen from unutilized rhubarb leaves using *Thermoanaerobacter thermohydrosulfuricus* strain AK91 isolated from Icelandic geothermal spring. The effects of various environmental factors were investigated to maximize both ethanol and hydrogen production. Additionally, tolerance of the strain towards various inhibitory compounds, namely aldehydes generated from hexoses and pentoses, and carboxylic acids were investigated. Finally, the ability of strain AK91 to produce bioethanol from lignocellulosic biomass hydrolysates, including oxalate-rich rhubarb, was evaluated.

2. Materials and Methods

2.1. Culture Media and Organisms

All materials were purchased from Sigma Aldrich, except for $^{13}\text{C}_1$ -labeled propionic acid which is from Cambridge Isotope Laboratories (Tewksbury, MA, USA). Pure nitrogen gas (<5 ppm O_2) was used in all cases.

Thermoanaerobacter strain AK91, was isolated from a hot spring 67 °C, pH 7.4) in Iceland with methods as previously described [15]. The medium used (BM) was according to [15]. Briefly, the medium was boiled for 15 min, chilled on an ice bath under nitrogen flushing, and dispersed to the experimental bottles to give the appropriate volume during active nitrogen flushing. Finally, the bottles were closed with butyl rubber stopper and aluminum caps, and autoclaved at 121 °C for 15 min. Glucose, vitamins, and trace elements were added from a syringe-filtered nitrogen-flushed stock bottle after autoclaving.

Hungate tubes or serum bottles were used for cultivations without agitation. Temperature and pH were 70 °C and 7.0, respectively. In all cases, a liquid–gas phase ration (L-G ratio) of 1.00 (1:1 ratio of liquid and gas) was used for a period of 5 days unless stated otherwise. All experiments were conducted in triplicate unless stated otherwise. The inoculation volume was 2% (*v/v*) in all experiments.

2.2. Collection of Rhubarb Biomass and Preparation of Hydrolysates

The Rhubarb (*Rheum rhabarbarum*) and Timothy grass (*Phleum pratense*) biomasses were collected from Eyjafjörður during summer 2015. The leaves were separated from the rhubarb stalks and used. The harvested biomass was dried in an incubator at 45–50 °C for 24 h, ground in a Waring blender and subsequently milled to <2 µm particles. The milled biomass was stored in airtight containers at ambient temperature. Then, 25 g of dry, milled biomass were weighed into screw cap bottles and approximately 600 mL of 0.5% (*v/v*) H_2SO_4 was added. The bottles were autoclaved for 60 min and titrated to pH 4.5 with 6 M NaOH.

The pre-treatment of Whatman paper, Rhubarb leaf, and Timothy grass was as described earlier [17]. Briefly, the biomass received Cellulase (1 mL, 700 U/mL, Sigma) via aseptic addition and was incubated at 47 °C for 96 h. The hydrolysates were centrifuged (20 min, 4700 rpm) the resultant supernatant collected, its pH adjusted to 7 with 6 M NaOH, and final volume adjusted to 1 L. The hydrolysates were sequentially vacuum filtered through a 53 µm nylon filter, Whatman #1 filter paper (11 µm), 5 µm nylon filter, and a 0.45 µm filter. Finally, the hydrolysates were sterilized by filtering through a syringe filter (Whatman, PES 0.22 µm) into sterile, nitrogen flushed bottles.

2.3. Characterization and Substrate Spectra

To determine temperature optimum for strain AK91, 117.5 mL serum bottles containing BM medium (pH 7.0) and glucose (20 mM) were used according to [15]. The pH optima of the strain was determined at the T_{opt} by cultivating at initial pH values from 3 to 9 in 0.5 pH unit increments.

2.4. Influence of Initial Glucose Concentration and Liquid–Gas Phase Ratio

To investigate the effect of different initial substrate concentrations, the strain was cultivated between 5 and 400 mM glucose. To evaluate the influence of L-G ratio the strain

was cultivated in 117.5 mL serum bottles containing different liquid volumes to yield L-G ratios of 0.017, 0.044, 0.093, 0.34, 1.04, and 3.27 as previously described [16]. As an example, a L-G ratio of 1.0 is prepared by adding 59.25 mL of BM medium leaving 59.25 mL of headspace while a L-G ratio of 3.16 can be obtained by using 90.0 mL of medium leaving 28.5 mL of headspace.

2.5. Effect of Inhibitory Compounds on Glucose Fermentation

The influence of inhibitory compounds during fermentation was evaluated for 11 potential inhibitors (acetate, propionate, butyrate, lactate, ethanol, malate, oxalate, levulinic acid, *p*-coumaric acid, 2-furfuraldehyde, and 5-HMF) at various concentrations (10–100 mM) during glucose (20 mM) fermentation. Experiments were performed in Hungate tubes (16 × 150 mm) at a L-G ratio of 1:1 with stock solutions of inhibitory compounds having been adjusted to pH 7. The cultures were incubated at 70 °C for five days under anaerobic conditions without stirring. Finally, end-products were analyzed.

2.6. Kinetic Study of Selected Inhibitory Compounds on Glucose Fermentation by *Thermoanaerobacter* Strain AK91

The influence of different concentrations (25, 50, 100 mM) of *n*-propionate on glucose fermentation kinetics by strain AK91 were performed in Hungate tubes (16 × 150 mm) at pH 7.0 and a L-G ratio of 1.00. Samples were collected after 24, 48, and 168 h for chemical analysis.

2.7. Fermentation of Biomass Hydrolysates

The fermentation of biomass HLs were conducted in 117.5 mL bottles with biomass loadings equivalent to 2.5, 5, and 10 g/L. In one case, the effect of different L-G ratios was tested for 2.5 g/L of biomass. Three different ratios were used: 0.04, 1.00, and 3.29. Then, 1 mL of fermentation broth was collected after 168 h for analysis.

2.8. Analytical Methods

Proximate analysis of dried biomass (ash, protein, and fat) was performed using standard AOAC methods, namely ash residue by heating in an ashing oven, protein by Kjeldahl method, and fat by Soxhlet extraction [18]. The determination of cell biomass was determined by optical density (OD) of cultures using Shimadzu UV-1800 UV-Visible spectrophotometer (600 nm, *l* = 1 cm). Hydrogen and volatile end-products were analysed using a Clarus 580 (PerkinElmer) gas chromatograph equipped with a thermal conductivity detector (TCD) and flame ion detector (FID), respectively, as reported previously [19]. Glucose concentration was determined colorimetrically using the Anthrone method as described previously [19]. Spectra for ¹³C NMR were obtained as previously reported [20].

3. Results and Discussion

3.1. Biomass Composition

The protein, ash, fat, and carbohydrate content of the Rhubarb leaves and Timothy grass are summarized in Table 1. The amounts of carbohydrates and fats in the Rhubarb leaves were similar to the values obtained for Timothy grass while the protein content was lower in Timothy grass.

3.2. Strain Characterization

Thermoanaerobacter strain AK91 was isolated from Icelandic hot spring (temperature 67 °C; pH 7.4) with already described techniques [15]. The strain has more than 99% similarity to *Thermoanaerobacter thermohydrosulfuricus* based on the 16S rRNA gene (KR007665) as previously reported [21]. Species of the genus *Thermoanaerobacter* are strictly anaerobic, fermenting numerous proteins and carbohydrates to various volatile fatty acids, alcohols, carbon dioxide, and hydrogen [22–25]. As of 2020, there are 15 species within the genus [26]. The strain grows from 55 °C to 75 °C with *T*_{opt} of 70 °C and between pH 4.5 to 8.0 with

pH_{opt} of pH 7.0 (Figure 1A,B). Growth at 70 °C and pH 7.0 resulted in a generation time of 1.36 h.

Table 1. Biochemical composition of raw biomass; values are presented as the average of three replicates \pm standard deviation.

Biomass	Proximate Analysis (% on a Dry Weight Basis)			
	Fat	Protein	Ash	Carbohydrates ¹
Rhubarb leaf	3.29 \pm 0.90	10.07 \pm 2.32	10.78 \pm 0.19	75.87
Timothy grass	3.73 \pm 0.28	15.72 \pm 0.16	5.96 \pm 0.04	74.59
Whatman paper	0.00 \pm 0.00	0.00 \pm 0.00	0.00 \pm 0.00	100.00

¹ Calculated by difference of other analytes subtracted from 100%.

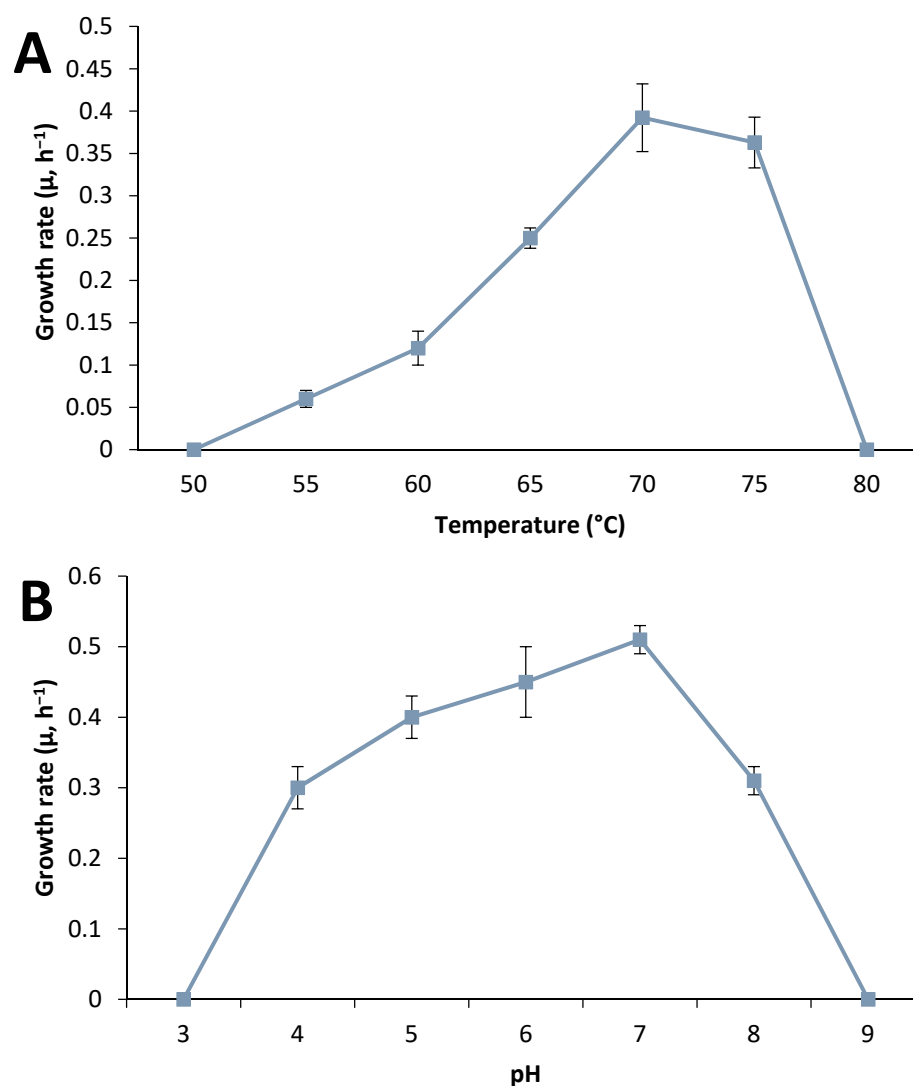


Figure 1. Characteristics of cultivation temperature (A) and pH at 70 °C (B) for *Thermoanarobacter* strain AK91.

Increased interest in the use of thermophiles for biofuel production is mainly because of their ability to degrade a wide variety of substrates [6,14,15]. Thus, the strain was cultivated on the main substrates present in lignocellulose in addition to several other compounds (Figure 2). The strain degrades many substrates like hexoses, xylose and several disaccharides, the trisaccharide raffinose, starch, grass and rhubarb hydrolysates, serine, and pyruvate (Figure 2). The main fermentation products were ethanol and acetate

(together with CO₂ and H₂) but lactate was also found in minor amounts (results not shown). The ratio of ethanol and acetate produced from sugars was close to 50:50, with exception of starch where ethanol was found to be the dominant end-product. Serine and pyruvate degradation resulted mainly in the production of acetate, most likely because of their higher oxidation states compared with sugars. No growth above controls was observed on arabinose, rhamnose, sucrose, cellulose, carboxymethylcellulose (CMC), and avicel. This is in common agreement with members of the genus generally not being capable of cellulose degradation but possessing the ability to degrade other polysaccharides such as starch, xylan, and pectin.

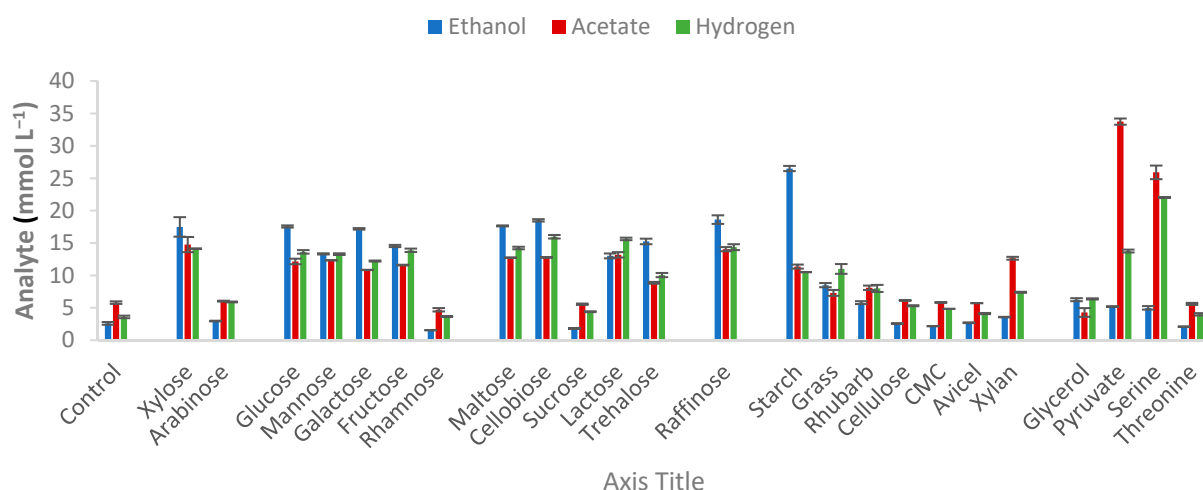


Figure 2. Formation of end-products after 5 day fermentation by *Thermoanaerobacter* strain AK91 on main substrates (70 °C, pH 7.0). The concentration for all substrates evaluated was 20 mM or in case of polymeric substrates 0.2% (*w/v*). Standard deviation are presented as error bars.

3.3. Effect of Culture Conditions on End-product Formation

To gain insight into the effect of various environmental factors the strain was cultivated at various initial glucose concentrations and L-G ratios, as shown in Figure 3A,B. Relatively low initial substrate concentrations have been shown to inhibit sugar degradation by thermophilic bacteria. This has indeed been pointed out as the main obstacle to utilizing thermophiles for bioethanol production compared with yeasts. The reasons for this sensitivity may be caused by several factors [4,6]. In addition to ethanol production, thermophiles also produce acids (acetate, lactate) that may cause pH lowering in closed systems with limited buffer capacity. This pH drop may inhibit cell activity and thus substrate degradation.

Figure 3A shows a good correlation of increased end-product (ethanol and acetate) formation between 5.0 and 20.0 mM initial glucose concentrations. At increased loadings (>20 mM initial glucose concentration), a clear inhibition is observed leading to a less portion of the glucose being degraded and levelling off or a decrease in end-product formation. With the highest glucose initial concentration applied (400 mM), less than 5% of the glucose was degraded. This is well known; other thermophilic anaerobic bacteria are very sensitive towards relatively low initial sugar concentrations, often being inhibited at concentrations above 20–30 mM [6,17].

Another reason for insufficient substrate utilization is the accumulation of hydrogen in closed batch culture systems. The increased partial pressure of hydrogen (p_{H_2}) may also change the flow of electrons leading to different end-product formation [16]. Thus, at high L-G ratios, at which p_{H_2} is higher relative to lower L-G ratios the formation of end-products is directed to more reduced compounds (ethanol, lactate), but away from oxidized products (acetate, hydrogen) and vice versa at low p_{H_2} [14–16]. To investigate the

influence of pH_2 on hydrogen production, the strain was grown in serum bottles at various L-G ratios. The maximum hydrogen yield from 1 mole of glucose is 4 moles of hydrogen when acetate is the only volatile end-product produced. Figure 3B shows that the strain produces maximally 2.8 mol H_2 per mole glucose (70.0% of the theoretical yield) at the lowest L-G ratio, but drops to 0.2 mol H_2 per mol glucose (5.0% of theoretical yields) at the highest L-G ratio used. Maximum ethanol yield is 2 mol from 1 mol of glucose. The highest ethanol yields were obtained at the highest L-G ratio used, or 72.5% of the theoretical yield. Thus, as stated before, it is more likely that the effect of culture conditions can have a more dramatic effect on determining if a strain is a good ethanol or good hydrogen producer rather than intrinsic features of the strain.

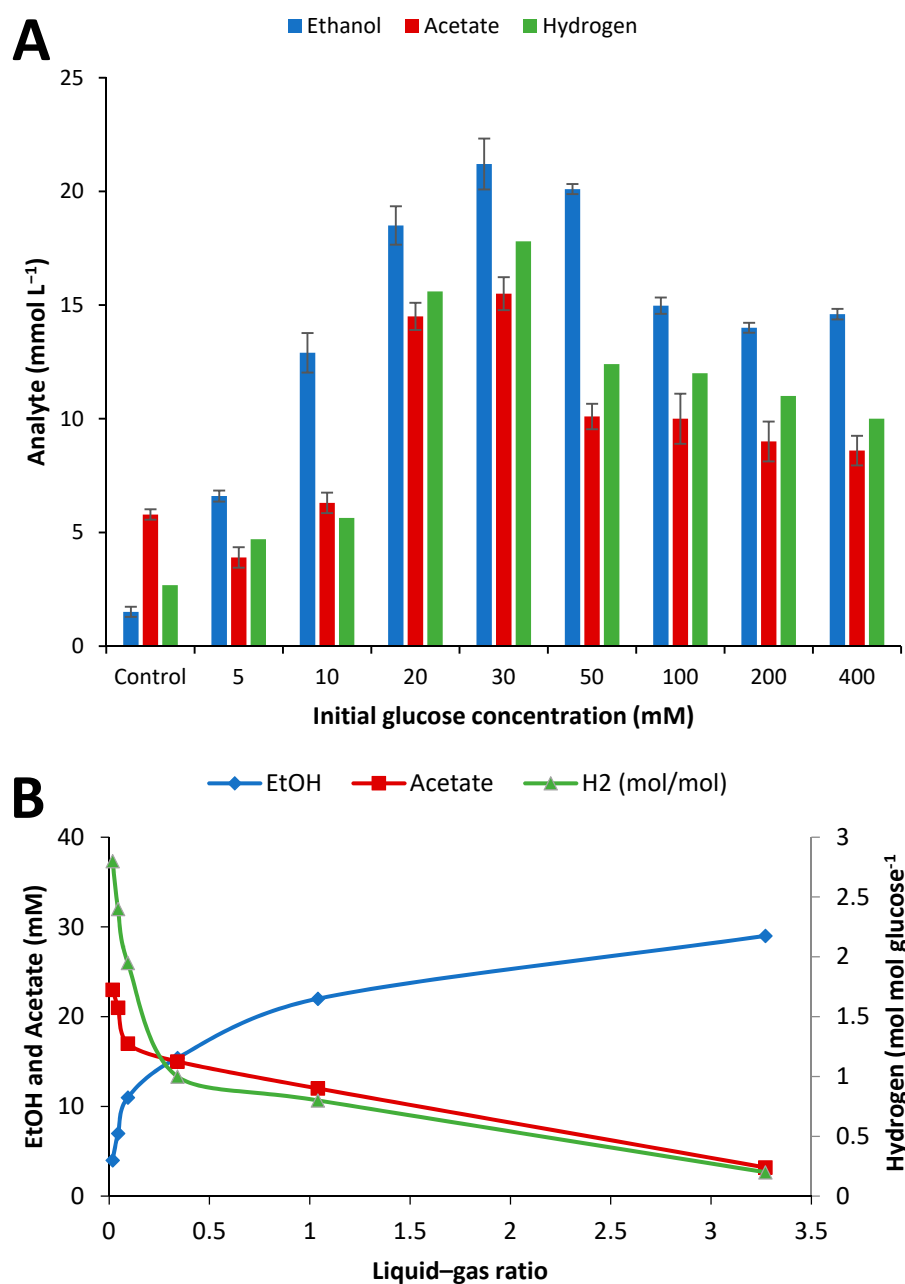


Figure 3. The effect of initial glucose concentration (A) and L-G ratio (at 20 mM glucose) (B) on fermentation by *Thermoanaerobacter* strain AK91. End-products were quantified after 5 days. Values represent averages of triplicate fermentations \pm standard deviation ($n = 3$).

3.4. Effect of Inhibitory Compounds on Growth

The formation of end-products by *Thermoanaerobacter* strain AK91 at 20 mM glucose initial concentration was investigated in the presence of selected exogenous inhibitory compounds. The compounds tested were acetate, propionate, butyrate, lactate, ethanol, malate, oxalate, levulinic acid, *p*-coumaric acid, 2-furfuraldehyde, and 5-HMF. Most of these substrates are well known as inhibitory for microorganisms and some originate directly from biomass pre-treatment [13]. The alkanolic carboxylic acids can be liberated from the pre-treatment of hemicellulose while the dicarboxylic acids, oxalate and malate, are abundant in rhubarb leaves. Ethanol and lactate are common fermentation end-products, and were included as *Thermoanaerobacter* strains produce both compounds. As a proxy for lignin degradation products, *p*-coumaric acid was selected while the inclusion of 5-HMF, 2-furfuraldehyde, levulinic acid are commonly associated with the degradation of hexoses and pentoses under acidic conditions [4,6]. Based on end-product formation the strain was insensitive to most of the organic acids tested up to the maximum tested value, but showed high sensitivity towards *p*-coumaric acid, levulinic acid, 2-furfuraldehyde, and 5-HMF (Table 2).

Table 2. Minimum inhibitory concentrations of model inhibitory compounds based on growth (optical density) and end-product formation of *Thermoanaerobacter* strain AK91.

	Minimum Inhibitory Concentration (mM)
Acetate	>80 mM
Propionate	>80 mM
<i>n</i> -Butyrate	>80 mM
Lactate	>50 mM
Ethanol	>100 mM
Malate	>40 mM
Oxalate	>80 mM
Levulinic acid	>20 mM
<i>p</i> -Coumaric acid	<10 mM
2-furfuraldehyde	<20 mM
5-HMF	<30 mM

There is rather little data available on the effects of inhibitory compounds on thermophilic bacteria. *Thermoanaerobacterium* strain AK17 was shown to be inhibited completely by 2-furfuraldehyde and 5-HMF at concentrations of 20 mM and 32 mM respectively, when grown on glucose [15].

Figure 4 shows the effect of increased propionate concentrations on glucose catabolism and formation of end-products by *Thermoanaerobacter* strain AK91.

Interestingly, production of acetate and hydrogen are not inhibited, their production actually increases a little, by increased initial concentrations of propionate, while ethanol production gradually decreases (Figure 4). Surprisingly, increased amounts of 1-propanol were observed to be produced with the addition of propionate although the ratio of 1-propanol to propionate added decreased above 20 mM. There is no simple explanation for the increase in hydrogen and acetate up to 20 mM but it is well known that the production of acetate and hydrogen are ATP yielding reactions whereas the production of ethanol does not give energy. A similar phenomenon was found for *n*-butyrate addition; 1-butanol was formed (results not shown). This seeming conversion of the acid to alcohol was thus tested in an NMR study (see below).

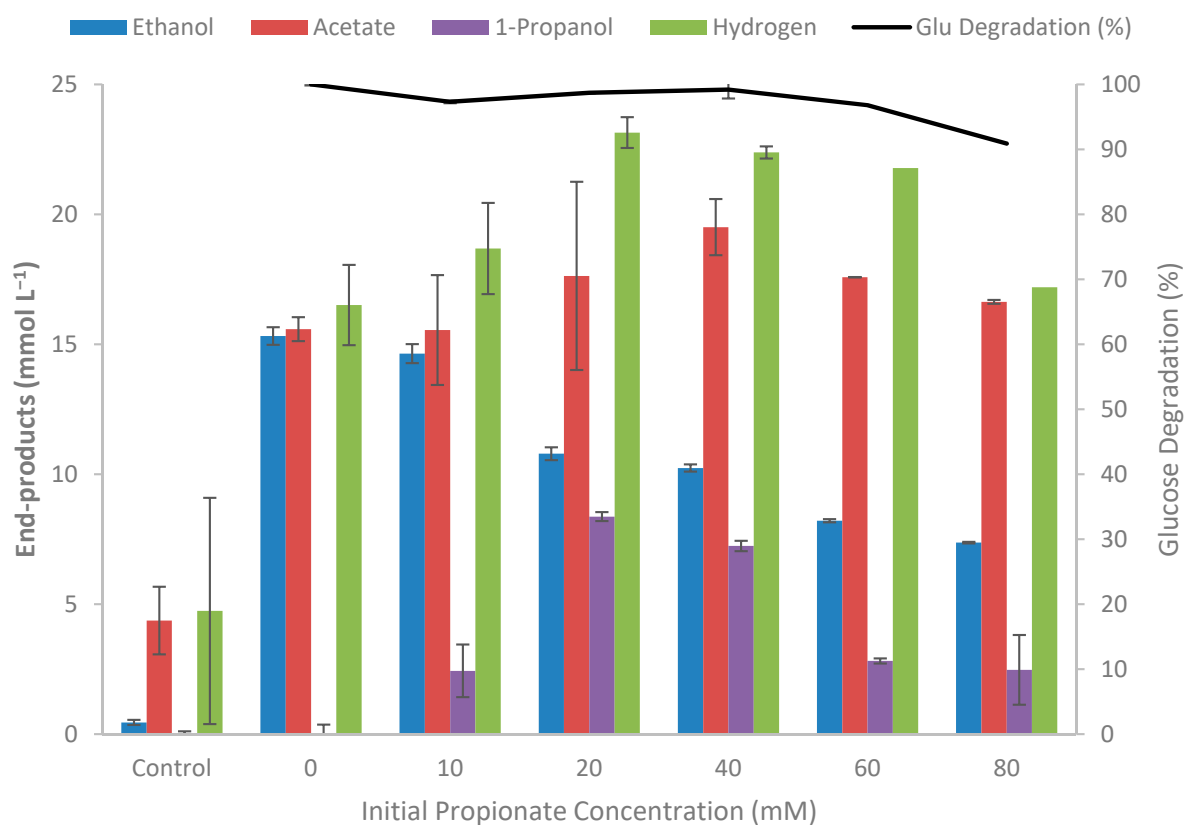


Figure 4. Influence of different initial propionate concentrations of end-product formation from glucose (20 mM) after 5 days of cultivation. Standard deviation are presented as error bars.

3.5. Kinetic Experiment on Glucose and Propionate

To examine the impact of both inhibitory effects of propionate during glucose fermentation, three different concentrations of the acids were used (20, 50, and 100 mM) and growth followed kinetically over a period of 7 days. During growth on 20 mM propionate, end-product formation was similar as without any addition of an acid (data with only glucose; Figure 2), namely formation of both ethanol (17.0 mM) and acetate (13 mM) together with hydrogen (Figure 5A). By increasing propionate to 50 and 100 mM, less ethanol (10.8 mM and 6.2 mM, respectively) was produced but slightly higher acetate concentrations were observed (15.2 and 18.8 mM, respectively). This is in good agreement with the data presented in Figure 4, where ethanol decreased with increasing propionate concentrations but acetate increased. Most interesting, however, was the conversion of propionate to propanol. Increased propionate concentrations resulted in increased formation of propanol in all cases. Recently, our research group has shown the capacity of *Thermoanaerobacter* species to convert fatty acids to their corresponding alcohols [20,27,28] under specific conditions. Instead of dispersing reducing equivalents to pyruvate and produce only ethanol (or lactate), these bacteria used the electrons produced to reduce fatty acids to alcohols. A similar trend was observed on glucose using three increasing concentrations of butyrate; less ethanol was produced with higher butyrate concentrations but acetate production remained similar or was slightly higher (results not shown). Finally, butyrate was converted to butanol as was the case for propionate conversion to propanol.

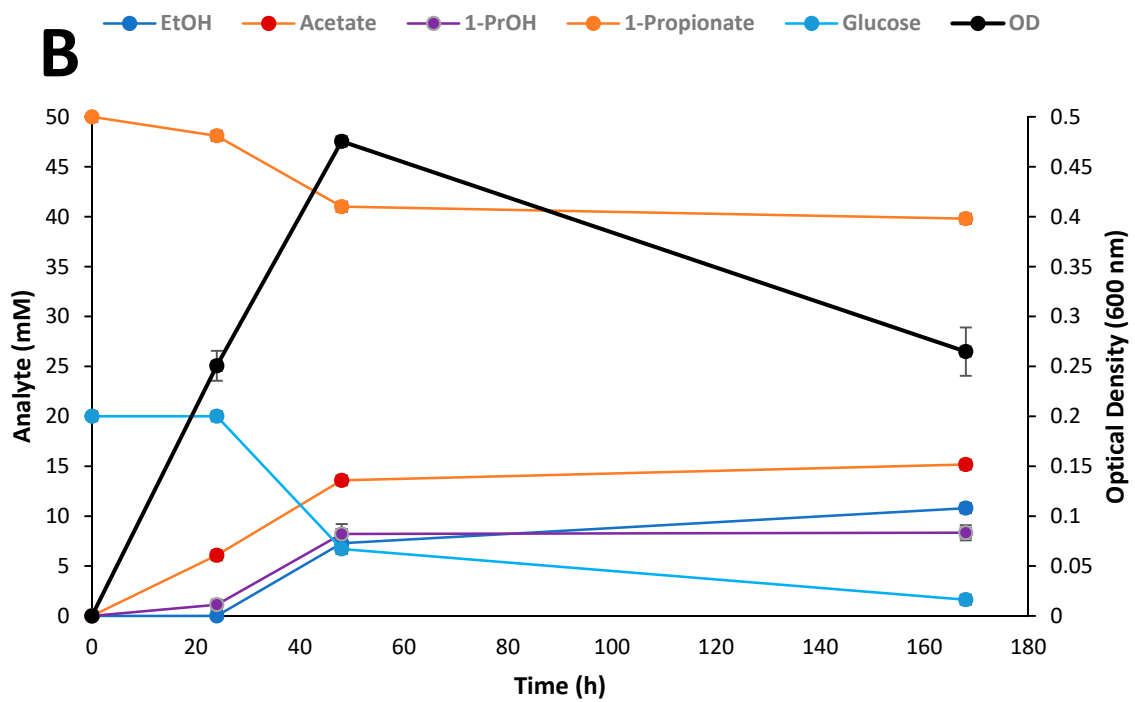
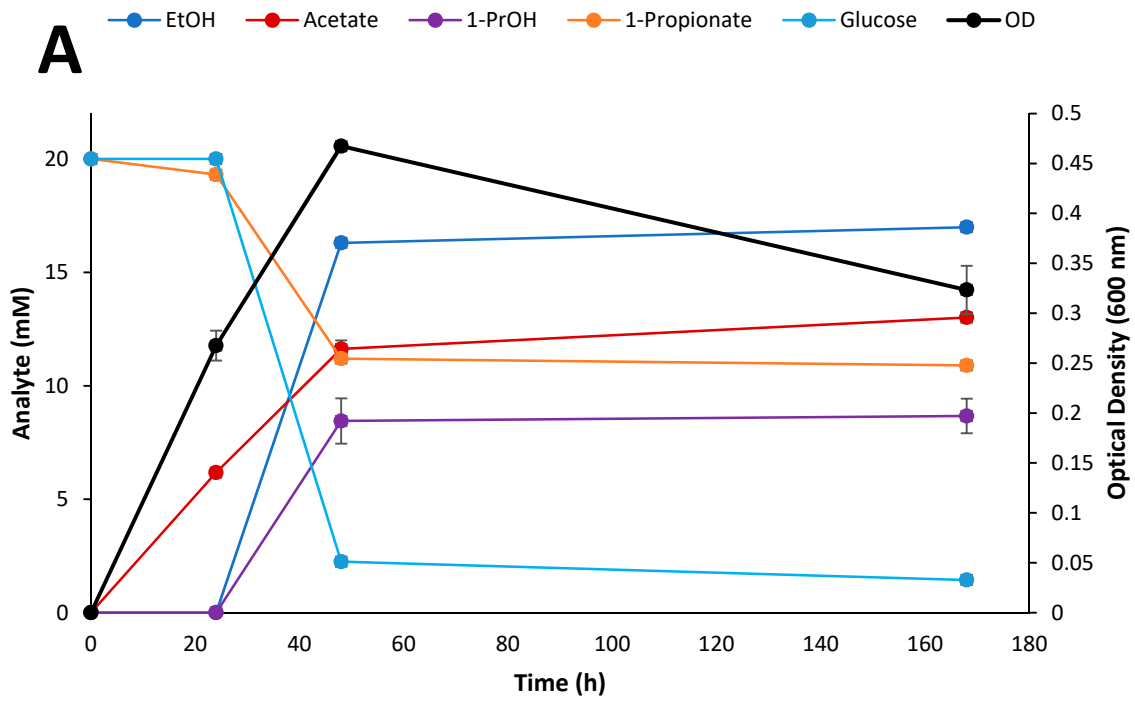


Figure 5. Cont.

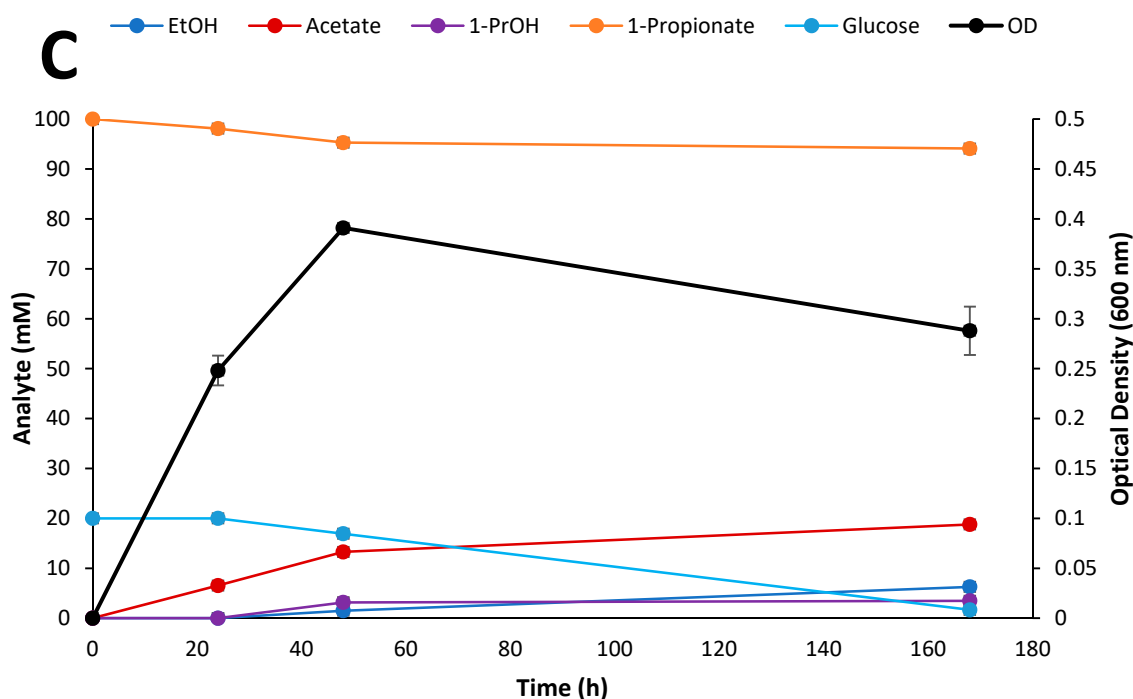


Figure 5. End-product formation from glucose (20 mM) in the presence of 20 mM *n*-propionate (A), 50 mM *n*-propionate (B) and 100 mM *n*-propionate (C).

3.6. Fermentation of Biomass Hydrolysates

Two types of lignocellulosic biomass were tested in the present investigation together with a control, Whatman paper, grass (*P. pratense*) and rhubarb (*R. rhubarbarum*). The focus was on rhubarb as a potential raw material for biofuel production as the rhubarb leaves are an agricultural waste material from the rhubarb industry. The grass *P. pratense*, was also chosen as a reference since there are substantial data available for both ethanol and hydrogen yields on this substrate. Based on the results above, experiments using all three types of biomass were performed at three different concentrations, 2.5, 5.0, and 10 g L⁻¹, of which the lowest concentrations were also tested at three different L-G ratios: 0.04, 1.0, and 3.29.

The reason for using different initial biomass loadings was the sensitivity of the strain towards increased glucose concentrations. In an experiment using three different concentrations of Whatman paper (2.5, 5.0, and 10.0 g L⁻¹), assuming it was completely hydrolysed to glucose, means that the concentration of glucose available should be between 15.4 to 61.7 mM. As observed earlier, *Thermoanaerobacter* strain AK91 is severely inhibited between 20–30 mM initial glucose concentration (Figure 3A). Thus, it is not surprising to see that the highest yields of ethanol from Whatman paper are observed at the lowest biomass loading used (2.5 g L⁻¹), or 14.0 mM (45.4% yields) (Figure 6A). The main reason for these low yields is due to a large fraction of the sugar ending up being converted to acetate under these culture conditions (11.0 mM). Together, acetate and ethanol amount to 25 mM of end-products, or 81.2% of theoretical carbon yields. The rest is presumably lactate and carbon stored in cells (not analyzed). Carbon yields of ethanol and acetate on Whatman paper dropped to 55.2 and 26.9% at 5.0 and 10.0 g L⁻¹ hydrolysate concentrations, respectively (Figure 6A), most likely due to inefficient glucose degradation at higher substrate loadings. Ethanol yields were thus 45.5, 27.6, and 13.4% at 2.5, 5.0 and 10.0 g L⁻¹ hydrolysate loadings, respectively. Yields for acetate at these concentrations were 35.7, 27.6 and 13.4%, respectively (Figure 6A). Yields of hydrogen were 23.6, 14.2, and 7.5%, respectively. However, by using a high L-G ratio for the lowest hydrolysate concentrations,

ethanol yields increased from 45.5 to 75% (Figure 6B). Similarly, hydrogen yields were improved from 23.6 to 55.7% by lowering the L-G ratio (Figure 6B).

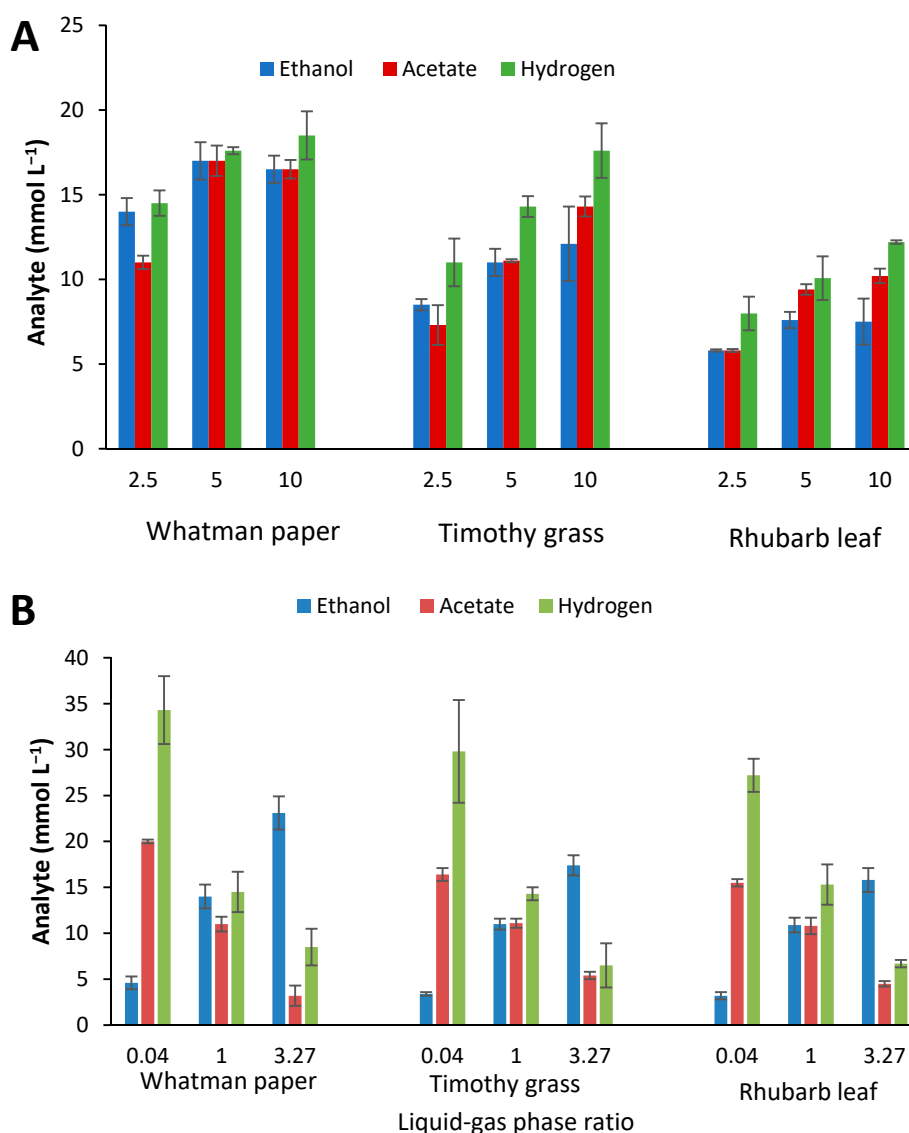


Figure 6. End-product formation of *Thermoanaerobacter* strain AK91 grown on cellulose, grass and rhubarb at three different hydrolysate concentrations (2.5, 5.0 and 10.0 g/L) (A) and at three different L-G ratio using 2.5 g/L hydrolysates (B). Values represent averages of triplicates with standard deviation represented by error bars.

It is clear from Figure 6A that the amounts of end-products do not increase linearly with increased substrate loadings in the case of Whatman paper. Since both grass and rhubarb contain less glucose but more of other varieties of sugars this “levelling off” phenomenon is not as apparent for this type of biomass. There seems to be less substrate inhibition when using the complex biomass as compared with the glucose present in the homogenous Whatman paper. However, both ethanol and hydrogen yields are lower on grass and rhubarb compared with yields from the Whatman paper hydrolysate. Ethanol concentrations on grass and rhubarb hydrolysates ranged from 8.6 to 12.1 mM from the three different concentrations used, with the highest yields being obtained on the lowest hydrolysate loading of 2.5 g L⁻¹. Similar values for hydrogen were 11.0 to 17.6 mmol L⁻¹ (Figure 6A). Values for rhubarb hydrolysates were similar or a little lower as compared with grass hydrolysates. Ethanol ranged from 5.8 to 10.9 mM, and hydrogen from 8.0 to 15.3 mmol L⁻¹. However, in the experiment using the lowest hydrolysate concentration

(2.5 g L⁻¹) and different L-G, these values shifted depending on the compounds and conditions tested (Figure 6B). As for the Whatman paper experiment, ethanol yields on grass hydrolysates were increased to a maximum of 17.4 mM (7.0 mM g dw⁻¹) at the highest L-G phase ratio examined and hydrogen to a maximum of 29.8 mmol/L (1.36 mol mol g dw⁻¹). Similarly, for rhubarb, the highest ethanol concentration obtained was 15.8 mM (6.3 mM g dw⁻¹) and hydrogen of a maximum of 27.2 mmol L⁻¹.

The maximum yields of ethanol and hydrogen are comparable with other similar strains. The strain seems to be more sensitive towards high glucose concentrations as compared with *Thermoanaerobacter* strain J1 [29]. *Thermoanaerobacter* strain J1 produced 35 mM ethanol from a Whatman paper hydrolysate (4.5 gL⁻¹) but this strain is highly ethanologenic compared to strain AK91. Another strain, *Thermoanaerobacterium* AK54, that has been investigated for both ethanol and hydrogen production, produced 29.2 mM of ethanol, 18.1 mM of acetate and 37.1 mmol L⁻¹ of hydrogen from grass hydrolysate [30]. Ethanol yields in the current study on grass and rhubarb were maximized by cultivating the strain at low substrate concentration and high p_H2 to 7.0 and 6.3 mM g⁻¹ biomass. These yields are 63 and 57% of theoretical yields from complex biomass. Hydrogen yields were also maximized by using low substrate concentrations and L-G ratios, to 11.9 and 10.9 mmol L⁻¹ g⁻¹ biomass. Thus, the strain can be a good choice whether to use it as an ethanol or hydrogen producer from complex biomass. This is to our knowledge the first time that rhubarb is used as a potential biofuel feedstock.

Maximum theoretical yields of ethanol from pure cellulose is 2 mol ethanol from 1 mol of glucose, or 11.1 mM g⁻¹. However, lower yields are typically observed from lignocellulosic biomass because of the variety of sugars present and because a portion of the sugars are lost in the pre-treatment steps of the biomass. Examples of high ethanol yields from various lignocellulosic biomass are shown in Table 3. *Thermoanaerobacter* species have been shown to produce high ethanol yields from complex biomass in the literature. *Thermoanaerobacter* strain BG1L1 produces between 8.5–9.2 mM g⁻¹ sugar consumed from wheat straw and corn stover [31,32] in continuous culture. Examples of other high yields from lignocellulose are that of *Thermoanaerobacter mathranii* on wheat straw [33] and *Thermoanaerobacter* strain J1 on various lignocellulosic biomasses [29]. Other genera, such as *Thermoanaerobacterium*, are also good ethanol producers when grown on carbohydrate biomass. As an example, *Thermoanaerobacterium* strain AK17 produces 8.6 mM g⁻¹ cellulose hydrolysate and 5.5 mM g⁻¹ grass hydrolysate at very low initial substrate (2.5 g L⁻¹) concentrations [14] and *Clostridium thermocellum* on paddy straw [34]. Rhubarb has to our knowledge not been used for bioethanol or biohydrogen production before. Some strains within the genus of *Thermoanaerobacter* have also been shown to be good hydrogen producers, such as *T. tengcongensis* which can reportedly product up to 4 mol hydrogen per mole glucose using continuous nitrogen flushing [35].

Table 3. Selected examples of ethanol production from lignocellulosic biomass by thermophilic bacteria. Ethanol yields are reported as mM g⁻¹ substrate degraded along with substrate concentrations and incubation temperatures. Ac—acid; Alk—alkaline; E—enzymatic; and WO—wet oxidation.

Organisms	Substrate	Conc. (g L ⁻¹)	Pre-Treatment	Ethanol Yields (mM g ⁻¹)	References
<i>Thermoanaerobacter</i> strain AK 91	Timothy grass	2.5	Ac/E	7.0	This study
<i>Thermoanaerobacter</i> strain AK 91	Rhubarb leaf	2.5	Ac/E	6.3	This study
<i>Clostridium thermocellum</i>	Paddy straw	8.0	None	6.10–8.00	[34]
<i>Thermoanaerobacter mathranii</i>	Wheat straw	6.7	WO/E	2.61	[33]
<i>Thermoanaerobacter</i> BG1L1	Corn stover	25.0–150.0	WO/E	8.50–9.20	[32]
<i>Thermoanaerobacter</i> BG1L1	Wheat straw	30.0–120.0	WO/E	8.50–9.20	[31]
<i>Thermoanaerobacter</i> strain J1	Hemp	4.5	Ac/E	4.3	[29]
<i>Thermoanaerobacterium</i> strain AK17	Grass	2.5	Ac/Alk/E	5.5	[14]

3.7. NMR Studies

To conclusively demonstrate n-propanol production from exogenously added propionate to glucose containing culture, $^{13}\text{C}_1$ -labeled propionate was used as a model compound. As has been demonstrated for other *Thermoanaerobacter* strains, strain AK91 also has the ability to convert carboxylic acids to their corresponding primary alcohols as evidenced by the appearance of a peak at 63.8 ppm which can be attributed to $^{13}\text{C}_1$ -labelled propanol (Figure 7).

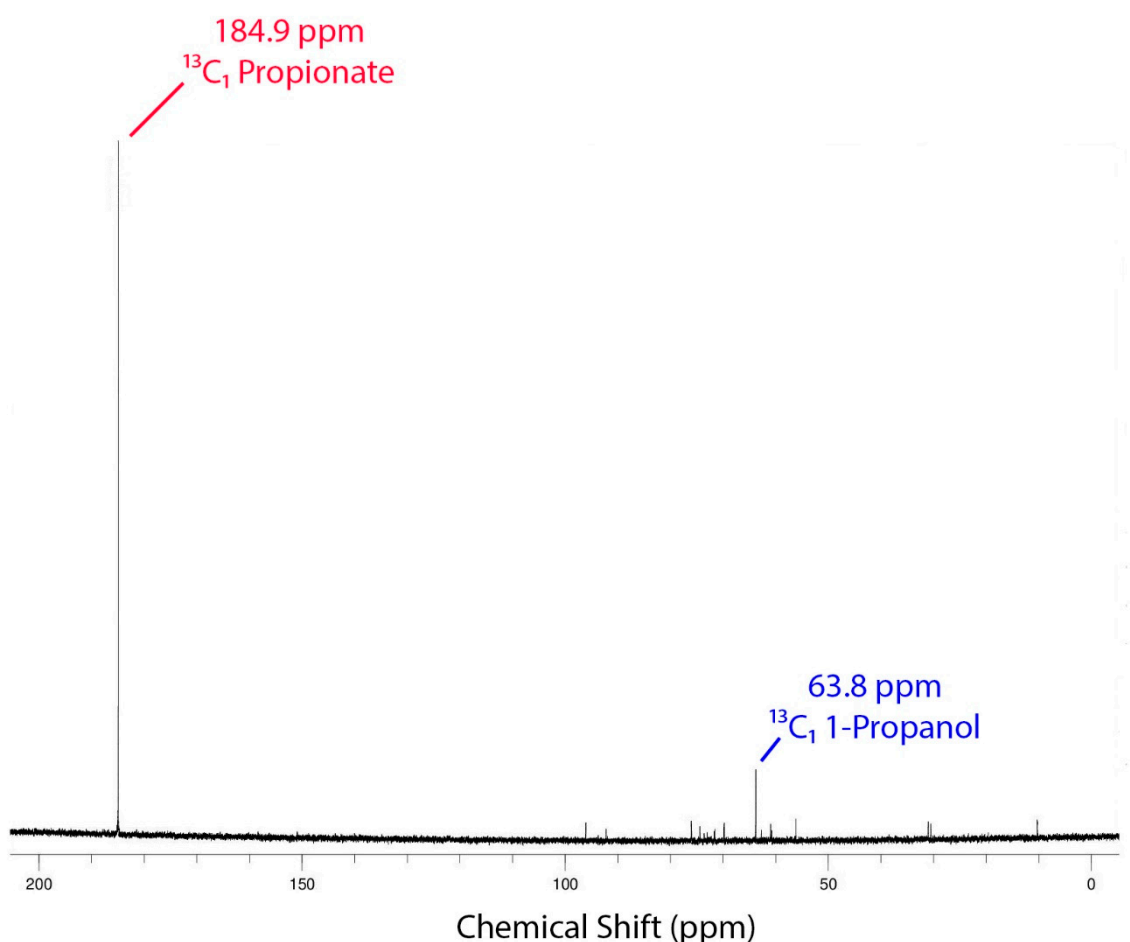


Figure 7. NMR spectrum of glucose (20 mM) degradation with $^{13}\text{C}_1$ 1-propionate by *Thermoanaerobacter* strain AK91.

While this strain produces less of the alcohols from carboxylic acids than other *Thermoanaerobacter* strains investigated to date, this physiological strategy may be useful for dealing with carboxylic acids liberated during the pre-treatment of lignocellulosic biomass. In the case of biomasses containing dicarboxylic acids such as oxalate, the production of a diol such as ethylene glycol may present a potentially useful route to generating these commodity chemicals as a co-product during biofuel production in addition to primary alcohols such as propanol and butanol being useful biofuels in their own right.

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