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Influence of Redox Potential on Product Distribution in *Clostridium thermosuccinogenes*

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Abstract

Clostridium thermosuccinogenes are the only known anaerobic thermophilic bacteria that ferment inulin to succinate and acetate as major products and formate, lactate, and ethanol as minor products. In this study, organic acid production in 2-L fermentations having an initially low (–300 to –330 mV) or high (–220 to –250 mV) redox potential was compared for two strains of *C. thermosuccinogenes* (DSM 5808 and DSM 5809). Although DSM 5809 consistently provided higher succinate yield, high variability in results was attributed to the absence of redox control during the fermentations, and, therefore, fermentations at three controlled redox potentials (–240, –275, and –310 mV) were conducted. At an intermediate redox potential (–275 mV), the succinate yield was the greatest (0.36 g of succinate/g of hexose unit), whereas ethanol yield was the least (0.02 g/g). Redox potential did not significantly affect acetate or lactate formation. At a controlled redox potential of –275 mV, the growth of DSM 5809 on three substrates was also compared: inulin, fructose, and glucose. DSM 5809 had similar growth rates when inulin (0.20/h) or glucose (0.21/h) was the carbon source but grew more slowly when fructose (0.16/h) was the carbon source. Also, the specific rate of utilization of fructose by DSM 5809 was higher (0.89 g of fructose/[g of biomass·h]) compared to glucose (0.53 g/[g·h]) or inulin (0.55 g/[g·h]). Succinate was the major product formed by DSM 5809 fermenting inulin (0.50 g/[g·h]) or glucose (0.36 g/[g·h]), and ethanol was the principal product when DSM 5809 fermented fructose (0.54 g/[g·h]).

Index Entries: *Clostridium thermosuccinogenes*; inulin; succinic acid; anaerobic fermentation; thermophile.

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Introduction

Inulin is a plant polymer found in the roots or tubers of plants and commonly consists of 20–30 fructose molecules connected to a terminal glucose residue by β(2→1) linkages. High concentrations of inulin (dry wt fraction) are found in Jerusalem artichoke (80%), chicory (75%), and dahlia (72%) *(1)*. Anaerobic bioconversion of renewable materials such as inulin to fuels and chemicals can have advantages compared to aerobic bioconversions because of the straightforward scale-up, higher productivity, and product yields of these fermentations *(2)*.

There are anaerobic microorganisms that ferment inulin to ethanol *(3)*, acetone, and butanol *(4)*, as well as to acetic, formic, and lactic acids *(5)*. However, *Clostridium thermosuccinogenes* are the only species of anaerobic thermophilic bacteria known to ferment inulin to succinic acid as a major product. Drent et al. *(6)* isolated four strains of *C. thermosuccinogenes* (DSM 5806–5809) from mesobiotic environments that ferment inulin to generate acetate and succinate as major products and lactate, formate, and ethanol as minor products.

Succinic acid is a four-carbon aliphatic dicarboxylic acid having a pK_{a1} of 4.2 and a pK_{a2} of 5.6 that finds applications in the manufacture of specialty chemicals, agriculture, food, medicine, textiles, plating, and waste-gas scrubbing *(7)*. Industrially, succinic acid is currently produced by hydrogenation of maleic anhydride to succinic anhydride followed by hydration to succinic acid *(7,8)*. The deprotonated form (succinate) can be produced by many anaerobic microorganisms usually near neutral pH *(9)*. For example, under optimal conditions, the strict anaerobe *Anaerobiospirillum succiniciproducens (9–12)* can produce succinic acid with glucose as the carbon source with a yield of 87% and a final concentration of 35 g/L *(9,13)*. Recently, Guettler et al. *(14)* isolated the facultative anaerobic Gram-negative bacterium *Actinobacillus* sp. 130Z, which achieved a final succinate concentration of 50 g/L while growing on a complex medium. With the exception of formate produced by *C. thermosuccinogenes*, the catabolic products of *C. thermosuccinogenes* growing on inulin are the same as those observed with *A. succiniciproducens* on glucose *(15)*. The two organisms may therefore have similar biochemical pathways.

One method of optimizing a fermentation is to understand how environmental parameters such as redox potential affect product distribution and alter influential variables so that formation of a desired product is maximized. Culture redox potential has been previously shown to correlate with the partitioning of fermentation products in *Clostridium acetobutylicum (16)* and has been experimentally associated with rates of NAD(P)H formation in these bacteria *(17)*. Although redox potential should play an integral role in NAD(P)H/NAD(P) ratios *(17)*, surprisingly few previous studies have focused on the effect of redox potential on product distribution in anaerobic organic acid fermentations. The principal objective of the present study was to elucidate how redox potential affects growth

and product distribution for two strains of *C. thermosuccinogenes* (DSM 5808 and DSM 5809). Also, we compared the growth of *C. thermosuccinogenes* on three substrates: inulin, and its constituent monomers, glucose and fructose.

Materials and Methods

C. thermosuccinogenes

C. thermosuccinogenes strains DSM 5808 and DSM 5809 were routinely cultivated using 5 g/L of inulin as a carbon source in a modified basal medium with the following composition (pH 7.2) *(6)*: 1.2 g/L of NaCl; 0.056 g/L of $MgCl_2 \cdot 6H_2O$; 0.3 g/L of KCl; 0.056 g/L of $CaCl_2 \cdot 2H_2O$; 0.27 g/L of NH_4Cl; 0.21 g/L of KH_2PO_4; 0.21 g/L of Na_2SO_4; 0.2 g/L of Na_2HPO_4; 1 g/L of yeast extract; 0.03 g/L of casamino acids; 1.5 mg/L of $FeCl_2 \cdot 4H_2O$; 0.07 mg/L of $ZnCl_2$; 0.1 mg/L of $MnCl_2 \cdot 4H_2O$; 0.006 mg/L of H_3BO_3; 0.19 mg/L of $CoCl_2 \cdot 6H_2O$; 0.002 mg/L of $CuCl_2 \cdot 2H_2O$; 0.024 mg/L of $NiCl_2 \cdot 6H_2O$; 0.036 mg/L of $Na_2MoO_4 \cdot 2H_2O$; 0.02 g/L of biotin; 0.02 mg/L of folic acid; 0.1 mg/L of pyridoxine-HCl; 0.05 mg/L of thiamine-HCl; 0.05 mg/L of nicotinic acid; 0.05 mg/L of calcium pantothenate; 0.001 mg/L of vitamin B_{12}; 0.05 mg/L of *p*-aminobenzoic acid; 0.05 mg/L of lipoic acid; 1 mg/L of resazurin; 2.5 g/L of $NaHCO_3$; 0.15 g/L of $Na_2S \cdot 9H_2O$.

Fermentations

Chicory inulin (Fructafit IQ, Imperial Suiker Unie, Sugarland, TX) was used as the sole carbon source. All batch fermentations were carried out in 2-L fermentors at a controlled pH of 6.75 and at 58°C. A first series of fermentations was conducted to compare the growth and product formation of the two strains. For this study, 10 g/L of inulin was used as the carbon source, and the initial redox potential was set at either high (–220 to –250 mV) or low potential (–300 to –330 mV). Redox potential was not adjusted after the onset of the fermentations. A second series of experiments was conducted in which redox potential was controlled during the course of each fermentation. In this case, 5 g/L of inulin was used as the carbon source, and the redox potential was controlled (±5 mV) at high (–240 mV), intermediate (–275 mV), or low potential (–310 mV). In all cases, redox potential was monitored on-line using an ORP sensor and transmitter (Ingold; Mettler-Toledo, Wilmington, MA). Redox potential was also measured off-line with a platinum electrode with a calomel reference electrode (Accumet; Fisher, Pittsburgh, PA). For redox control, a solution of 35 g/L of $Na_2S \cdot 9H_2O$ was added either manually or automatically with a peristaltic pump (Scilog, Madison, WI). The headspace of the fermentors was maintained anaerobically by bubbling 95 mL/min of 15% oxygen-free CO_2 in N_2.

Analyses

A Shimadzu HIC-6A ion chromatography system (Shimadzu, Columbia, MD) was used to analyze monosaccharides, organic acids, and ethanol

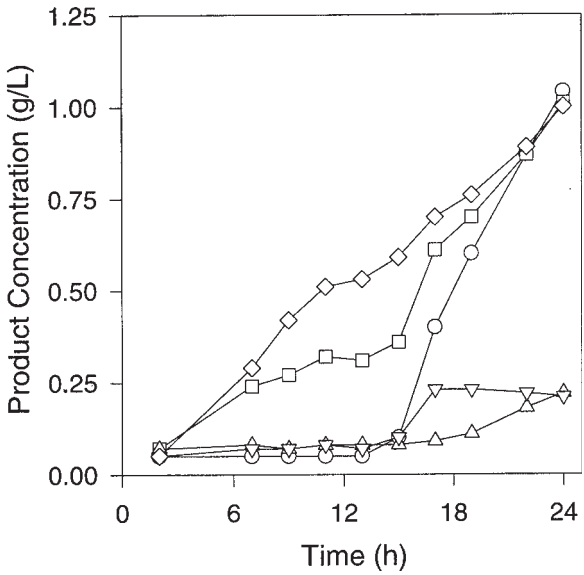

Fig. 1. Concentrations of succinate (○), acetate (□), lactate (△), formate (▽), and ethanol (◇) for *C. thermosuccinogenes* DSM 5808 fermenting 10 g/L of inulin at pH 6.75 and initial redox potential of –220 to –250 mV.

as previously described *(18)*. Cell growth was monitored by optical density (OD) at 620 nm (DU 650 Spectrophotometer; Beckman, Fullerton, CA). An OD_{620} of 1.0 was found to correspond to 0.44 g of dry cell weight. Hexose units of inulin were determined by hydrolyzing a 2.5-mL sample with 100 µL of 37% HCl and analyzing reducing sugars using the dinitrosalicylic acid assay *(19)*. Statistical analysis of data was accomplished with SAS using separate-variance *t*-tests and paired *t*-tests *(20)*.

Results and Discussion

Comparison of DSM 5808 and DSM 5809

C. thermosuccinogenes strains DSM 5808 and DSM 5809 required an initial redox potential below –200 mV for growth and grew poorly at redox potentials below –335 mV. Both DSM 5808 and DSM 5809 fermented inulin to succinate, acetate, formate, lactate, and ethanol (typical 2-L fermentations shown in Figs. 1 and 2). Final product concentrations for 24-h fermentations of DSM 5808 varied widely: succinate (1.04–1.70 g/L), acetate (1.01–1.59 g/L), lactate (0.22–0.51 g/L), formate (0.00–0.21 g/L), and ethanol (0.02–1.00 g/L). The final concentrations for DSM 5809 also varied widely: succinate (1.24–2.33 g/L), acetate (1.22–1.62 g/L), lactate (0.46–1.56 g/L), formate (0.31–0.62 g/L), and ethanol (0.17–1.29 g/L). The total quantity of soluble products was significantly higher for DSM 5809 (5.62 g/L) compared to DSM 5808 (3.78 g/L) for all fermentations ($\alpha = 0.05$). Also, within 24 h, DSM 5809 utilized significantly higher inulin (7.19 g/L) than DSM 5808 (4.12 g/L)

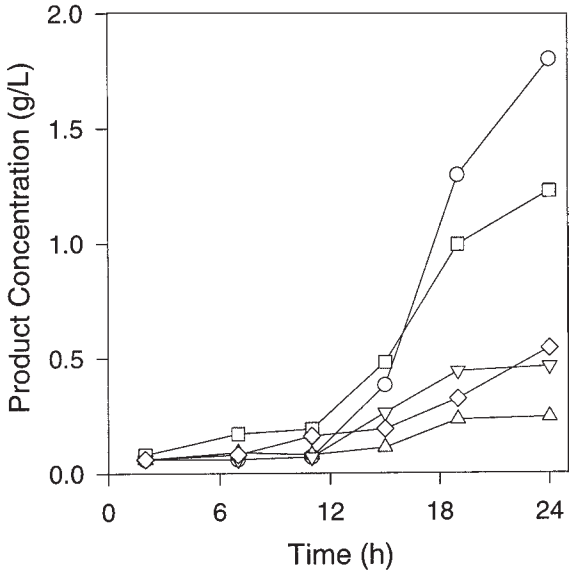

Fig. 2. Concentrations of succinate (○), acetate (□), lactate (△), formate (▽), and ethanol (◇) for *C. thermosuccinogenes* DSM 5809 fermenting 10 g/L of inulin at pH 6.75 and initial redox potential of –300 to –330 mV.

Table 1
Final Product Yields from the Fermentation
of 10 g/L of Inulin by *C. thermosuccinogenes* DSM 5808 and DSM 5809
at Low and High Initial Redox Potentials[a]

	DSM 5808		DSM 5809	
Product	–300 to –330 mV	–220 to –250 mV	–300 to –330 mV	–220 to –250 mV
Succinate	0.31 (0.04)	0.30 (0.04)	0.25 (0.03)	0.29 (0.06)
Acetate	0.29 (0.05)	0.29 (0.04)	0.19 (0.02)	0.21 (0.02)
Lactate	0.11 (0.00)	0.06 (0.01)	0.10 (0.03)	0.16 (0.03)
Formate	0.00 (0.00)	0.05 (0.01)	0.05 (0.01)	0.08 (0.02)
Ethanol	0.02 (0.01)	0.24 (0.06)	0.10 (0.03)	0.09 (0.04)
Biomass	0.08 (0.02)	0.11 (0.03)	0.07 (0.01)	0.07 (0.01)

[a]Product yields are in grams of product per gram of hexose units consumed. Initial redox potentials: low = –300 to –330 mV; high = –220 to –250 mV. Standard errors of measurements are included in parentheses.

(α = 0.05). Table 1 summarizes the yields obtained from these multiple fermentations. The results obtained for fermentations with initial redox poising showed high standard errors, which indicates high variability (Table 1). Therefore, few statistical comparisons could be made to assess the differences between the two strains and two initial redox potentials. The only significant results were that DSM 5808 produced no formate and low ethanol (0.02 g of ethanol/g of hexose unit) at low initial redox poten-

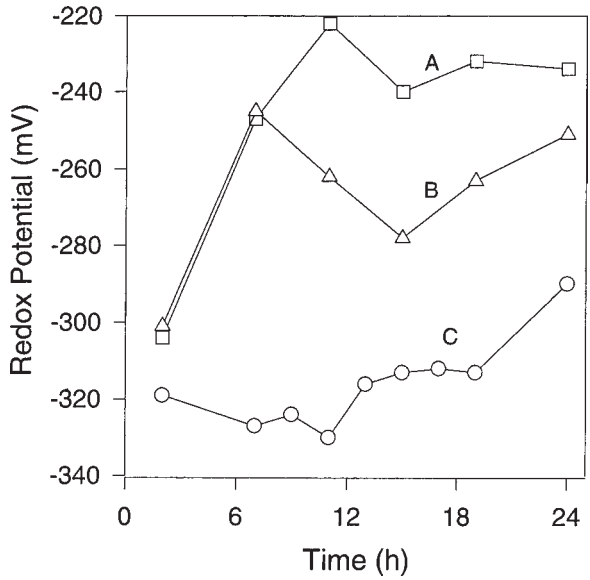

Fig. 3. Measured redox potential for three fermentations of *C. thermosuccinogenes* DSM 5809 fermenting 10 g/L of inulin at pH 6.75 and initial redox potential of –300 to –330 mV. A, B, and C: three separate fermentations at these identical conditions.

tials, but greater yields of formate (0.05 g/g) and ethanol (0.24 g/g) at high initial redox potentials. For DSM 5809, the yield of lactate (0.16 g/g) at high initial redox potentials was greater than the lactate yield (0.10 g/g) at low initial redox potentials.

We suspected that the high variability in these results were due to the absence of redox potential control during the fermentations. This proposition was supported by trends observed in measured redox potential. For example, Fig. 3 shows the measured redox potential during the three fermentations (labeled A, B, and C) of DSM 5809 subject to an initially low redox potential. As can be seen, the path of the redox potential for each of the fermentations was unique, even though pH was controlled and gas sparging was identical. The corresponding final product concentrations also differed in these three fermentations. For example, final succinate concentrations for these fermentations were 2.33 g/L (A), 1.24 g/L (B), and 1.97 g/L (C), whereas final ethanol concentrations were 0.56 g/L (A), 1.29 g/L (B), and 0.29 g/L (C). Merely setting the redox potential at an initial value seems insufficient to guarantee the path of the redox potential during the fermentation. Although the results demonstrated higher product formation and inulin consumption with DSM 5809, redox potential clearly must be controlled to clarify the effect of this parameter on product distribution.

Comparison of Three Controlled Redox Potentials

Fermentations were conducted with DSM 5809 and controlled redox potential to determine whether the high variability was attributable to the

Table 2
Effect of Controlled Redox Potential
on Product Yield in *C. thermosuccinogenes* DSM 5809
Fermenting 5 g/L of Inulin at pH 6.75[a]

	Controlled redox potential		
Product	–240 mV	–275 mV	–310 mV
Succinate	0.27 (0.01)	0.36 (0.00)	0.30 (0.01)
Acetate	0.18 (0.00)	0.30 (0.04)	0.20 (0.00)
Lactate	0.15 (0.04)	0.10 (0.00)	0.09 (0.01)
Formate	0.04 (0.02)	0.09 (0.02)	0.12 (0.01)
Ethanol	0.09 (0.01)	0.02 (0.00)	0.07 (0.00)
Biomass	0.13 (0.00)	0.11 (0.00)	0.10 (0.01)

[a]Product yield is in grams of product per gram of hexose units utilized. Standard errors of measurements are included in parentheses.

absence of control and to investigate how redox potential influenced product distribution. Three values of redox potential (±5 mV) were chosen: –240, –275, and –310 mV. Because our initial fermentations with DSM 5809 utilized only 7.19 g/L of inulin, 5 g/L of inulin was used as the carbon source for these fermentations.

Table 2 shows the product yields from these redox-controlled fermentations. The product concentrations and yields from the fermentations in which redox potential was controlled were far less variable than those obtained earlier, permitting detailed statistical analysis. Succinate yield was significantly greater at –275 compared with –240 and –310 mV ($\alpha = 0.10$), and ethanol yield was significantly greater at –240 and –310 than at –275 mV ($\alpha = 0.05$). The yield of formate increased significantly with a decrease in redox potential ($\alpha = 0.05$). The lactate and acetate yields did not differ significantly with redox potential.

Redox potential has been previously demonstrated to have significant influence on product formation in other obligate anaerobes. For example, *C. acetobutylicum* had maximum butyrate yield (0.35 g/g) and minimum butanol yield (0.10 g/g) at a controlled redox potential of –250 mV *(21)*. However, on decreasing the redox potential to –300 mV, the butyrate yield decreased (0.05 g/g) and the butanol yield (0.20 g/g) increased *(21)*. Thus, redox potential was concluded to be an indicator of fermentation product partitioning in *C. acetobutylicum (21)*. Another study with *C. acetobutylicum* demonstrated that decreasing the redox potential from –298 to –320 mV increased the specific rate of butanol production from 2.11 to 2.53 mmol/(g·h) with a corresponding decrease in butyrate production from 2.13 to 1.91 mmol/(g·h) *(17)*. Studies with *Methanosarcina barkeri* and *Methanobacterium thermoautotrophicum* showed that controlled redox potential values between –370 and –520 mV were optimal for methane production, with the growth rate and specific methane production rate decreasing sharply between –315 and –350 mV *(22,23)*.

Many obligate anaerobic bacteria form succinate, ethanol, or lactate to recycle reduced NADH to regenerate the oxidized form (NAD^+) so that glycolysis may continue *(2)*. The oxidation:reduction ratio (O:R) is often used to determine whether the total quantity of hydrogen (in the form of H_2 or NADH) generated and consumed are equal *(2)*. The O:R value of formaldehyde is arbitrarily taken as 0. Any compound more oxidized than formaldehyde has a positive O:R ratio, and compounds more reduced than formaldehyde have a negative O:R ratio. For example, carbon dioxide has an O:R ratio of +2 whereas succinate, ethanol, and lactate have O:R ratios of –2, –2, and 0, respectively *(2)*. Since lactate has a greater O:R ratio than succinate or ethanol, bacteria might produce more succinate or ethanol in preference to lactate to balance the O:R ratio at lower redox potential. Indeed, a linear relationship was previously found between NADH concentration and culture redox potential for *C. acetobutylicum* with lower redox potential corresponding to higher NADH concentrations *(17)*. In fermentations of *C. thermosuccinogenes*, a higher concentration of NADH may similarly be present at –310 than at –275 mV or than at –240 mV. In the controlled redox potential fermentations completed in the present study, the fraction of reduced end-products that were succinate and ethanol (i.e., ([succinate + ethanol]/[succinate + ethanol + lactate]) was 0.81 mol/mol at –310 mV, 0.76 mol/mol at –275 mV, and 0.71 mol/mol at –240 mV. These results are consistent with low redox potential favoring the generation of the most reduced fermentation products.

The observations in formate production are consistent with previous studies showing that the activity of pyruvate formate lyase, which commonly catalyzes formate generation from pyruvate in anaerobes, increases with a decrease in redox potential *(24)*. Acetate production is commonly a significant means of energy generation (through the formation of one ATP) in anaerobes, and its synthesis is therefore beneficial for cell maintenance. Since acetate has an O:R ratio of 0, and its formation does not involve oxidation of reducing equivalents, a change in redox potential or intracellular NADH concentration would not be expected to affect acetate formation in DSM 5809. Previous studies with *C. acetobutylicum* also demonstrated that changes in culture redox potential did not affect acetate yield *(16)*.

In summary, *C. thermosuccinogenes* DSM 5809 altered final product distribution at different controlled redox potentials. Control of redox potential during a fermentation would therefore seem vital to optimize fermentations using *C. thermosuccinogenes* DSM 5809 for succinate production.

Comparison of Growth on Inulin, Glucose, and Fructose

Using native inulinase, *C. thermosuccinogenes* DSM 5809 is able to break down inulin into fructooligosaccharides (polymers of less than 10 fructose units), fructose, and glucose. Although the inulinase of *C. thermosuccinogenes* DSM 5807 is well characterized *(6)*, a comparison of fermentation products of DSM 5809 from inulin, fructose, and glucose has not been completed. To understand the relative rates of consumption of these three

Table 3
Specific Growth, Specific Substrate Consumption, and Specific Product Formation Rates of *C. thermosuccinogenes* DSM 5809 Fermenting Inulin, Fructose, or Glucose at Controlled Redox Potential of –275 mV and pH 6.75[a]

Parameter	Inulin	Fructose	Glucose
Specific growth rate (h^{-1})	0.20 (0.00)	0.16 (0.04)	0.21 (0.03)
Specific substrate consumption rate (g substrate/[g biomass·h])	0.55 (0.06)	0.89 (0.05)	0.53 (0.06)
Specific product formation rate (g product/[g biomass·h])			
Succinate	0.50 (0.03)	0.10 (0.02)	0.36 (0.05)
Acetate	0.36 (0.01)	0.18 (0.00)	0.30 (0.05)
Lactate	0.32 (0.02)	0.27 (0.07)	0.32 (0.03)
Formate	0.17 (0.03)	0.00 (0.00)	0.27 (0.07)
Ethanol	0.02 (0.00)	0.54 (0.10)	0.18 (0.00)

[a]Standard errors of measurements are included in parentheses.

substrates, a series of 5 g/L fermentations was completed at pH 6.75, 58°C, and controlled redox potential of –275 mV (Table 3). All rates were calculated during the mid-log phase.

The specific rates of utilization for glucose and inulin were quite similar (0.55 g of glucose/[g of biomass·h] vs 0.53 g/[g·h]). The specific rate of fructose utilization (0.89 g/[g·h]) was greater than that of both glucose and inulin. However, the growth rates of *C. thermosuccinogenes* DSM 5809 on both inulin and glucose (0.20 and 0.21/h) were much greater than the growth rate on fructose (0.16/h). A similar observation has been previously reported for *Corynebacterium glutamicum*, which had a higher growth rate on glucose than on fructose, but utilized fructose more rapidly than glucose *(25)*. Although the growth rates and substrate utilizations during glucose and inulin fermentations were similar, the specific rate of succinate production was greater when DSM 5809 fermented inulin (0.50 g/[g·h]) compared to glucose (0.36 g/[g·h]). By contrast, the specific rate of ethanol formation was greater when DSM 5809 fermented glucose (0.18 g/[g·h]) compared to inulin (0.02 g/[g·h]). When fermenting fructose, DSM 5809 produced succinate more slowly (0.10 g/[g·h]) and ethanol more quickly (0.54 g/[g·h]). Interestingly, formate was not observed during growth of DSM 5809 on fructose. These observations of product formation suggest that regulation is similar when inulin or glucose is the sole carbon source but different when fructose is the sole carbon source.

Previous studies demonstrated that the optimum growth temperature of DSM 5807 is 58°C when inulin is the carbon source but 70°C when fructose is the carbon source *(6)*. Here, the lower growth rate of DSM 5809 on fructose compared to inulin is consistent with the growth behavior of

DSM 5807 and may be a reason for the different product distributions. The greater fructose utilization rate compared to the inulin or glucose utilization rates suggests that the rate of glycolytic flux may be a factor in altering product distribution during growth on fructose. This proposition is supported by previous studies with *Lactococcus lactis (26)* and *Enterobacter agglomerans* CNCM1210 *(27)* in which high glycolytic flux led to accumulation of glyceraldehyde-3-phosphate and dihydroxyacetone-phosphate, which inhibit pyruvate formate lyase. If pyruvate formate lyase catalyzes formate formation in *C. thermosuccinogenes* and these two glycolysis intermediates are inhibitory, then a high rate of fructose consumption may be a cause of the absence of formate under these conditions. In a study by Dijkerman et al. *(28)* with the anaerobic fungi *Neocallismastix* sp. strain L2, growth on inulin, fructose, and glucose resulted in significant differences in formate, lactate, ethanol, and hydrogen formation. However, in their study the rates of substrate utilization and product formation for *Neocallismastix* sp. strain L2 fermenting inulin, fructose, and glucose were not calculated.

In summary, *C. thermosuccinogenes* DSM 5809 has similar rates of utilization of inulin and glucose but greater fructose utilization rates. The greater fructose utilization rate and lower growth rate may contribute to differences in product distribution.

Conclusion

Although *C. thermosuccinogenes* DSM 5809 utilized significantly more inulin and had significantly higher total final product concentrations than DSM 5808, the results obtained from fermentations with initial redox poising had high variability, attributed to the absence of redox control. For fermentations of DSM 5809 in which redox potential was controlled, lower redox potential favored the generation of the more reduced products succinate and ethanol at the expense of lactate. Also, at a redox potential of –275 mV, DSM 5809 showed similar specific growth rates on inulin and glucose but a lower growth rate on fructose. However, the specific rate of fructose consumption was greater than the specific rate of glucose or inulin consumption. A higher glycolytic flux during growth on fructose may cause accumulation of glycolysis intermediates, which are known in other organisms to inhibit pyruvate formate lyase and lead to the absence of the product formate. Such an effect with fructose would likely serve to explain the observed differences in other end products. To elucidate the molecular bases for these observations, the detailed biochemical pathways used by DSM 5809 in the breakdown of these compounds will first have to be established.

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