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# Metabolic Flux Analysis of *Clostridium thermosuccinogenes*

## *Effects of pH and Culture Redox Potential*

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## Abstract

*Clostridium thermosuccinogenes* are anaerobic thermophilic bacteria that ferment various carbohydrates to succinate and acetate as major products and formate, lactate, and ethanol as minor products. Metabolic carbon flux analysis was used to evaluate the effect of pH and redox potential on the batch fermentation of *C. thermosuccinogenes*. In a first study, the effects of four pH values (6.50, 6.75, 7.00, and 7.25) on intracellular carbon flux at a constant redox potential of –275 mV were compared. The flux of carbon toward succinate and formate increased whereas the flux to lactate decreased significantly with a pH increase from 6.50 to 7.25. Both specific growth rate and specific rate of glucose consumption were unaffected by changes in pH. The fraction of carbon flux at the phosphoenolpyruvate (PEP) node flowing to oxaloacetate increased with an increase in pH. At the pyruvate node, the fraction of flux to formate increased with increasing pH. At the acetyl CoA node, the fraction of flux to acetate increased significantly with an increase in pH. A second study elucidated the effect of four controlled culture redox potentials (–225, –250, –275, and –310 mV) on metabolic carbon flux at a constant pH of 7.25. Lower values of culture redox potential were correlated with increased succinate, acetate, and formate fluxes and decreased ethanol and hydrogen fluxes in *C. thermosuccinogenes*. Lactate formation was not significantly influenced by redox potential. At the PEP node, the fraction of carbon to oxaloacetate increased with a decrease in redox potential. At the pyruvate node, the fraction of carbon to formate increased, while at the acetyl CoA node, the fraction of carbon flux to acetate increased with reduced redox potential. The presence of hydrogen in the headspace or the addition of nicotinic acid to the growth media resulted in increased hydrogen and ethanol fluxes and decreased succinate, acetate, formate, and lactate fluxes.

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**Index Entries:** *Clostridium thermosuccinogenes*; anaerobic fermentation; culture redox potential; NADH; metabolic flux; organic acids.

## Introduction

Several succinate-forming mesophilic bacteria have been isolated and their biochemical pathways elucidated *(1–3)*. For example, the strict anaerobe *Anaerobiospirillum succiniciproducens* under optimal conditions ferments glucose to form succinate with a yield of 87% and a final concentration of 35 g/L *(1,4–6)*. More recently, facultative anaerobic Gram-type negative bacteria *Actinobacillus* sp. 130Z have been isolated that achieve a final succinate concentration of 50 g/L while growing on complex media *(7)*. *Clostridium thermosuccinogenes* is the only known succinate-forming thermophilic anaerobic bacterium, and this microorganism ferments glucose, fructose, or inulin as carbon sources and generates acetate and succinate as major products and lactate, formate, and ethanol as minor products *(8)*.

Several studies have focused on generating more of a particular product from anaerobic fermentations by optimizing parameters such as pH and carbonate *(9,10)*. Comparatively few studies have used culture redox potential (CRP) as a parameter to optimize a particular product of microaerobic or anaerobic fermentation. Nevertheless, different controlled CRP values have been shown to lead to different product distributions. In *Bacillus subtilis* fermentations, lactate was the principal product at a CRP of –229 mV, 2,3-butanediol at –195 mV, and acetoin at –160 mV *(11)*. Studies with *Methanosarcina barkeri* and *Methanobacterium thermoautotrophicum* demonstrated that controlled redox potential values between –370 and –520 mV were optimal for methane production with the growth rate and specific methane production decreasing sharply between –315 and –350 mV *(12,13)*. A strong correlation between the culture redox potential and the metabolic state has been demonstrated for *Clostridium acetobutylicum (14)*. Specifically, a decrease in redox potential from –250 to –300 mV led to higher butanol yields and lower butyrate yields.

Changes in pH and redox potential constitute environmental perturbations to bacteria that would likely influence the flow of intracellular carbon. Metabolic flux analysis is a tool to study alteration in intracellular carbon flux in response to changes in environmental or genotypic conditions *(15–19)*. Flux analysis has been used for studying anaerobic fermentations. For example, flux analyses have been completed on the acetone-butanol fermentation by *C. acetobutylicum* and metabolic pathway rates correlated with intracellular NAD(P)H fluorescence measurements *(20)*. In another study, *Clostridium butyricum* fermentations at increasing dilution rates during growth on glycerol and glucose resulted in increased accumulation of intracellular acetyl CoA and a shift in carbon flux from butyrate formation to acetate formation *(21)*. Metabolic flux analysis has also elucidated the effects of pH and lactate concentration on lactate production by *Streptococcus lactis*, demonstrating that pH affected only the rate

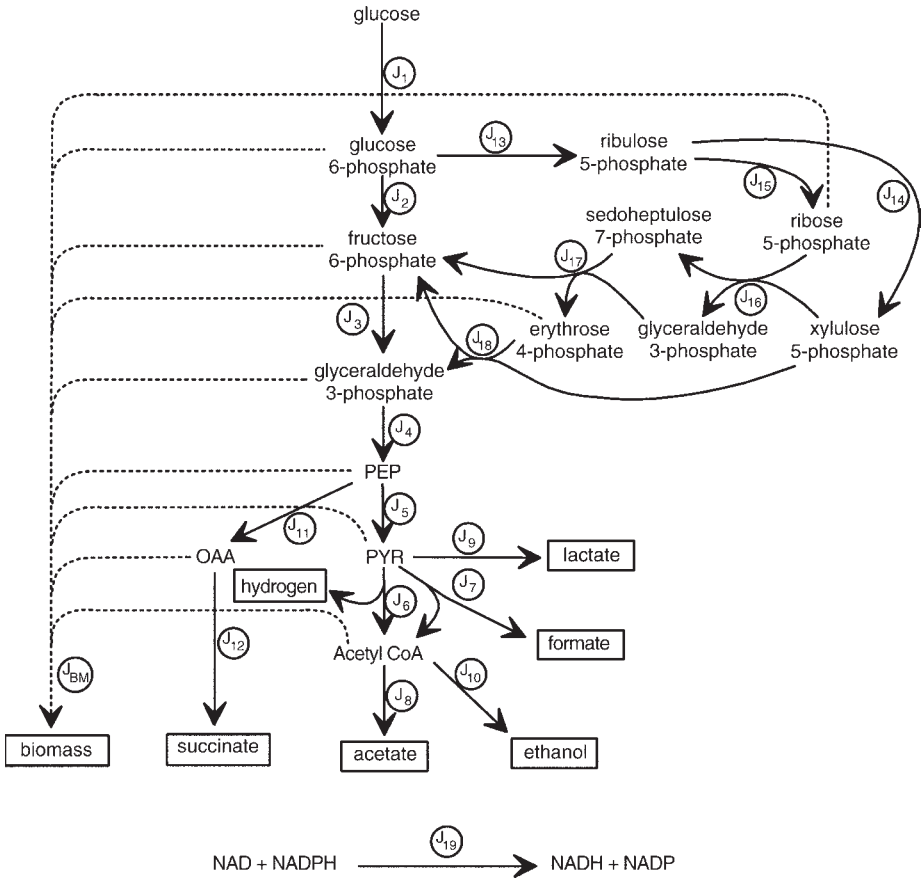


Fig. 1. Fermentative pathways of *C. thermosuccinogenes* DSM 5809. Dotted lines indicate contribution of metabolites to biomass synthesis. PYR, pyruvate; OAA, oxaloacetate.

of lactose uptake, and a high lactate concentration caused accumulation of phosphoenolpyruvate (PEP) and starvation *(22)*. Insertion of genes coding for pyruvate decarboxylase and alcohol dehydrogenase also affected intracellular metabolic flux in *Escherichia coli* grown anaerobically on glucose *(17)*.

The fermentative pathways of *C. thermosuccinogenes* utilizing glucose have recently been established *(23)*. From PEP, the pathway network consists of five branches (Fig. 1) leading to the production of succinate, acetate, lactate, ethanol, formate, and hydrogen. The pathways leading to production of succinate, ethanol, hydrogen, and lactate each involve the oxidation of excess reducing equivalents (NAD[P]H). Acetate production occurs with the formation of one adenosine triphosphate (ATP). Although the fermentative pathways have been elucidated, and redox potential is known to affect product distributions with *C. thermosuccinogenes (24)*, the effect of environmental variables such as pH and redox potential on metabolic flux distributions remains unknown. The objectives of the present study were to investigate *C. thermosuccinogenes* fermentations in response to several

controlled values of pH (6.50–7.25) and culture redox potential (–225 to –310 mV). Metabolic flux analysis was used to elucidate the effect of these environmental variables on carbon flux distributions in *C. thermosuccinogenes*.

## Materials and Methods

### Strain and Medium

*C. thermosuccinogenes* strain DSM 5809 was routinely cultivated at a pH of 7.2 using 5 g/L of glucose as a carbon source in a modified basal medium with the following composition *(8)*: 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 $KH_2PO_4$, 0.1 g/L of $Na_2SO_4$, 0.2 g/L of $Na_2HPO_4$, 2.5 g/L of $NaHCO_3$, 0.15 g/L of $Na_2S \cdot 9H_2O$, 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 B12, 0.05 mg/L of *p*-aminobenzoic acid, 0.05 mg/L of lipoic acid, 1 mg/L of resazurin, 1 g/L of yeast extract, and 0.03 g/L of casamino acids.

### Fermentations

All batch fermentations were conducted at 58°C in 2-L fermentors with 5 g/L of glucose. In a first set of experiments, culture redox potential was controlled at –275 mV during the fermentation, and pH was controlled at one of four values: 6.50, 6.75, 7.00, or 7.25. In a second set of experiments, pH was controlled at 7.25, and culture redox potential was controlled at one of four values: –225, –250, –275, or –310 mV. Redox potential was monitored online with an ORP sensor and transmitter (Ingold; Mettler-Toledo, Wilmington, MA). Redox potential was also measured offline with a platinum electrode with calomel reference electrode (Accumet; Fisher, Pittsburgh, PA). The pH was controlled with 2 *M* $Na_2CO_3$. For redox control, a solution of 35 g/L of $Na_2S \cdot 10H_2O$ was added either manually or automatically with a peristaltic pump (Scilog, Madison, WI). The headspace of the fermentors was maintained anaerobically by sparging 95 mL/min of 15% oxygen-free carbon dioxide in nitrogen.

### Analyses

A Shimadzu HIC-6A ion chromatography system (Shimadzu, Columbia, MD) was used to analyze glucose, organic acids, and ethanol as previously described *(25)*. 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. Two or three fermentations were completed for each pH and redox potential studied. Statistical analyses of data were accomplished with SAS using paired *t*-tests

*(26)*, and $\alpha = 0.05$ was used as the criterion for significance. To calculate the carbon balance, the Gottschalk *(27)* method was used, and the biomass molecular weight was taken to be 26 g/mol *(28)*.

### *Metabolic Flux Analysis*

The methodology followed has been previously described in detail *(17,19,29)*. Mass balance equations were formulated from the biochemical network of *C. thermosuccinogenes* shown in Fig. 1 (see equations in Appendix). Because *C. thermosuccinogenes* is able to grow on pentose sugars *(8)*, the pentose phosphate pathway (PPP) was included in the biochemical network. Furthermore, a PEP-dependent phosphotransferase system (PTS) was not included in the current pathway network because studies with *Clostridium paradoxum* demonstrated the absence of a PEP-dependent PTS *(30)*, and, indeed, no thermophilic anaerobes are known to have a PEP-dependent PTS *(31)*.

The biochemical network for *C. thermosuccinogenes* consisted of 20 fluxes and 14 mass balance equations and was well poised with a condition number of 15 *(32)*. The stoichiometric coefficients of the metabolites necessary to produce monomers for cell building blocks were adapted for growth in rich media *(33)*. Seven of the fluxes ($J_1$, $J_7$, $J_8$, $J_9$, $J_{10}$, $J_{12}$, and $J_{BM}$) were directly calculated from experimental data by dividing the net change in concentration of the metabolite during a 2-h period of the exponential phase (data at 30-min intervals) by the time of duration of that phase. These rates were converted to millimoles/liter·hour and then divided by the mean biomass during that phase. Since 7 fluxes were determined from the experimental data, the system reduced to 13 unknown fluxes and 14 mass balance equations; hence, the system was overdetermined. The least-square estimates for both the measured and unmeasured fluxes were determined using a previous method *(34)*.

To understand how the environmental variables affect carbon flux, flux partitioning at key branch points, or nodes, was studied. Such nodal analysis was accomplished at a specific node by scaling the carbon flux entering that node to 100. Each scaled flux exiting the specific node therefore represents the fraction of the incoming carbon flux exiting by that route. Flux partitioning provides a means to observe how multiple enzymes compete with a single substrate under a set of environmental conditions.

## Results

### *Influence of pH on Metabolism*

To investigate the effect of pH on product formation and intracellular carbon flux distribution, fermentations were conducted at a controlled redox potential of –275 mV and at four different controlled levels of pH: 6.50, 6.75, 7.00, and 7.25. Table 1 summarizes the product yields, specific growth rates, and specific glucose consumption rates at these different pH levels. The yield of succinate (grams/gram) increased significantly from

Table 1
Product Yields (g of product formed/g of glucose utilized), Specific Growth Rates, and Specific Glucose Consumption Rates from *C. thermosuccinogenes* Fermentations Using 5 g/L of Glucose as Carbon Source at Four Different Controlled pH Values and at CRP of –275 mV

| | Controlled pH[a] | | | |
|---|---|---|---|---|
| Parameter | 6.50 | 6.75 | 7.00 | 7.25 |
| Product yield (g/g) | | | | |
| Succinate | 0.12 (0.01) | 0.25 (0.03) | 0.35 (0.03) | 0.54 (0.01) |
| Acetate | 0.10 (0.00) | 0.18 (0.00) | 0.23 (0.02) | 0.22 (0.01) |
| Lactate | 0.73 (0.02) | 0.28 (0.02) | 0.21 (0.00) | 0.00 (0.00) |
| Formate | 0.02 (0.00) | 0.07 (0.00) | 0.15 (0.02) | 0.21 (0.00) |
| Ethanol | 0.03 (0.00) | 0.06 (0.01) | 0.04 (0.00) | 0.01 (0.00) |
| Biomass | 0.11 (0.00) | 0.10 (0.00) | 0.11 (0.00) | 0.12 (0.00) |
| Carbon balance | 1.12 | 0.98 | 1.01 | 0.92 |
| Specific growth rate ($h^{-1}$) | 0.20 (0.01) | 0.20 (0.02) | 0.24 (0.01) | 0.21 (0.00) |
| Specific glucose consumption rate (g/[g·h]) | 0.59 (0.01) | 0.54 (0.03) | 0.67 (0.00) | 0.66 (0.03) |

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

0.12 at a controlled pH of 6.50 to 0.54 at a pH of 7.25 ($\alpha = 0.05$). As the pH level of the fermentations was increased, the formate yield also significantly increased (0.02–0.21) whereas lactate yield decreased (0.73–0.00) ($\alpha = 0.05$). The yield of acetate more than doubled between the pH values of 6.50 and 7.00 but remained unchanged between pH 7.00 and 7.25. Ethanol, biomass yields, and specific growth rates appeared to be unaffected by the pH level of the fermentation. Carbon balances ranged from 0.92 to 1.12.

### *Influence of pH on Metabolic Fluxes*

Metabolic flux analysis was used to elucidate the effect of pH on carbon flux in *C. thermosuccinogenes*. In general, negligible flux through the PPP occurred at each of the four levels of pH studied at –275 mV. This result suggests that the cells were able to obtain the cellular precursors ribose 5-phosphate and erythrose 4-phosphate from the small quantity of yeast extract or casamino acids present in the media at this CRP. Individual fluxes varied as a function of pH only after the PEP node in the pathway. Therefore the PEP, pyruvate, and acetyl CoA branch points were the key nodes studied; Table 2 gives the results of these flux-partitioning calculations. Also, to demonstrate the consistency of the flux network, R/O values were calculated. R/O is equal to the sum of the calculated fluxes leading to NAD(P)H formation divided by the sum of the calculated fluxes leading to NAD(P) formation. For these pH studies, the R/O values were very close

Table 2
Effect of pH on Carbon Partitioning at PEP, Pyruvate, and Acetyl CoA Nodes in *C. thermosuccinogenes* Fermentations[a]

| | | Flux at PEP node ($J_4$ = 100) | | Flux at pyruvate node ($J_5$ = 100) | | | Flux at acetyl CoA node ($J_6 + J_7$ = 100) | |
|---|---|---|---|---|---|---|---|---|
| pH | R/O | $J_5$ | $J_{11}$ | $J_6$ | $J_7$ | $J_9$ | $J_8$ | $J_{10}$ |
| 6.50 | 1.03 | 89 | 11 | 19 | 0 | 81 | 96 | 4 |
| 6.75 | 0.98 | 75 | 25 | 36 | 25 | 39 | 72 | 28 |
| 7.00 | 0.99 | 71 | 29 | 25 | 46 | 30 | 75 | 25 |
| 7.25 | 1.00 | 56 | 44 | 2 | 94 | 4 | 89 | 11 |

[a]All 2-L fermentations were carried out at a CRP of –275 mV. The flux entering into each of these nodes was normalized to 100. R/O is equal to the total NAD(P)H formed divided by the total NAD(P) formed according to the flux results.

to 1.0, indicating that the organism maintained a redox balance at these conditions.

At the PEP node, the fraction of carbon flux to oxaloacetate ($J_{11}$) consistently increased with an increase in pH (with a simultaneous decrease in the fraction to pyruvate). In other words, as the *C. thermosuccinogenes* fermentation was carried out at progressively greater controlled levels of pH (but an identical CRP), the cells increasingly sent carbon at the PEP node to oxaloacetate at the expense of the carbon to pyruvate. At the pyruvate node, the fraction of carbon flux to lactate ($J_9$) consistently decreased with an increase in pH from 6.50 to 7.25. By contrast, the fraction of carbon flux to formate ($J_7$) consistently increased. The fraction of flux to acetyl CoA with hydrogen generation ($J_6$) at first increased and then decreased as the pH of a fermentation progressed from a value of 6.50 to 7.25. At the acetyl CoA node, the fraction of the carbon flux to acetate ($J_8$) was highest at pH 6.50, was decreased at pH 6.75 and 7.00, and then increased at pH 7.25. Of course, the carbon flux to ethanol ($J_{10}$) was the remaining fraction.

### *Influence of CRP on Metabolism*

Anaerobes form reduced end products (e.g., lactate) as a means to regenerate NAD necessary for glycolysis. Results obtained showing that end-product distribution was significantly influenced by pH suggest that NADH/NAD may also influence end-product formation and flux partitioning. Since intracellular NADH is correlated with CRP *(35)*, we investigated the effect of CRP on product formation and intracellular carbon flux distribution. Fermentations were conducted at pH 7.25 at four controlled CRPs: –225, –250, –275, and –310 mV. Table 3 summarizes the product yields, specific growth rates, and specific glucose consumption rates at the four different values of CRP. Succinate yield (grams/gram) increased significantly from 0.08 at a CRP of –225 mV to 0.44 at –310 mV. Acetate yield was greater at the two low CRPs compared with the two high CRPs. Formate yield was similarly greater at the two low CRPs than at the two high

Table 3
Product Yields (g of product formed/g of glucose utilized), Specific Growth Rates, and Specific Glucose Consumption Rates from *C. thermosuccinogenes* Fermentations Using 5 g/L of Glucose as Carbon Source at Four Different Controlled CRP Values and at Controlled pH of 7.25

| | Controlled CRP[a] | | | |
|---|---|---|---|---|
| Parameter | –225 mV | –250 mV | –275 mV | –310 mV |
| Product yield (g/g) | | | | |
| Succinate | 0.08 (0.03) | 0.13 (0.01) | 0.54 (0.01) | 0.44 (0.03) |
| Acetate | 0.11 (0.02) | 0.15 (0.01) | 0.22 (0.01) | 0.25 (0.00) |
| Lactate | 0.05 (0.02) | 0.13 (0.01) | 0.00 (0.00) | 0.06 (0.03) |
| Formate | 0.05 (0.01) | 0.06 (0.01) | 0.21 (0.00) | 0.22 (0.01) |
| Ethanol | 0.34 (0.06) | 0.25 (0.00) | 0.01 (0.00) | 0.01 (0.00) |
| Biomass | 0.09 (0.00) | 0.09 (0.01) | 0.12 (0.00) | 0.12 (0.01) |
| Carbon balance | 1.04 | 1.05 | 0.92 | 1.05 |
| Specific growth rate ($h^{-1}$) | 0.21 (0.03) | 0.17 (0.03) | 0.21 (0.00) | 0.24 (0.00) |
| Specific glucose consumption rate (g/[g·h]) | 0.52 (0.06) | 0.77 (0.06) | 0.66 (0.03) | 0.61 (0.03) |

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

CRPs. Ethanol yield decreased significantly with a decrease in CRP whereas the biomass yield was greater at lower CRPs. Lactate yield did not follow a trend with a change in CRP. The specific rate of glucose consumption was the greatest at a CRP of –250 mV (0.77 g of glucose/[g of biomass·h]) and lowest at a CRP of –225 mV (0.52 g/[g·h]). The carbon balances for fermentations at these four controlled CRPs ranged between 0.92 and 1.05.

### *Influence of CRP on Metabolic Fluxes*

Metabolic flux analysis was again used to elucidate the effect of redox potential on carbon flux in *C. thermosuccinogenes*; Table 4 gives the R/O values and the flux-partitioning results at the three key nodes. The R/O value was inexplicably low (0.79) at a CRP of –225 mV, suggesting that the rate of NAD(P) formed was substantially greater than the rate of NAD(P)H formed.

At the PEP node, the fraction of the carbon from PEP to oxaloacetate increased with a decrease in CRP. Of course, the fraction of the carbon from PEP to pyruvate correspondingly decreased. At the pyruvate node, the fraction of carbon to acetyl CoA with hydrogen production via pyruvate ferredoxin oxidoreductase ($J_6$) decreased significantly from a CRP of –225 mV to a CRP of –310 mV. Indeed, at the lowest CRP (–310 mV), the cells were calculated to consume hydrogen. Because hydrogen was not present in the gas phase for these fermentations, the cells more likely obtained reducing

Table 4
Effect of CRP on Carbon Partitioning at PEP, Pyruvate, and Acetyl CoA Nodes in *C. thermosuccinogenes* Fermentations[a]

| CRP (mV) | R/O | Flux at PEP node ($J_4$ = 100) | | Flux at pyruvate node ($J_5$ = 100) | | | Flux at acetyl CoA node ($J_6 + J_7$ = 100) | |
|---|---|---|---|---|---|---|---|---|
| | | $J_5$ | $J_{11}$ | $J_6$ | $J_7$ | $J_9$ | $J_8$ | $J_{10}$ |
| –225 | 0.79 | 98 | 2 | 83 | 9 | 8 | 17 | 83 |
| –250 | 0.88 | 82 | 18 | 63 | 16 | 21 | 41 | 59 |
| –275 | 1.00 | 56 | 44 | 2 | 94 | 4 | 89 | 11 |
| –310 | 1.00 | 53 | 47 | –12 | 94 | 18 | 100 | 0 |

[a]All 2-L fermentations were carried out at pH 7.25. The flux entering each of these nodes was normalized to 100. R/O is equal to the total NAD(P)H formed divided by the total NAD(P) formed according to the flux results.

equivalents from some media components (i.e., yeast extract) at this lowest CRP. This possibility was not observed in the calculated R/O value of 1.0. The fraction of carbon to acetyl CoA with formate production via pyruvate formate lyase ($J_7$) increased significantly with this decrease in CRP. The fraction of carbon flux to lactate did not follow a trend. At the acetyl CoA node, the fraction of the flux from acetyl CoA to acetate increased with a decrease in CRP, with the fraction to ethanol decreasing correspondingly.

### *Influence of NADH:NAD Ratio on Metabolism*

Numerous studies have been completed concerning the relationship between the intracellular NADH:NAD ratio and microbial physiology. Several approaches have been used to affect the intracellular NADH:NAD ratio. One approach to influence NADH:NAD has been to culture the cells in a complex medium compared with a minimal medium. For example, a complex medium used to culture *Clostridium cellulolyticum* was shown to have an NADH:NAD ratio of 42–57 compared with 0.29–2.08 obtained on a synthetic medium *(36)*. A second approach to affect the NADH:NAD ratio has been by the degree of reduction in the carbon source. For example, growth of *E. coli* on the more reduced carbon source sorbitol resulted in a greater measured NADH:NAD ratio, greater expression of the *adhE* gene, and greater ethanol production compared to growth on more oxidized carbon sources *(37,38)*. A third approach to affect the NADH:NAD ratio has been by the addition of hydrogen into the fermentor headspace. The addition of hydrogen in the gas phase has been shown to increase the NADH:NAD ratio in *Clostridium thermohydrosulfuricum* with a corresponding increase in ethanol production and decrease in acetate production *(39)*. In facultative organisms, a shift from aerobic to anaerobic conditions is a fourth method to influence the NADH:NAD ratio. For example, altering the growth conditions from aerobic to anaerobic increased the NADH:NAD ratio in *Enterococcus faecalis* NCTC 775 *(40)*. As a fifth approach, the total

Table 5
Product Yields (g of product formed/g of glucose utilized), Specific Growth Rates, and Specific Glucose Consumption Rates from *C. thermosuccinogenes* Fermentations Using 5 g/L of Glucose as Carbon Source with Atmosphere of 85% $N_2$/15% $CO_2$, 85% $H_2$/15% $CO_2$, or 85% $N_2$/15% $CO_2$ Plus 0.4 g/L of Nicotinic Acid[a]

| | Fermentation condition[b] | | |
|---|---|---|---|
| Parameter | 85% $N_2$/15% $CO_2$ | 85% $H_2$/15% $CO_2$ | 85% $N_2$/15% $CO_2$ + 0.4 g/L of nicotinic acid |
| Product yield (g/g) | | | |
| Succinate | 0.25 (0.03) | 0.01 (0.00) | 0.01 (0.00) |
| Acetate | 0.18 (0.00) | 0.14 (0.01) | 0.13 (0.02) |
| Lactate | 0.28 (0.02) | 0.04 (0.00) | 0.06 (0.02) |
| Formate | 0.07 (0.00) | 0.01 (0.00) | 0.00 (0.00) |
| Ethanol | 0.06 (0.01) | 0.33 (0.01) | 0.39 (0.02) |
| Biomass | 0.10 (0.00) | 0.08 (0.00) | 0.10 (0.01) |
| Carbon balance | 0.98 | 1.00 | 1.13 |
| Specific growth rate ($h^{-1}$) | 0.20 (0.02) | 0.16 (0.03) | 0.20 (0.00) |
| Specific glucose consumption rate (g/[g·h]) | 0.54 (0.03) | 0.80 (0.02) | 1.19 (0.05) |

[a]All 2-L fermentations were carried out at a controlled CRP of –275 mV and at pH 6.75.
[b]Standard errors of measurements are indicated in parentheses.

pool of NAD + NADH may be altered, a procedure that has also been shown to influence gene expression under anaerobic conditions. For instance, the addition of 0.4 g/L of nicotinic acid in the media for *E. coli* fermentations resulted in a fourfold increase in the NAD + NADH pool and a doubling of *adhE* gene expression *(38)*. Similarly, the addition of 1.5 mg/L of nicotinic acid in the media caused a fourfold increase in NAD(P) in *E. coli (41)*.

To investigate the influence of the NADH:NAD ratio and the total NAD + NADH pool on metabolic carbon flux in *C. thermosuccinogenes*, two additional sets of fermentations were conducted. In a first set of experiments, *C. thermosuccinogenes* was cultured in an atmosphere of 85% $H_2$/15% $CO_2$. In a separate set of fermentations, 0.4 g/L of nicotinic acid was added to the fermentation media. Each of these fermentations was controlled at a pH of 6.75 and a CRP of –275 mV, and results were compared with those from fermentations conducted previously under an atmosphere of 85% $N_2$/15% $CO_2$ at the same pH and CRP conditions.

Table 5 gives the product yields (grams/gram), specific growth rate, and specific glucose consumption rates of *C. thermosuccinogenes* fermentations having an atmosphere of 85% $H_2$/15% $CO_2$ and with 0.4 g/L of nicotinic acid. Succinate, lactate, and formate yields were significantly lower in fermentations with $H_2$ atmosphere or with the addition of nicotinic acid

Table 6
Effect of Fermentation Conditions on Carbon Partitioning at PEP, Pyruvate, and Acetyl CoA Nodes in *C. thermosuccinogenes* Fermentations[a]

| Condition | R/O | Flux at PEP node ($J_4$ = 100) | | Flux at pyruvate node ($J_5$ = 100) | | | Flux at acetyl CoA node ($J_6 + J_7$ = 100) | |
|---|---|---|---|---|---|---|---|---|
| | | $J_5$ | $J_{11}$ | $J_6$ | $J_7$ | $J_9$ | $J_8$ | $J_{10}$ |
| 85% $N_2$/15% $CO_2$ | 0.98 | 75 | 25 | 36 | 25 | 39 | 72 | 28 |
| 85% $H_2$/15% $CO_2$ | 0.84 | 96 | 4 | 95 | 0 | 5 | 33 | 67 |
| 85% $N_2$/15% $CO_2$ + 0.4 g/L of nicotinic acid | 0.80 | 97 | 3 | 92 | 0 | 8 | 20 | 80 |

[a]Two-liter fermentations were carried out with an atmosphere of 85% $N_2$/15% $CO_2$, 85% $H_2$/15% $CO_2$, or 85% $N_2$/15% $CO_2$ plus 0.4 g/L of nicotinic acid. All fermentations were carried out at a controlled CRP of –275 mV and at pH 6.75. The flux entering each of these nodes was normalized to 100. R/O is equal to the total NAD(P)H formed divided by the total NAD(P) formed according to the flux results.

compared with those with $N_2$ atmosphere. Ethanol yield was significantly greater in the fermentation with 85% $H_2$ or with nicotinic acid. The specific rate of glucose consumption was significantly greater with $H_2$ (0.80 g of glucose/[g of biomass·h]) or with nicotinic acid (1.19 g/[g·h]) compared with fermentations with $N_2$ (0.54 g/[g·h]). Biomass and acetate yields, as well as specific growth rates, were not significantly affected by the atmosphere or by this change in media composition.

### *Influence of NADH:NAD Ratio on Metabolic Fluxes*

Metabolic flux analysis was used to study the effect of an 85% $H_2$ atmosphere or adding 0.4 g/L of nicotinic acid to the media on carbon flux in *C. thermosuccinogenes*. R/O calculations and nodal analyses at three key nodes were again performed; Table 6 gives these results. The R/O values for both the hydrogen atmosphere and the nicotinic acid fermentations were significantly lower than 1.0. At the PEP node, the fraction of carbon flowing from PEP to oxaloacetate ($J_{11}$) was greatly diminished for the fermentations with 85% $H_2$ or 0.4 g/L of nicotinic acid compared with fermentations with 85% $N_2$. Naturally, the fraction of flux from PEP to pyruvate was greater. At the pyruvate node, the fraction of carbon flux to acetyl CoA by pyruvate ferredoxin oxidoreductase ($J_6$) was greater under an 85% $H_2$ atmosphere or 0.4 g/L of nicotinic acid than under an 85% $N_2$ headspace. The fraction of carbon through pyruvate formate lyase and lactate dehydrogenase ($J_7$ and $J_9$, respectively) reduced significantly in fermentations with $H_2$ or nicotinic acid. At the acetyl CoA node, the fraction of carbon flux to acetate reduced significantly when the cells were fermented under a hydrogen atmosphere or with nicotinic acid. Of course, the fraction of carbon flowing to ethanol increased correspondingly.

## Discussion

### *Effect of pH*

We studied batch fermentations of *C. thermosuccinogenes* at four different controlled pH levels and at a CRP of –275 mV. Our results indicate that the product distribution and metabolic fluxes change significantly with external culture pH. Studies with other clostridial species have demonstrated that internal pH decreases with a decrease in culture pH. In both *Clostridium pasteurianum (42)* and *C. acetobutylicum (43)*, the internal pH was maintained 0.4–0.5 pH units above the extracellular pH when the external pH was between 4.0 and 7.0. If *C. thermosuccinogenes* regulates its internal pH in a similar manner, then the external (controlled) pH of *C. thermosuccinogenes* should correlate with its internal pH.

Carbon partitioning at the PEP node between oxaloacetate and pyruvate formation was affected by pH, with lower pH favoring oxaloacetate formation. This result indicates that the competition between the enzymes mediating these conversions is affected by pH. The enzymes catalyzing

oxaloacetate formation in succinate-producing anaerobes are PEP carboxylase and PEP carboxykinase. One difference between these two enzymes lies in the form of the cosubstrate required: PEP carboxykinase uses dissolved $CO_2$ as a cosubstrate whereas PEP carboxylase requires the bicarbonate ion ($HCO_3^-$) *(44)*. Of course, pH plays an important role in the equilibrium between $CO_2$ and $HCO_3^-$, with a pH between 6.0 and 7.5 increasingly favoring bicarbonate *(45)*. Specifically, at the lowest studied pH of 6.50, approx 42% of the species would be $CO_2$, whereas at pH 7.25 about 11% of the species would be $CO_2$. With a fixed gas composition in an open system, the dissolved concentration of bicarbonate would have been about six times greater at pH 7.25 than at pH 6.50. Although 15% $CO_2$ was used for sparging, the gas flow rate was only 0.05 vvm and concentrated $Na_2CO_3$ was used to control pH, so that the dissolved $CO_2$/bicarbonate concentrations may have been close to saturation for an atmosphere of pure $CO_2$. The actual solubility of carbonate species was not calculated nor was it determined for the complex media at the elevated temperature, nor could the intracellular concentration of $CO_2$ be estimated.

*C. thermosuccinogenes* has previously been shown to have PEP carboxylase activity but no PEP carboxykinase activity *(23)*. The increasing carbon partition toward oxaloacetate (and on to succinate) with increasing pH correlates with the greater availability of bicarbonate in the media. Interestingly, in contrast to *C. thermosuccinogenes*, the succinate-producing anaerobe *A. succiniciproducens* mediates PEP carboxylation principally via PEP carboxykinase and generates more succinate at pH 6.2 than at pH 7.2 *(10)*. Other factors may cause oxaloacetate formation to be preferred at lower pH in *C. thermosuccinogenes*. For example, the optimal PEP carboxylase activity may occur at a pH >7.5, as has been shown for the thermophilic bacteria *Rhodothermus obamensis (46)*. If such a pH/activity maximum is similar to this enzyme in *C. thermosuccinogenes*, then lower pH levels could contribute to reduced oxaloacetate generation compared to pyruvate generation.

Partitioning at the pyruvate node was also dramatically affected by pH. *C. thermosuccinogenes* underwent a metabolic shift from almost complete lactate synthesis at pH 6.50 to formate synthesis at pH 7.25. Principally three enzymes compete for pyruvate in *C. thermosuccinogenes*: lactate dehydrogenase (LDH), pyruvate formate lyase (PFL), and pyruvate ferredoxin oxidoreductase (PFO) *(23)*. Studies with other organisms also indicate that relatively low culture pH favors lactate formation. With *C. thermohydrosulfuricum* the optimal pH for LDH activity was 6.0; however, LDH activity decreased rapidly with increasing pH with no activity detected at pH 8.0 *(47)*. Also, the lactate yield (moles/mole) was greater from *E. coli* maintained at pH 6.2 (0.84) than at pH 7.8 (0.62) *(48)*, and higher specific rates of lactate formation in *Enterococcus faecalis* NCTC 775 occurred at pH 5.5 compared with pH 8.5 *(49)*. Our results similarly indicate that lactate formation is progressively favored over acetyl CoA formation (via either PFL or PFO) with lower values of media pH. In contrast to

LDH activity, several studies have demonstrated decreased PFL activity with lowered pH. For example, several strains of *E. coli (48)* yielded (moles/mole) much greater formate at pH 7.8 (0.86) than at pH 6.2 (0.02), although this organism shows formate hydrogenase lyase activity whereas *C. thermosuccinogenes* does not *(23)*. In *C. butyricum*, greater PFL activity was observed at pH 7.5 than at lower pH values *(50)*. Formate production and PFL activity in *E. faecalis* NCTC 775 were similarly greater at pH 7.5 than at pH 5.5 *(49)*.

The third route for carbon flux to exit the pyruvate node is to the formation of acetyl CoA (and hydrogen) via PFO *(23)*. PFO must compete with LDH and PFL for their mutual substrate pyruvate. The results indicate that PFO is outcompeted by LDH at the lowest pH studied (6.50) and by PFL at the greatest pH studied (7.25). At the intermediate pH levels, the greatest partitioning via PFO was observed.

Partitioning at the acetyl CoA node was moderately influenced by pH. Two sequential enzymes are actually represented by the flux to acetate ($J_8$) in *C. thermosuccinogenes*: phosphotransacetylase and acetate kinase *(23)*. Although the pH-dependent kinetic behavior of these enzymes in *C. thermosuccinogenes* is unknown, these enzymes in other *Clostridium* have been studied. For example, phosphotransacetylase from *Clostridium kluyveri* showed a narrow maximum in activity at pH 7.4 *(51)*. The optimal acetate kinase activity in *C. acetobutylicum* P262 occurred at pH 8.0 with only 30% of activity present at pH 5.5 *(52)*. If these enzymes in *C. thermosuccinogenes* follow similar pH behavior, higher pH values would be expected to favor acetate formation, excluding other influences. Aldehyde dehydrogenase and alcohol dehydrogenase together sequentially constitute $J_{10}$, the flux from acetyl CoA to ethanol. Aldehyde dehydrogenase isolated from *Clostridium beijerinckii* NRRL B592 had a narrow pH optimum between 6.5 and 7.0 *(53)*. Alcohol dehydrogenase obtained from *C. beijerinckii* NRRL B592 showed an optimal pH at 6.0 with a gradual increase in activity to a pH of 9.0 *(54)*. Similar activities of these two dehydrogenases in *C. thermosuccinogenes* would suggest that lower pH values should favor ethanol formation. The results of comparing the relative flux between ethanol and acetate formation in *C. thermosuccinogenes* were consistent with these explanations for the pH values of 6.75, 7.00, and 7.25. However, at the lowest studied pH of 6.50, the fraction of the flux from acetyl CoA to acetate was the greatest while the fraction to ethanol was the least. This result might be owing to the extremely high flux to lactate at this lowest pH. Since ethanol serves as another means (in addition to lactate) for cells to regenerate oxidized cofactors (e.g., NAD), lactate synthesis might be sufficient at a pH of 6.50 to meet this demand for NAD, causing *C. thermosuccinogenes* to favor ATP and acetate formation by some regulatory mechanism at this lowest of the studied pH values.

### *Effect of CRP*

To examine the effect of CRP on metabolic flux distributions, we studied batch fermentations of *C. thermosuccinogenes* at four different controlled

CRP levels and at pH 7.25. Our results indicate that the product distribution and metabolic fluxes also change significantly with CRP.

Carbon partitioning at the PEP node was markedly influenced by CRP, with lower CRP (i.e., more reduced conditions) favoring oxaloacetate formation. The effect of CRP or NADH on the two enzymes in *C. thermosuccinogenes* competing for PEP, PEP carboxylase, and pyuvate kinase is not known. In another study, the PEP carboxylase of *Pseudomonas* MA was activated 50-fold by 0.2 m*M* NADH *(55)*. Since lower CRP leads to greater intracellular NADH *(35)*, if the PEP carboxylase of *C. thermosuccinogenes* is similarly activated by NADH, the observed increase in partitioning to oxaloacetate could be explained by PEP carboxylase activation at lower CRP. However, the maximum succinate yield occurred at a CRP of –275 mV, not –310 mV (Table 3). Three other enzymes beyond PEP carboxylase, two of which involve reduction, are in the path to succinate formation. Reduced CRP also influences other pathways that compete with the succinate branch, so that the relationship is likely to be much more complicated. Of course, flux analyses represent rates and were calculated over a small (2 h) portion of the fermentations whereas yield calculations represent overall conversions without regard to rate and were based on end products.

Carbon partitioning at the pyruvate node was also affected by CRP, with lower CRP favoring formate formation. In other organisms such as *E. coli (56)*, *E. faecalis* NCTC 775 *(49)*, and *Streptococcus faecalis (57)*, PFL activity has been shown to be sensitive to CRP with highly reduced environments favoring its activity. If PFL is similarly affected by CRP in *C. thermosuccinogenes*, then PFL could increasingly outcompete PFO and LDH for their mutual substrate pyruvate as CRP was decreased. Similarly, in *C. acetobutylicum* in vivo hydrogenase activity decreased with increased intracellular NADH and ATP concentrations *(58)*. Greater NADH concentrations expected at low CRPs with *C. thermosuccinogenes* might also inhibit hydrogenase activity. Our results with decreased hydrogen production relative to formate production at lower CRPs are consistent with these explanations. An interesting observation is that flux via PFO decreases remarkably as the environment becomes more reduced, to the point that the calculated flux is in the direction of pyruvate synthesis at –310 mV. This result may be a mere mathematical consequence of the least-square analysis or may indicate an unsteady-state flow during the 2-h interval for which flux calculations were based. The variation in the flux $J_9$ with CRP suggests that lactate formation is principally used by cells for flexibility needed to balance the NADH:NAD ratio owing to other biochemical reactions.

Carbon partitioning at the acetyl CoA was also significantly affected by CRP, with lower acetate formation. In a previous study with *C. acetobutylicum*, acetate kinase activity was observed to increase with an increase in NADH fluorescence *(59)*. Similar acetate kinase activity in *C. thermosuccinogenes* would explain the increased acetate partitioning. Of course, the regulation between acetate and ethanol synthesis is a means for a cell to balance NAD requirements. Since the acetate flux is involved in ATP

generation, the cell may be meeting ATP demand by affecting acetate generation by some regulation mechanism.

### *Effect of NADH:NAD Ratio*

Our results with the replacement of nitrogen with hydrogen in the atmosphere and with the addition of nicotinic acid both resulted in dramatic metabolic shifts. In either case the cells used ethanol synthesis as the primary means to regenerate NAD(P), and succinate, formate, and lactate synthesis was minimal. Increased ethanol production with greater NADH:NAD ratios has been observed in *C. acetobutylicum (14)*, *C. thermohydrosulfuricum (39)*, and *E. coli (38)*. In the present study, the result is particularly noteworthy because reducing CRP led to more succinate at the expense of ethanol, while the addition of $H_2$ or nicotinic acid led to more ethanol at the expense of succinate. If a high expression and activation of alcohol dehydrogenase occurs with *C. thermosuccinogenes*, as has been observed with *E. coli (38)*, then the high ethanol formation observed here may be the result of drawing carbon away from succinate (and lactate) pathways or a result of some regulatory action on genes or enzymes in those pathways. These observations also imply that $H_2$ and nicotinic acid have different effects on the cells than reduced CRP, sufficient to change the flux partitioning at each of the three principal nodes. Evidence that the physiologic state of the organism has changed with the addition of $H_2$ or nicotinic acid is indicated by the reduced R/O values to about 0.80 for these fermentations, values observed elsewhere only in the –225 mV and pH 7.25 fermentations, which similarly showed very low succinate flux and high ethanol flux. Indeed, the flux-partitioning results with $H_2$ or nicotinic acid are all very consistent with the results of the –225 mV and pH 7.25 fermentations (Table 4). Like the fermentations having $H_2$ or nicotinic acid, in this fermentation pyruvate was the preferred product from PEP, PFO was the principal path for pyruvate utilization, and ethanol with NAD(P) regeneration was greatly favored over acetate formation with ATP generation.

A previous study with *C. thermohydrosulfuricum* demonstrated that in the presence of $H_2$ an increase in the NADH:NAD ratio was associated with increased hydrogen production *(39)*. A similar increase in intracellular NADH:NAD ratio in *C. thermosuccinogenes* with hydrogen or nicotinic acid would explain the observed trend in PFO partitioning. In addition, high NADH:NAD ratios in *Lactococcus lactis (60)* and in *C. acetobutylicum (61)* resulted in the inhibition of glyceraldehyde 3-phosphate dehydrogenase, leading to accumulation of glyceraldehyde 3-phosphate and dihydroxyacetone phosphate, compounds that inhibit PFL. If *C. thermosuccinogenes* is similarly regulated, the observed decreased flux through PFL would also be expected. However, increased flux through PFL was observed at the more reduced CRP in fermentations without $H_2$ or nicotinic acid (Table 4).

## Conclusion

A change in the environmental variables of pH and CRP or affecting NADH:NAD by introducing either a hydrogen atmosphere or nicotinic

acid significantly influenced intracellular carbon flux in *C. thermosuccinogenes*. In general, higher pH or decreased CRP resulted in greater generation of succinate, acetate, and formate. The presence of a hydrogen atmosphere in the fermentor headspace or the addition of nicotinic acid into the media resulted in much greater ethanol formation and decreased generation of succinate and formate. Analysis of flux partitioning at the three principal fermentative nodes (PEP, pyruvate, and acetyl CoA) demonstrated that cells modulate the relative activities of enzymes competing for the same substrate in response to environmental conditions. Clearly, control of CRP is an important aspect of conducting anaerobic fermentations. Although this study did not elucidate the actual regulatory mechanisms present in cells, it suggests several avenues for further study to clarify the environmental response of the key enzymes.

## Acknowledgment

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## Appendix

*Embden-Meyerhof-Parnas Pathway*

($J_1$) Glucose + ATP = Glucose 6-phosphate + ADP
($J_2$) Glucose 6-phosphate = Fructose 6-phosphate
($J_3$) Fructose 6-phosphate + ATP = 2 Glyceraldehyde 3-phosphate + ADP
($J_4$) Glyceraldehyde 3-phosphate + ADP + NAD = NADH + ATP + PEP + $H_2O$
($J_5$) PEP + ADP = ATP + Pyruvate

*Pyruvate Dissimilation*

($J_6$) Pyruvate + CoA = Acetyl CoA + $H_2$ + $CO_2$
($J_7$) Pyruvate + CoA = Acetyl CoA + Formate
($J_9$) Pyruvate + NADH = Lactate + NAD

*Acetyl CoA Dissimilation*

($J_8$) Acetyl CoA + ADP = Acetate + CoA + ATP
($J_{10}$) Acetyl CoA + NADH + NADPH = Ethanol + NADP + NAD + CoA

*Succinate Formation*

($J_{11}$) PEP + $CO_2$ = Oxaloacetate
($J_{12}$) Oxaloacetate + 2 NADH = Succinate + 2 NAD + $H_2O$

*Pentose Phosphate Pathway*

($J_{13}$) Glucose 6-phosphate + $H_2O$ + 2 NADP = Ribulose 5-phosphate + $CO_2$ + 2 NADPH
($J_{14}$) Ribulose 5-phosphate = Xylulose 5-phosphate

($J_{15}$) Ribulose 5-phosphate = Ribose 5-phosphate
($J_{16}$) Xylulose 5-phosphate + Ribose 5-phosphate = Glyceraldehyde 3-phosphate + Sedoheptulose 7-phosphate
($J_{17}$) Sedoheptulose 7-phosphate + Glyceraldehyde 3-phosphate = Fructose 6-phosphate + Erythrose 4-phosphate
($J_{18}$) Xylulose 5-phosphate + Erythrose 4-phosphate = Fructose 6-phosphate + Glyceraldehyde 3-phosphate

*Transhydrogenation Reaction*

($J_{19}$) NADPH + NAD = NADH + NADP

*Biomass Synthesis*

($J_{BM}$) 0.0016 Glucose 6-phosphate + 0.0055 Fructose 6-phosphate + 0.0547 Glyceraldehyde 3-phosphate + 0.0028 PEP + 0.0115 Acetyl CoA + 0.0371 Oxaloacetate + 0.0730 Ribose 5-phosphate + 0.7957 ATP = Biomass + 0.7957 ADP

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