RESEARCH ARTICLE

Acetate formation during recombinant protein production in *Escherichia coli* K-12 with an elevated NAD(H) pool

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Abstract

Acetate formation is a disadvantage in the use of *Escherichia coli* for recombinant protein production, and many studies have focused on optimizing fermentation processes or altering metabolism to eliminate acetate accumulation. In this study, *E. coli* MEC697 (MG1655 *nadR nudC mazG*) maintained a larger pool of NAD(H) compared to the wild-type control, and also accumulated lower concentrations of acetate when grown in batch culture on glucose. In steady-state cultures, the elevated total NAD(H) found in MEC697 delayed the threshold dilution rate for acetate formation to a growth rate of 0.27 h⁻¹. Batch and fed-batch processes using MEC697 were examined for the production of β -galactosidase as a model recombinant protein. Fed-batch culture of MEC697/pTrc99A-*lacZ* compared to MG1655/pTrc99A-*lacZ* at a growth rate of 0.22 h⁻¹ showed only a modest increase of protein formation. However, 1 L batch growth of MEC697/pTrc99A-*lacZ* resulted in 50% lower acetate formation compared to MG1655/pTrc99A-*lacZ* and a two-fold increase in recombinant protein production.

KEYWORDS

acetate, batch process, chemostat, NAD(H) pool, β -galactosidase

1 | INTRODUCTION

Escherichia coli is often used for the commercial production of chemicals and recombinant proteins. However, acetate formation in aerobically grown cultures of *E. coli* is undesirable because the chemical inhibits cell growth and protein formation [1,2] and induces stress responses even at a low concentration [3]. Moreover, the formation of acetate constitutes a diversion of carbon that might have contributed to biomass or protein synthesis [4].

Under aerobic batch growth on glucose as the sole carbon source, acetate accumulates as a result of overflow metabolism [5,6]. Comparison of several common *E. coli* strains led to a range of 0.88–5.12 g/L acetate when grown in batch cultures with 20 g/L glucose [7]. Subsequent comprehensive analysis of *E. coli* B compared to *E. coli* K-12 strains

indicates that the former has more active acetate/acetyl-CoA assimilation pathways, particularly the glyoxylate shunt [8,9]. The complete oxidation of 1 mol of glucose via glycolysis and the tricarboxylic acid (TCA) cycle in *E. coli* generates 10 mol of NAD(P)H and 2 mol of FADH₂ [10]. Those reduced cofactors can theoretically be reoxidized within the electron transport chain to maintain a redox balance and generate ATP by oxidative phosphorylation. However, if the capacity to oxidize reduced cofactors is lower than the rate of glucose consumption, cells respond as though they are oxygen limited [11]. Specifically, NADH accumulates [12], and this cofactor acts as an allosteric inhibitor of citrate synthase [13], reducing the flux of acetyl-CoA into the TCA cycle [14]. The redox ratio of NADH/NAD⁺ correlates with acetate overflow in *E. coli*, with a ratio greater than a critical redox ratio marking the onset of acetate formation. Acetate formation can be delayed by reducing the NADH/NAD⁺ ratio, for example, by overexpression of a water-forming NADH oxidase [12]. Acetate is generated from acetyl-CoA mainly via the enzymes phosphotransacetylase and acetate kinase when carbon is directed

Abbreviations: DCW, dry cell weight; PEP, phosphoenolpyruvate; TCA, tricarboxylic acid.

to acetate, although other unknown pathways contribute [8], and acetate re-assimilation through acetyl-CoA synthase is curtailed under conditions of high growth rate [15]. Complete oxidation of acetyl-CoA to CO₂ through the TCA cycle generates 3 mol NAD(P)H/mol acetyl-CoA, conversion of acetyl-CoA to oxaloacetate via the glyoxylate shunt generates 1 mol NAD(P)H/mol acetyl-CoA, while conversion of acetyl-CoA to acetate generates no NAD(P)H. Thus, both acetate formation and anaplerotic pathways such as the glyoxylate shunt are means to modulate the redox balance either by regulatory means or by simple mass action [11,16,17].

Various strategies have been applied to reduce acetate formation, including metabolic engineering [18–20] and process control [21–25]. As noted above, acetate formation commences when the cell growth rate or glucose uptake rate surpasses a threshold [12], and since the oxygen consumption rate also reaches a maximum at this threshold [26], many metabolic engineering and process strategies address the balance between growth rate and oxygen utilization.

Because of the relationship between acetate formation and NAD(H), we hypothesized that curtailing the natural degradation of NAD(H) by the cell would directly impact acetate formation and thus would affect recombinant protein production. An *E. coli* strain (MEC697) with *nadR nudC mazG* gene knockouts associated with NAD(H) degradation pathway elevates the NAD(H) pool during the cell growth [27], and an increase of total NAD(H) leads to a lower NADH/NAD⁺ ratio [28]. In this study we compared the growth and metabolism of MEC697 and wild-type *E. coli* on several carbon sources. A decrease of acetate formation was indeed found during growth on glucose. We furthermore examined MEC697 for the production of β -galactosidase, which is often used as a model recombinant protein [29,30].

2 | MATERIALS AND METHODS

2.1 | Strains and plasmids

Escherichia coli MG1655 and MEC697 (MG1655 *nadR nudC mazG*) were used in this study [27]. The plasmid pTrc99A-*lacZ* expressing β -galactosidase [4] was transformed into each strain. The plasmid was sequenced, and the results for the *lacZ* gene are shown in Supporting Information Figure S1.

2.2 | Growth medium

The defined medium contained (per L): 2.8 g KH₂PO₄, 5.1 g K₂HPO₄, 2.5 g K₂SO₄, 4.38 g NH₄Cl, 25.0 mg Na₂(EDTA)·2H₂O, 0.15 g MgSO₄·7H₂O, 20 mg citric acid, 0.25 mg ZnSO₄·7H₂O, 0.125 mg CuCl₂·2H₂O, 1.25 mg MnSO₄·H₂O, 0.875 mg CoCl₂·6H₂O, 0.06 mg H₃BO₃, 0.25 mg Na₂MoO₄·2H₂O, 5.5 mg FeSO₄·7H₂O, and 20 mg thiamine-HCl. The pH of the medium was adjusted to 7

PRACTICAL APPLICATION

Escherichia coli is often used for the commercial production of chemicals and recombinant proteins. However, acetate formation in aerobically grown cultures of *E. coli* is undesirable because the chemical inhibits cell growth and protein formation and induces stress responses even at a low concentration. This research investigates the relationship between NAD(H) cofactor decomposition, acetate formation, and recombinant protein production. These results will provide guidance for the design of processes for the production of recombinant proteins.

with 30% w/v NaOH. This medium was supplemented with 20 g/L glucose, gluconate, or sorbitol, or with 10 g/L glucose for recombinant protein production. For a fed-batch study, the bioreactor initially contained this defined medium with 2.5 g/L glucose; and a feed solution contained (per L): 250 g glucose, 7 g MgSO₄·7H₂O, 2 mg CoCl₂·6H₂O, 7.85 mg MnCl₂·4H₂O, 0.8 mg CuCl₂·2H₂O, 1.67 mg H₃BO₃, 4 mg Na₂MoO₄·2H₂O, 21 mg FeSO₄·7H₂O, and 4.5 mg EDTA. Cultures using strains harboring the pTrc99A-*lacZ* plasmid contained 50 mg ampicillin/L.

2.3 | Batch processes

Shake flask cultures (50 mL) grown to an OD of 3 (600 nm) in defined medium were used to inoculate a 2.5 L bioreactor (Bioflo 2000, New Brunswick Scientific, Edison, NJ) containing 1.3 L of defined medium to compare carbon sources or 1 L for β -galactosidase production. In both cases, agitation was maintained at 400 rpm, and temperature was 37°C. Air flow rate was controlled at 1 L/min with oxygen mixed as necessary to ensure dissolved oxygen (DO) concentration above 40% of saturation. The pH was maintained at 7.0 with automatic addition of 15% KOH/5% NH₄OH. Additionally, cultures for β -galactosidase production were induced with 0.75 mM IPTG when the OD reached 0.5.

2.4 | Steady-state processes

Carbon-limited chemostats were initiated in batch mode in the 2.5 L bioreactor containing 1 L of defined medium with 5 g/L glucose. When the OD reached 4, the continuous process commenced using a selected constant dilution rate with a feed of 10 g/L glucose. A steady-state condition was assumed after five residence times, at which time the oxygen and CO₂ concentrations in the effluent gas remained unchanged. Five dilution rates were examined for both MG1655 (0.10–0.27 h^{−1}) and MEC697 (0.10–0.37 h^{−1}). During the entire process, the pH was maintained at 7.0 with 15% KOH/5% NH₄OH, the

temperature at 37°C, and the agitation at 400 rpm. An airflow rate of 1.0 L/min using mass flow controllers (Unit Instruments, Orange, CA) ensured the DO concentration remained above 40% of saturation.

2.5 | Fed-batch processes

Fed-batch processes were carried out in the 2.5 L bioreactor (Bioflo 310, New Brunswick Scientific, Edison, NJ) with 0.8 L initial volume. When the initial glucose was nearly exhausted under batch conditions, the fed-batch mode was commenced by exponentially feeding the medium so that cell growth was at a constant specific rate of 0.22 h⁻¹. The pH was maintained at 7.0 with 15% NH₄OH, the temperature at 37°C, and the agitation at 400 rpm. Air and oxygen were mixed as necessary at 1.0–1.5 liter/min flow rate to maintain a DO concentration above 40% of saturation.

2.6 | Quantification of NAD⁺/NADH and pyruvate/phosphoenolpyruvate

NAD⁺ and NADH concentrations were measured by a cycling enzymatic method [31], where alcohol dehydrogenase uses NAD⁺ to oxidize ethanol into acetaldehyde, and NAD⁺ or NADH transfers the reducing equivalents from ethanol ultimately to 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, which is measured at 570 nm.

Cell extracts for intracellular pyruvate and phosphoenolpyruvate (PEP) measurements were prepared by a modified perchloric acid method [32]. Briefly, 10 mL culture was promptly combined with 30 mL of 60% v/v methanol (–50°C), and then centrifuged (10 300 × g for 5 min at –20°C). The pellet was resuspended in 2 mL of 35% w/v HClO₄ (–25°C). After 10 min in ice, the HClO₄ treated samples were centrifuged (42 000 × g for 30 min at 4°C) and neutralized with 5 M K₂CO₃ to pH 7.0. After centrifugation (42 000 × g for 10 min at 4°C), the supernatant was assayed enzymatically to determine the concentration of pyruvate or PEP. For pyruvate measurement, the reaction mixture contained 50 mM TEA/EDTA buffer (adjusted with 20% NaOH to pH 7.6), 12 mM MgSO₄, 44 mM KCl, 1 mM ADP, 0.3 mM NADH, and 50 U/mL lactate dehydrogenase (Sigma-Aldrich, St. Louis, MO). For PEP measurement, 50 U/mL pyruvate kinase was also added (Sigma-Aldrich, St. Louis, MO). The rate of decline of NADH measured at 420 nm is proportional to the quantity of pyruvate or PEP.

2.7 | Enzyme activity

In this study, a whole cell measurement was used to measure the activity of β -galactosidase [33]. Briefly, 100 μ L of a cell sample was added into 900 μ L of 0.1 M sodium phosphate buffer (pH 7.0), 10 mM KCl, 10 mM MgSO₄, 0.9 μ M SDS, 0.3 mM β -mercaptoethanol, and 0.6 mM chloroform. After incubation at 30°C for 5 min, the measurement was initiated

by adding 200 μ L of 13.2 mM ortho-nitrophenyl- β -D-galactoside, while 500 μ L of 1 M Na₂CO₃ terminated the reaction. One unit of β -galactosidase was defined as the amount of enzyme to generate 1 μ mol of o-nitrophenol per min at 30°C.

2.8 | Analytical methods

The OD at 600 nm was used to monitor cell growth (UV-650 spectrophotometer, Beckman Instruments, San Jose, CA). LC with a refractive index detector and a Coregel 64-H ion-exclusion column (Transgenomic, Glasgow, United Kingdom) with a mobile phase of 4 mM H₂SO₄ was used for analysis of organic chemicals [34]. For dry cell weight (DCW) measurement, three independent 10 mL samples were centrifuged (8400 × g for 10 min), and the cell pellets washed with 30 mL deionized (DI) water three times then dried at 60°C for 24 h. Oxygen uptake rate and CO₂ evolution rate were calculated by measuring the influent and effluent concentrations of oxygen and CO₂ (Ultramat 23 gas analyzer, Siemens, Germany).

3 | RESULTS

3.1 | The growth of *E. coli* MG1655 and MEC697 on various carbon sources

In a previous study, *E. coli* MEC697 having knockouts in three genes associated with NAD(H) degradation (MG1655 *nadR mudC mazG*) showed an increased intracellular pool of NAD(H) [27]. Since the use of carbon sources with different oxidation states affects the intracellular NAD(H) pool (San et al., 2002), we examined the growth, acetate and NAD(H) (i.e. NAD⁺ plus NADH) for MEC697 and wild-type strain MG1655, each grown on 20 g/L gluconate, glucose, or sorbitol as the sole carbon source using controlled batch conditions.

With 20 g/L glucose as the carbon source, MG1655 attained an OD of 15.8 (6.5 g DCW/L) and 2.1 g/L acetate in 10.3 h with a specific growth rate of 0.53 h⁻¹ (Supporting Information Figure S2, Table 1). During the exponential growth phase, MG1655 maintained a total NAD(H) of 4.6 μ mol/g DCW. For MEC697 grown on glucose, an OD of 16.3 and 1.1 g/L acetate were obtained in 11 h with a specific growth rate of 0.60 h⁻¹. MEC697 maintained about 6.7 μ mol/g DCW NAD(H) (Supporting Information Figure S3).

Using gluconate as carbon source, the average NAD(H) for MG1655 was 11.6 μ mol/g DCW, and ultimately 2.5 g/L acetate accumulated in the culture (Supporting Information Figure S4, Table 1). For MEC697, the NAD(H) was 16.8 μ mol/g DCW, and about 2.9 g/L acetate had accumulated when gluconate was depleted (Supporting Information Figure S5). Using sorbitol as carbon source, a greater amount

TABLE 1 Growth rate, acetate formation, biomass formation, and NAD(H) concentrations during batch growth of MG1655 or MEC697 (MG1655 *nadR nudC mazG*) on 20 g/L gluconate, glucose, or sorbitol in controlled bioreactor

	Gluconate		Glucose		Sorbitol	
	MG1655	MEC697	MG1655	MEC697	MG1655	MEC697
Acetate (g/L)	2.5 ± 0.5	2.9 ± 0.0	2.2 ± 0.1	1.1 ± 0.1	0.11 ± 0.07	0.12 ± 0.01
Biomass (g/L)	5.3	4.8	6.5	6.7	6.6	6.6
Total NAD(H) (μmol/g DCW)	11.6 ± 0.5	16.8 ± 0.3	4.6 ± 0.4	8.2 ± 0.5	12.4 ± 1.3	18.2 ± 0.6
NAD ⁺ /NADH	1.9	1.9	3.5	3.9	1.1	1.1
Specific growth rate (h ⁻¹)	0.74	0.66	0.53	0.60	0.45	0.52
Biomass yield (g/g)	0.27	0.25	0.31	0.33	0.31	0.31

of NAD(H) was also observed during growth of MEC697: 12.4 μmol/g DCW for MG1655 compared to 18.2 μmol/g DCW for MEC697 (Supporting Information Figures S6 and S7). For both strains the acetate accumulation was very low: 0.11–0.12 g/L.

For each of these three carbon sources, MEC697 exhibited a greater total NAD(H) concentration than MG1655 during the exponential growth phase (Table 1). The culture grown on gluconate led both strains to accumulate more acetate than cultures grown on glucose or sorbitol. Although acetate accumulation from glucose was twice as great in MG1655 as in MEC697, no significant difference between acetate formation was observed for these two strains when grown on gluconate or sorbitol.

3.2 | The steady-state growth of MG1655 and MEC697 on glucose

A chemostat permits microbial growth to achieve a constant nutrient-limited growth rate, and therefore a constant specific glucose consumption rate. Such a system allows a comparatively precise analysis of metabolism. Previous research found that acetate formation correlates with specific glucose consumption rate: acetate formation (“overflow”) commenced only when the glucose consumption surpassed a threshold rate which itself correlated with NAD(H) [12]. To examine such overflow metabolism when NAD(H) degradation pathways were knocked out, we compared MG1655 and MEC697 under steady-state, glucose-limited growth at different dilution rates. As shown in Figure 1, no acetate accumulated when either strain grew at a low growth rate (low glucose consumption rate, q_S). When the glucose consumption rate exceeded a threshold value, acetate accumulation commenced, and this threshold occurred at a specific glucose consumption rate (q_S) of about 0.50 g/g DCW h for MG1655 and about 0.70 g/g DCW h for MEC697. Furthermore, as the specific glucose consumption rate further increased, MEC697 exhibited lower acetate formation. For example, at the dilution rate of 0.27 h⁻¹, MG1655 achieved a steady-state acetate concentration of 1.39 g/L ($q_S = 0.92$ g/g DCW h, $q_A = 0.13$ g/g DCW h), while MEC697 achieved

FIGURE 1 Steady-state physiological profiles of MG1655 and MEC697 (MG1655 *nadR nudC mazG*) on 10 g/L glucose: the biomass yield $Y_{X/S}$ (◆, ◇) and specific acetate formation rate q_A (●, ○) are shown for MG1655 (solid symbols) and MEC697 (open symbols) as functions of the specific glucose consumption rate (q_S)

a steady-state acetate concentration of only 0.11 g/L ($q_S = 0.70$ g/g DCW h, $q_A = 0.01$ g/g DCW h). Acetate accumulation in MG1655 led to a decrease in biomass yield ($Y_{X/S}$) from 0.42 to 0.29 (g DCW/g glucose consumed), while biomass yield for MEC697 was maintained above 0.40 g DCW/g even after acetate accumulated (Figure 1). The specific oxygen consumption rate (q_{O_2}) and CO₂ evolution rate (q_{CO_2}) for MG1655 and MEC697 increased with increasing q_S (or growth rate) (Figure 2), having no significant difference between MG1655 and MEC697. The carbon balance for both strains was 100–110% under all conditions except MG1655 at the highest growth rate (129%). For MG1655, the percentage of the total carbon in biomass (i.e. biomass C/total generated C) was found to be 50, 40, and 27% at the highest dilution rates ($q_S = 0.51$, 0.68, and 0.92 g/gh, respectively), whereas for MEC697, the percentage of carbon found in biomass was largely unchanged at 42, 50, and 53% at the highest dilution rates ($q_S = 0.70$, 0.74, and 0.85 g/gh, respectively). Thus, the onset of acetate formation for MG1655 correlates with a greater decrease in biomass formation than is observed for MEC697.

FIGURE 2 Steady-state respiration for MG1655 and MEC697 (MG1655 *nadR nudC mazG*): the specific oxygen consumption rate q_{O_2} ($\blacktriangle$, $\triangle$) and the specific CO_2 evolution rate q_{CO_2} ($\blacktriangledown$, $\triangledown$) are compared for MG1655 (solid symbols) and MEC697 (open symbols) as functions of the specific glucose consumption rate (q_s)

Since the batch processes revealed a greater NAD(H) concentration in MEC697 than in MG1655, the concentrations of these two cofactors were also measured in the steady-state experiments. At any given value of q_s , the total NAD(H) concentration in MG1655 was 6.5 to 9.0 $\mu\text{mol/g DCW}$ (Figure 3A), while the total NAD(H) concentration in MEC697 was 12.9 to 17.7 $\mu\text{mol/g DCW}$, consistently greater than MG1655 (Figure 3B). Although this same difference between total NAD(H) concentration was observed at low growth rates when acetate did not accumulate in either strain, lower acetate formation was found in the strain with greater NAD(H) availability.

We also measured the steady-state intracellular concentrations of two key glycolytic intermediates in order to identify imbalances that might exist in the two strains as the glucose consumption rate increased (Figure 4). Pyruvate and PEP participate in a large number of biochemical reactions, and a change in the intracellular level of these two important

intermediates could indicate the shift of fluxes in pathways using pyruvate or PEP [12]. For MG1655, acetate accumulation correlated with a sudden elevated concentration of pyruvate relative to PEP (Figure 4A). For MEC697, the steady-state concentrations for pyruvate and PEP did not change as abruptly with increasing glucose consumption (Figure 4B).

3.3 | Recombinant protein production in the exponential fed-batch process

An exponential fed-batch process is an operational mode which provides a constant growth rate while attaining a high cell density cultivation for recombinant protein production [35,36]. In order to compare MG1655 and MEC697 for recombinant protein production, we transformed the two strains with pTrc99A-*lacZ* expressing the model protein β -galactosidase and performed for each strain a single glucose-limited, fed-batch processes designed to maintain a specific growth rate of about 0.22 h^{-1} . After a short initial batch process with 2.5 g/L glucose, the process was switched to fed-batch growth using a feed of 250 g/L glucose. For both MG1655 and MEC697 (Figure 5), the biomass increased exponentially, and the glucose concentration remained at zero. MG1655 reached 53.7 g DCW/L biomass in 14.7 h with a biomass yield of 0.21 g DCW/g glucose consumed, and ultimately generated 302 kU/L β -galactosidase with a yield of 1.21 kU/g glucose consumed (Figure 5A). MEC697 reached 56.1 g DCW/L biomass in 16.6 h with a yield of 0.22 g DCW/g glucose consumed, and a slightly greater β -galactosidase concentration of 344 kU/L with a yield of 1.38 kU/g glucose consumed (Figure 5B). Interestingly, MG1655 did not accumulate more acetate than MEC697 at that moderate growth rate. The fed-batch process with MEC697 with a constant growth rate of 0.22 h^{-1} showed at best only a modest increase in recombinant protein production compared to fed-batch growth of MG1655 at that same growth rate.

FIGURE 3 Intracellular concentrations of NAD⁺ and NADH in MG1655 (A) and MEC697 (MG1655 *nadR nudC mazG*) (B) during steady-state growth. The specific acetate formation rate q_A ($\bullet$) is also shown as function of the specific glucose consumption rate (q_s)

FIGURE 4 Intracellular concentrations of glycolysis metabolites pyruvate and PEP in MG1655 (A) and MEC697 (MG1655 *nadR nudC mazG*) (B) during steady-state growth. The specific acetate formation rate q_A (●) is also shown as function of the specific glucose consumption rate (q_s)

FIGURE 5 Production of β -galactosidase during exponential fed-batch, glucose-limited growth of MG1655/pTrc99A-lacZ (A) and MEC697/pTrc99A-lacZ (B): biomass (●); acetate (○); β -galactosidase (■)

FIGURE 6 Production of β -galactosidase during batch growth of MG1655/pTrc99A-lacZ (A) and MEC697/pTrc99A-lacZ (B) on 10 g/L glucose: glucose (▲); OD (●); acetate (○); β -galactosidase (■). Error bars indicate standard deviation

3.4 | Recombinant protein production in a batch process

We were also interested in whether recombinant protein formation was different in MEC697 compared to MG1655 under maximum growth rate, batch conditions. Therefore, duplicate batch processes using 10 g/L glucose compared MG1655/pTrc99A-lacZ with MEC697/pTrc99A-

lacZ (Figure 6). MG1655/pTrc99A-lacZ reached an OD of 8.8 in 10 h with a yield of 0.33 g DCW/g glucose, and accumulated 1.1 g/L acetate (Figure 6A). In contrast, MEC697/pTrc99A-lacZ achieved an OD of 10.5 in about 13 h with a yield of 0.40 g DCW/g glucose, and accumulated 0.53 g/L acetate (Figure 6B). At the time glucose was exhausted, MG1655/pTrc99A-lacZ generated 34.3 kU β -galactosidase/g DCW with a yield of 3.28 kU/g

glucose consumed, while MEC697 generated 72.4 kU β -galactosidase/g DCW with a yield of 6.90 kU/g glucose (Figure 6). Although MEC697/pTrc99A-*lacZ* required about 30% more time to consuming 10 g/L glucose, less acetate and more biomass were generated using MEC697/pTrc99A-*lacZ*. Moreover, knockouts in the NAD(H) degradation pathway resulted in about twice the formation of a model recombinant protein.

4 | DISCUSSION

Because acetate formation is a challenge for utilizing *E. coli* in recombinant proteins production, many engineering approaches have focused on eliminating acetate to maximize the protein generation [37]. These approaches can be classified in two strategies: process modification or genetic modification.

For process modification, a simple and straightforward approach is a fed-batch process to maintain a low cell growth rate [1,38]. Alternatively, since acetate is an extracellular by-product, directly removing acetate also reduces the inhibitory effects of acetate on cell growth [3,39], though this approach does not prevent carbon being diverted unnecessarily to acetate rather than to biomass. The pH-stat or controlling DO serve as tools to reduce acetate formation [22,40]. In addition to these operational processes, the medium composition impacts the acetate formation. For example, replacing glucose with fructose [19], or supplementing with yeast extract or individual amino acids [41] can decrease acetate and increase protein yield. However, altering medium composition generally complicates downstream processes and comes at a greater cost.

Genetic modification to alter central metabolism in *E. coli* can reduce acetate. The key pathways involved in acetate formation are pyruvate oxidase which converts pyruvate to acetate and phosphotransacetylase/acetate kinase which together convert acetyl-CoA into acetate. Blocking those two pathways is effective in reducing the acetate [42–45], but an increase of pyruvate relative to PEP in the knockout strains leads to a much lower growth rate [46]. Because a high glucose consumption rate is strongly linked to acetate formation, another approach is to curtail glucose uptake. The deletion of *ptsG* and *ptsHI* genes in PEP:sugar phosphotransferase system decreased acetate formation but also significantly reduced specific growth rate [47,48]. Diversion of pyruvate, or its precursor PEP, directly to the TCA cycle can also eliminate the formation of acetate [4,49,50]. Expression of small RNAs, such as GadY and SgrS which respectively regulate acid resistance and sugar uptake, also reduce acetate formation [51–53].

The link between NADH/NAD⁺ and acetate generation has also been established. A greater redox ratio of NADH/NAD⁺ correlates with acetate overflow [12] and removing “excess”

NADH by overexpressing a NADH oxidase in an *arcA* strain eliminated acetate production and increased of recombinant protein production [54]. Overexpressing formate dehydrogenase gene from *Candida boidinii* similarly alters the NADH/NAD⁺ ratio and affects acetate formation under anaerobic growth [55]. Altering this redox ratio by introducing enzymes which directly utilize NADH seems to be a promising way to control acetate overflow. Similarly, an increased pool of those cofactors by overexpression of the *pncB* gene was correlated with a reduction of NADH/NAD⁺ ratio and reduced acetate formation during anaerobic *E. coli* growth [56]. Similarly, expression of hemoglobin increases consumption of NADH, which correlates with decreased acetate formation in batch culture [57]. The relationship between NAD(H) and recombinant protein production is complicated by observations that the NAD(H) pool or cell redox state may affect plasmid stability [58] as well as the global regulation of genes and thus recombinant protein production indirectly [59,60].

In the current study, we examined acetate formation in *E. coli* through increasing the NAD(H) pool. The approach was to lower the rate of NAD(H) turnover by using knockouts in the *nadR*, *nudC*, and *mazG* genes associated with NAD(H) degradation pathways [27]. This strain (MEC697) indeed did have an elevated cofactor pool, and less acetate formation with only a modest impact on the cell growth. The greater biomass formation we observed may simply be the result of reduced carbon shunted to acetate, which also corresponded to an improvement of recombinant protein production. Curtailing NAD(H) degradation pathway did not materially alter the NADH/NAD⁺ ratio but did significantly elevate the total NAD(H) concentration and reduce acetate formation during fully aerobic growth on glucose. Thus, the relationship between NAD(H) and acetate overflow appears to be more complicated than merely a correlation between NADH/NAD⁺ and q_A [12].

Recent analyses strongly implicate the changing proteome demands between generating energy and generating biomass in overflow metabolism, and serve as a unifying theory for the phenomenon [61,62]. Given the intimate relationship between energy biogenesis, the cells’ use of NADH for ATP generation (via oxidative phosphorylation), and the cells’ demand for the reduced cofactor NADPH directly for biomass synthesis, the present study on reducing the turnover of reduced cofactors likely shows one aspect of this complex metabolic and regulatory network.

Metabolite distribution can be influenced by carbon sources according to their oxidation states. Under anaerobic conditions, a more reduced carbon source results in more reduced products such as ethanol, whereas the more oxidative carbon sources yield more acetate [63]. In a comparison of anaerobic growth of *E. coli* on gluconate, glucose, or sorbitol, gluconate yielded the most acetate but the least ethanol, while

sorbitol yielded the least acetate but the most ethanol [64]. Similar observations were also found in either MG1655 or MEC697 when compared these three carbon sources in aerobic batch growth. However, knockouts in NAD(H) degradation genes in MEC697 resulted in greater acetate and lower biomass in the case of gluconate, while no difference in acetate and biomass between MG1655 and MEC697 was observed when sorbitol was the sole carbon source. Only for glucose did reducing the NAD(H) turnover result in less acetate and greater biomass formation, and a change in the NAD^+/NADH ratio (Table 1). Because metabolic regulation differs depending on the specific carbon source, very little comparison can be made with the same strain between carbon sources; the focus of comparison must be with a single carbon source between the two strains. Unsurprisingly, these results demonstrate the use of carbon sources with different oxidation states will change the intracellular environment and impact the metabolite distribution.

The concentration of PEP and pyruvate are important indicators for carbon fluxes in glycolysis. Steady-state pools of PEP and pyruvate increased with increasing glucose uptake rate [56]. An elevated intracellular pyruvate concentration was previously correlated with acetate overflow in wild-type *E. coli* [12] and was also observed in the current study (Figure 4). Pyruvate participates in a wide range of biochemical reactions and may contribute to acetate formation directly [65]. In this study, the triple knockout strain MEC697 minimized pyruvate accumulation as growth rate increased (Figure 4). Lowering pyruvate pool has previously been described as one approach for preventing acetate formation. For example, introducing pyruvate carboxylase which converts pyruvate to oxaloacetate resulted in more carbon flux directly enter in TCA cycle and a 60% reduction in acetate [4,49]. Similarly, diverting pyruvate into another benign product, such as acetolactate and acetoin using acetolactate synthase, led to lower acetate formation [66]. Instead of redirecting pyruvate into the TCA cycle or toward other by-products, the triple knockout *E. coli* allowed a high glucose uptake rate without this increase in the intracellular pyruvate concentration. An alternative approach to lower pyruvate pool is directing more carbon from PEP into oxaloacetate and consequently reduces acetate production. For example, overexpressing PEP carboxylase led to a reduction of 60% of acetate excretion in *E. coli* VJS632 aerobic cultures [50]. In our study, the triple knockout strain MEC697 was not influenced by changes in carbon fluxes resulting from increased glucose uptake rate but maintained a comparatively constant PEP concentration over a wide range of growth rate.

A fed-batch process was designed to differentiate the high cell density growth of MG1655 and MEC697 for the goal of recombinant protein generation. An exponential fed-batch process is a typical approach to prevent acetate formation and accumulate more biomass in carbon-limited cultures [1,35].

These two strains growing at 0.22 h^{-1} did not materially change their acetate and biomass formation: MEC697 and MG1655 were largely indistinguishable at this growth rate, and overflow metabolism was low. A higher fed-batch growth rate might have resulted in a greater difference between the strains. Indeed, batch growth (i.e. at each strain's maximum growth rate) resulted in 50% less acetate and two-fold greater recombinant protein generation in MEC697 compared to MG1655 (Figure 6). The results clearly distinguish between batch and fed-batch processes. Batch growth of *E. coli* is usually not preferred to achieve high cell density because of acetate formation [67,68], because of greater difficulty in oxygen transport (and, at large scale, heat transfer) at high growth rate, and because *E. coli* growth is inhibited when the initial nutrients are present above certain concentrations [69]. Fed-batch culture by maintaining a 1 g/L glucose concentration previously achieved a high cell density as well as a high growth rate, but significant acetate formation also occurred [7].

5 | CONCLUDING REMARKS

The triple deletion of genes associated with NAD(H) degradation increased the intracellular pool of NAD(H). An elevated pool of intracellular NAD(H) correlated with lower acetate formation in *E. coli* during batch growth on glucose as the carbon source. Considering the essential role of NAD(H) in aerobic metabolism, our study provides a new insight for *E. coli* metabolism and for the production of recombinant proteins.

Nomenclature

q_A [g/g DCW·h]	specific acetate formation rate
q_S [g/g DCW·h]	specific substrate uptake rate
$Y_{X/S}$ [g DCW/g]	biomass yield from substrate carbon source

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CONFLICT OF INTEREST

The authors have declared no conflict of interest.

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