

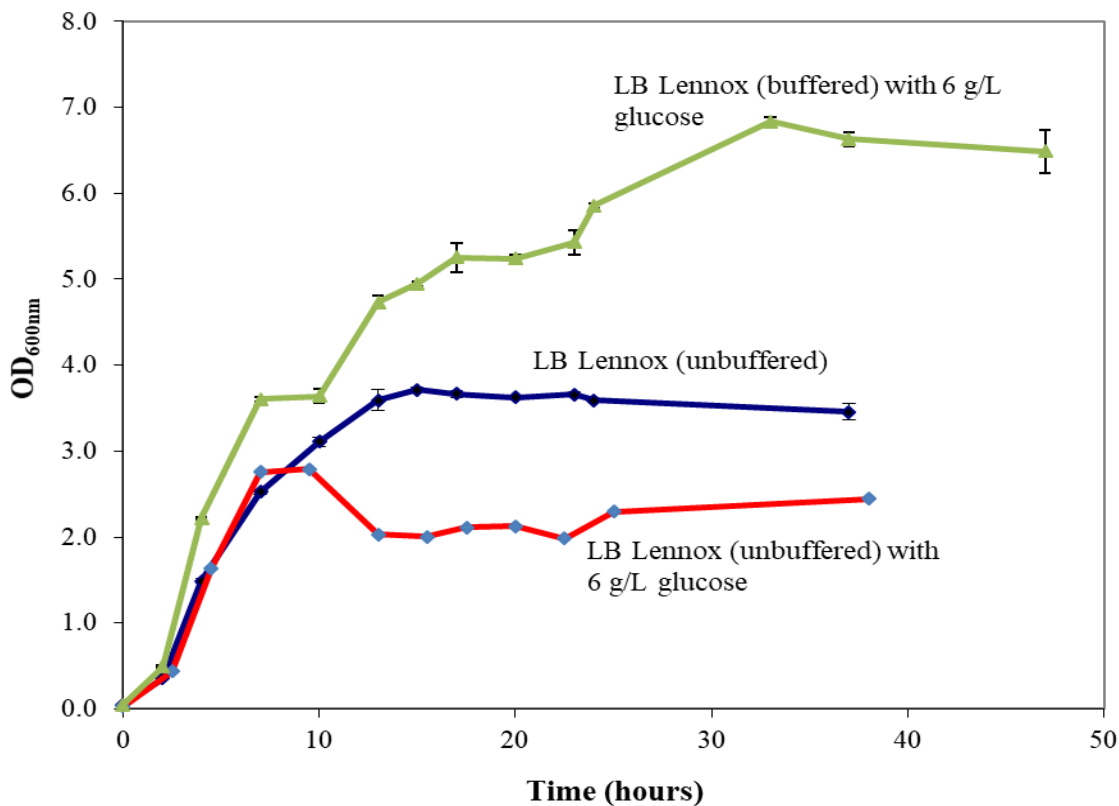
Effect of glucose and ammonium chloride supplementation and phosphate buffer on *Escherichia coli* DH5 α growth in LB Lennox medium

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Graphical abstract



Short description

Cultivation of bacteria in LB Lennox medium often generates insufficient biomass for applied microbiology studies such as biosorption. Thus, supplementation of the medium with glucose is commonly used to improve biomass formation and cell yield. However, this was often met with reduced biomass formation in the case of excess glucose supplementation beyond an optimal glucose concentration. This was primarily due to the secretion of acidic metabolites into the culture broth that depressed broth's pH, which was observed to be unfavourable for biomass formation. Furthermore, metabolism of excess glucose lead to overflow metabolism that generated acetate, the accumulation of which might be detrimental to biomass formation in addition to depressing the culture broth's pH. Thus, this study investigated the effect of glucose and ammonium chloride

supplementation on *Escherichia coli* DH5 α (ATCC 53868) growth in LB Lennox medium, and whether a high capacity phosphate buffer (89 mM phosphate) could prevent low broth pH that reduce biomass formation. Experiment results revealed that a high capacity phosphate buffer could moderate pH decrease and help improve biomass formation of *E. coli* DH5 α in LB Lennox medium supplemented with 2 g/L and 6 g/L glucose. On the other hand, supplementation of LB Lennox medium with 1 g/L and 4 g/L ammonium chloride did not affect growth performance and pH profile of *E. coli* DH5 α at 37 °C; thereby, indicating that LB Lennox medium was not deficient in nitrogen. Finally, no diauxic lag phase was observed during growth of *E. coli* DH5 α in buffered and unbuffered LB Lennox medium with 2 g/L glucose, which indicated that 2 g/L glucose supplementation did not alter the carbon and nitrogen balance of the medium significantly such that a diauxic shift was necessary.

Abstract

LB Lennox medium is commonly used for bacterial cell culture, but the amount of biomass obtained while sufficient for molecular biology and biochemical studies, is insufficient for applied microbiology research. Thus, supplementation of LB Lennox with glucose is commonly used in delivering more nutrients for biomass formation. However, an optimal concentration of glucose exists for each bacterial species beyond which biomass formation would be reduced due to the low culture broth's pH and acetate accumulation that arise from the metabolism of excess glucose. Hence, addition of a phosphate buffer system in LB Lennox medium might moderate pH fluctuation detrimental to biomass formation. Finally, while LB Lennox medium is rich in amino acids, supplementation with an easily assimilable nitrogen source may augment cell growth. Using *Escherichia coli* DH5 α (ATCC 53868) as model organism, this study aimed to understand the effect of glucose and ammonium chloride supplementation on growth performance of the bacterium at 37 °C, and whether a high capacity (89 mM phosphate) buffer system could help reduce pH fluctuation and improve biomass formation. Experiment results revealed that a high capacity phosphate buffer (89 mM phosphate) enabled higher optical density to be obtained in LB Lennox medium supplemented with 2 g/L and 6 g/L glucose. Specifically, maximal optical density of 6.0 was obtained in buffered LB Lennox with 2 g/L glucose compared to 5.0 in unbuffered LB Lennox with 2 g/L glucose. Similarly, 6.8 was the maximal optical density of *E. coli* DH5 α grown in buffered LB Lennox medium with 6 g/L glucose, while 2.8 was obtained in the unbuffered LB Lennox medium with the same glucose concentration. For comparison, the maximal optical density for *E. coli* DH5 α grown in unbuffered LB Lennox medium was 3.4. pH fluctuation was significantly reduced with incorporation of a high capacity phosphate buffer system in LB Lennox medium and correlated with higher biomass formation even for buffered LB Lennox medium without glucose supplementation (maximal optical density of 3.7). On the other hand, supplementation of unbuffered LB Lennox medium with 1 g/L and 4 g/L ammonium chloride did not affect growth performance and pH profile; thereby, indicating that the medium was not deficient in nitrogen. Finally, diauxic lag phase was not observed in growth of *E. coli* DH5 α in LB Lennox medium supplemented with 2 g/L glucose, with and without a phosphate buffer, which indicated that 2 g/L glucose supplementation did not significantly alter the carbon and nitrogen

balance of the medium. Collectively, high capacity phosphate buffer system helped moderate pH fluctuations; thereby, enabling greater biomass formation in LB Lennox medium with 2 g/L and 6 g/L of glucose supplementation. Supplementing LB Lennox medium with ammonium chloride did not alter growth performance and pH profile, which highlighted that the medium is not deficient in nitrogen.

Keywords: growth medium, phosphate buffer, *Escherichia coli*, LB Lennox, glucose, ammonium chloride, sugar deficiency, overflow metabolism, low pH, biomass formation,

Subject areas: bioengineering, biotechnology, biochemistry, cell biology, microbiology,

Highlights

- 1) LB Lennox medium could not support high cell density cultivation of most bacteria; thus, glucose supplementation was commonly used to provide more nutritional building blocks for biomass formation. However, excess glucose supplementation was known to reduce biomass formation due to ensuing low culture broth pH; thus, an optimal glucose concentration exists beyond which glucose supplementation decrease biomass yield.
- 2) Using *Escherichia coli* DH5 α (ATCC 53868) as model organism, the effect of glucose and ammonium chloride supplementation, and whether a high capacity phosphate buffer could moderate pH fluctuation for improving broth's conditions for biomass yield was investigated.
- 3) Experiment results revealed that a high capacity phosphate buffer (89 mM phosphate) was able to moderate pH fluctuations during growth of *E. coli* DH5 α in LB Lennox medium with 2 g/L and 6 g/L glucose, and delivered higher optical density compared to the unbuffered medium supplemented with the same glucose concentrations.
- 4) In unbuffered LB Lennox medium, supplementation with 2 g/L glucose enhanced biomass formation, while 6 g/L glucose supplementation reduced biomass formation.
- 5) More importantly, diauxic lag phase was not observed during growth of *E. coli* DH5 α in buffered and unbuffered LB Lennox medium with 2 g/L glucose, which indicated that 2 g/L glucose did not significantly alter the carbon and nitrogen balance of LB Lennox medium; thereby, negating the need for diauxic shift.
- 6) Finally, supplementation of LB Lennox medium with 1 g/L and 4 g/L ammonium chloride did not alter growth performance and pH profile; thereby, highlighting that the medium was not deficient in nitrogen.
- 7) Collectively, supplementation of glucose of concentration between 2 g/L and 6 g/L in LB Lennox medium with a high capacity phosphate buffer (89 mM phosphate) should help *E. coli* DH5 α achieve greater biomass formation.

Introduction

Glucose is commonly added to growth medium for increasing biomass formation.¹ However, excessive amount of glucose in medium was also shown to reduce biomass formation due possibly to secretion of acidic metabolites from glucose metabolism that depressed the culture broth's pH. Thus, an optimal concentration of glucose exists where biomass formation could be maximized without excessive impact on broth's pH and biomass formation.

Given that catabolism of excess glucose generates acetate, which in excessive concentrations, could depress broth's pH and thus serve as an environmental stressor for cells and negatively impact on biomass formation,^{2 3 4 5} addition of a buffer could possibly reduce the extent in which broth's pH could be depressed; thereby, enabling increased glucose supplementation which delivers more metabolic building blocks for biomass formation. Hence, many commercial growth media such as M9 incorporates a phosphate buffer system for reducing pH fluctuations during cultivation of the cells.

Comprising tryptone, yeast extract and sodium chloride, LB Lennox medium is a digest of proteins and is rich in amino acids, not sugars. Thus, glucose supplementation is the natural way forward for improving biomass formation in cells through the provision of more metabolic building blocks. However, could supplementation of LB Lennox medium with ammonium chloride provide an easily assimilable source of nitrogen that could improve biomass formation in cells?

Although LB Lennox medium is commonly used for the cultivation of *Escherichia coli*, one major problem concerning its use lies in the relative lack of biomass formation during growth of the bacterium in the medium. Specifically, without supplementation, the maximal optical density of *E. coli* DH5 α (ATCC 53868) that could be achieved in the medium is 3.4, which does not yield sufficient biomass for downstream characterization and applied microbiology studies. Thus, one possibility for increasing biomass formation in LB Lennox medium would be glucose supplementation coupled with addition of a phosphate buffer system into the medium for reducing pH fluctuation during growth of bacteria. Additionally, supplementation of ammonium chloride could also provide a source of easy to metabolize nitrogen source which may help improve biomass formation. The objective of this study is thus the use of *E. coli* DH5 α as a model organism for understanding the effect of glucose and ammonium chloride supplementation and addition of a phosphate buffer system on cell growth and biomass formation.

Materials and Methods

Materials

LB Lennox medium was purchased from Difco and used as is. Composition of LB Lennox medium (unbuffered) was [g/L]: Tryptone, 10.0; Yeast extract, 5.0; NaCl, 5.0. Composition of LB Lennox medium (buffered) was [g/L]: Tryptone, 10.0; Yeast extract, 5.0; NaCl, 5.0; K₂HPO₄, 12.54; KH₂PO₄, 2.31.

Growth of Escherichia coli DH5α in liquid medium

Stock cultures of *Escherichia coli* DH5α were prepared in 40% glycerol and stored at -70 °C before use. One glycerol stock culture of *E. coli* DH5α was used in inoculating 100 mL of LB Lennox medium in 250 mL glass conical flask as seed culture. Incubation conditions were 37 °C and 230 rpm rotational shaking in a temperature controlled incubator. After 24 hours of incubation, 1 mL of seed culture was used as inoculum for 100 mL of LB Lennox medium with different concentrations of glucose, ammonium chloride or phosphate buffer in 250 mL glass conical flasks. Incubation conditions were 37 °C and 230 rpm rotational shaking in a temperature controlled incubator (Yih Der LM-570RD, Taiwan). Three biological replicates were performed for each experiment.

Measurement of optical density and pH

At appropriate time points, aliquots were withdrawn from the cultures for optical density and pH measurements. Optical density was measured with a Shimadzu Biospec Mini UV-Visible spectrophotometer at 600 nm, using a quartz cuvette of pathlength 10 mm (volume: 3.5 mL). If optical density exceeded 1, deionized water was used to dilute the samples. pH was measured, without dilution, using an Orion 9156 BNWP pH probe fitted to a Mettler Toledo Delta 320 pH meter.

Results and Discussion

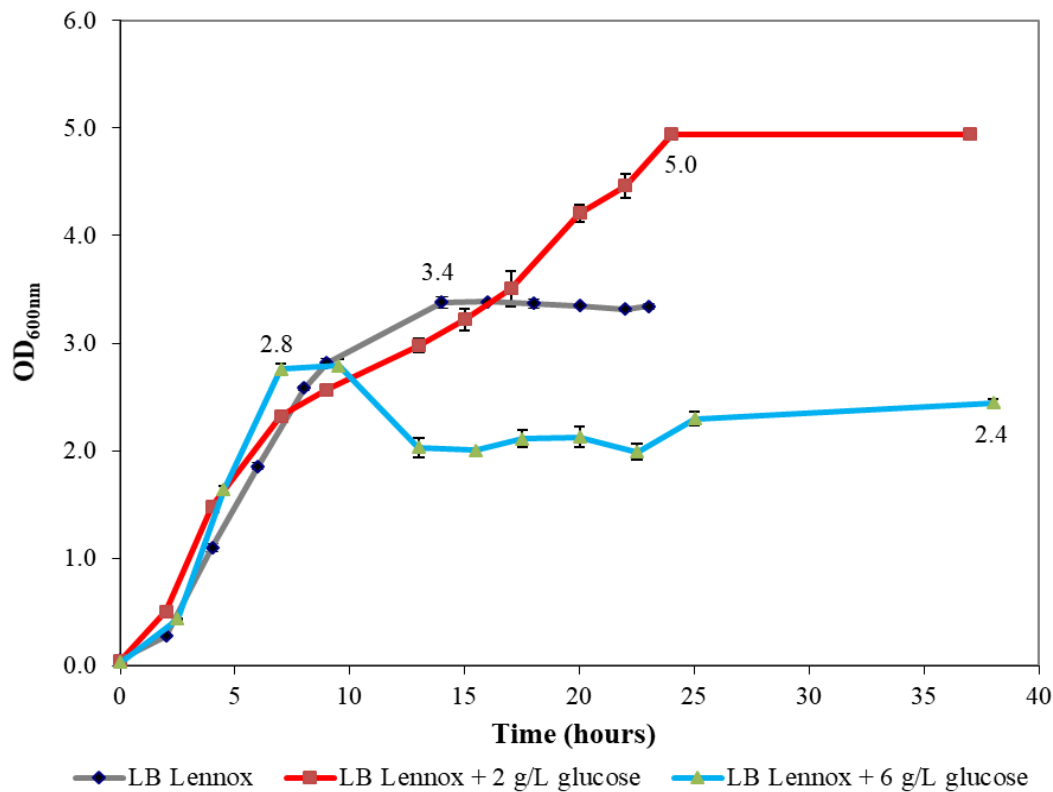


Figure 1a: Growth of *E. coli* DH5 α at 37 °C in LB Lennox medium with different concentrations of glucose supplementation. LB Lennox medium with 2 g/L of glucose yielded the highest biomass formation with no lag phase. On the other hand, supplementation of LB Lennox medium with 6 g/L of glucose reduced biomass formation.

Growth of *E. coli* DH5 α in LB Lennox medium with different concentrations of glucose supplementation resulted in different growth profiles that spoke of the effect of glucose on metabolism and biomass formation (Figure 1a). Specifically, excess glucose (i.e., 6 g/L) resulted in reduced biomass formation due possibly to excess flux in the glycolytic pathway that led to acetate accumulation and depression of broth's pH. Low culture broth's pH is a source of environmental stress that could reduce biomass formation. For example, cultivation of *E. coli* DH5 α in LB Lennox medium with 2 g/L of glucose yielded a maximal optical density of 5.0 with no lag phase during the culture. This highlighted that there was little imbalance in carbon and nitrogen content of the primary carbon source and secondary nutritional source of the medium, which meant that the cells did not need to spend time readjusting metabolism and metabolic pathways for coping with a nutrient source with different carbon and nitrogen content. On the other hand, growth of the bacterium in LB Lennox medium with 6 g/L glucose resulted in depressed biomass formation of optical density 2.8, which was lower than that during growth of *E. coli* DH5 α in LB Lennox medium (optical density of 3.4). Excess glucose was likely a reason for the reduced

biomass formation due to high glycolytic flux that led to acetate accumulation, which depressed the broth's pH and increased environmental stress on the cells.

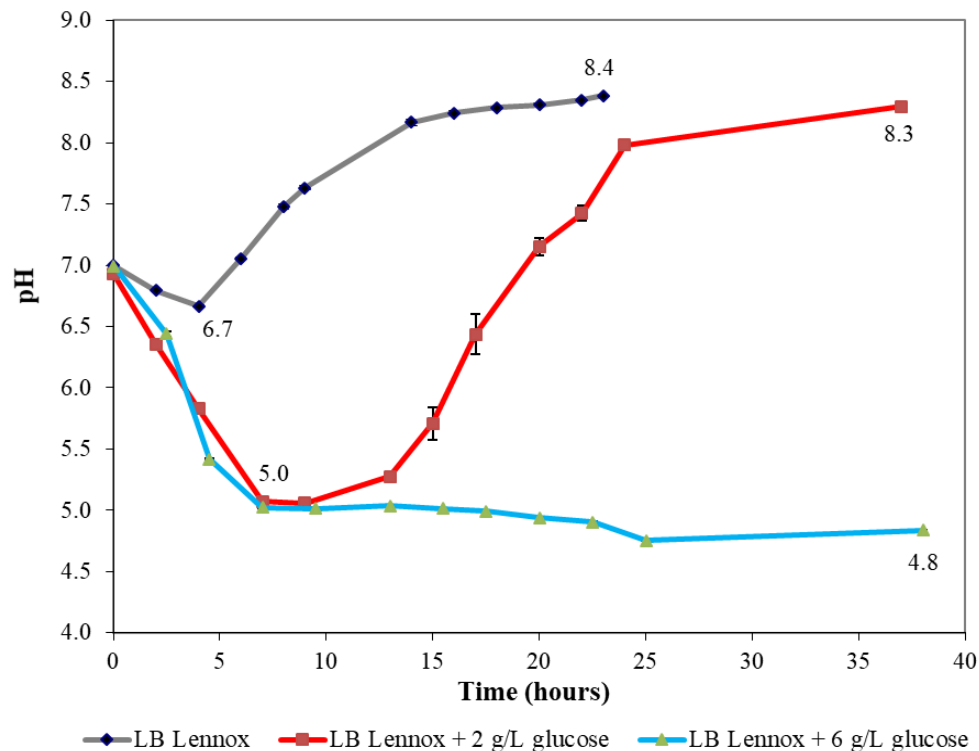


Figure 1b: Variation in culture broth's pH during growth of *E. coli* DH5 α at 37 °C in LB Lennox medium with different concentrations of glucose supplementation. Note that low pH might not be the sole reason for the inability to recover from overflow metabolism.

pH profiles of *E. coli* DH5 α grown in LB Lennox medium supplemented with different concentrations of glucose revealed that excess glucose supplementation such as 6 g/L resulted in reduced biomass formation, and the inability of the bacterium to recover from a low pH environment (Figure 1b). This could be due to the excess acetate generated from overflow metabolism through the central carbon pathway that acted as a source of environmental stress to the cells. On the other hand, observations of an increase in broth's pH after an initial decrease to pH 5.0 during *E. coli* DH5 α growth in LB Lennox medium with 2 g/L glucose suggested that low pH might not be the only trigger for reduced biomass formation in LB Lennox medium with 6 g/L of glucose. Specifically, growth of *E. coli* DH5 α in LB Lennox medium with 2 g/L glucose and 6 g/L glucose resulted in a pH decrease to pH 5.0, but the pH profile of the bacterium in LB Lennox medium with 6 g/L glucose remained at pH ~ 5.0 and resulted in low biomass formation, while that of the bacterium in LB Lennox medium with 2 g/L glucose was able to rebound to a final pH of 8.3 accompanied by biomass formation. Thus, acetate accumulation during induction of overflow metabolism likely generated significant cellular stress that could stop biomass formation. Finally, during growth of *E. coli* DH5 α in LB Lennox medium without glucose supplementation,

pH first decrease from 7.0 to 6.7 followed by a gradual increase to 8.2 at the end of the culture, which indicated that acidic metabolites were first secreted into the medium followed by the secretion of alkaline metabolites that resulted in a pH rise.

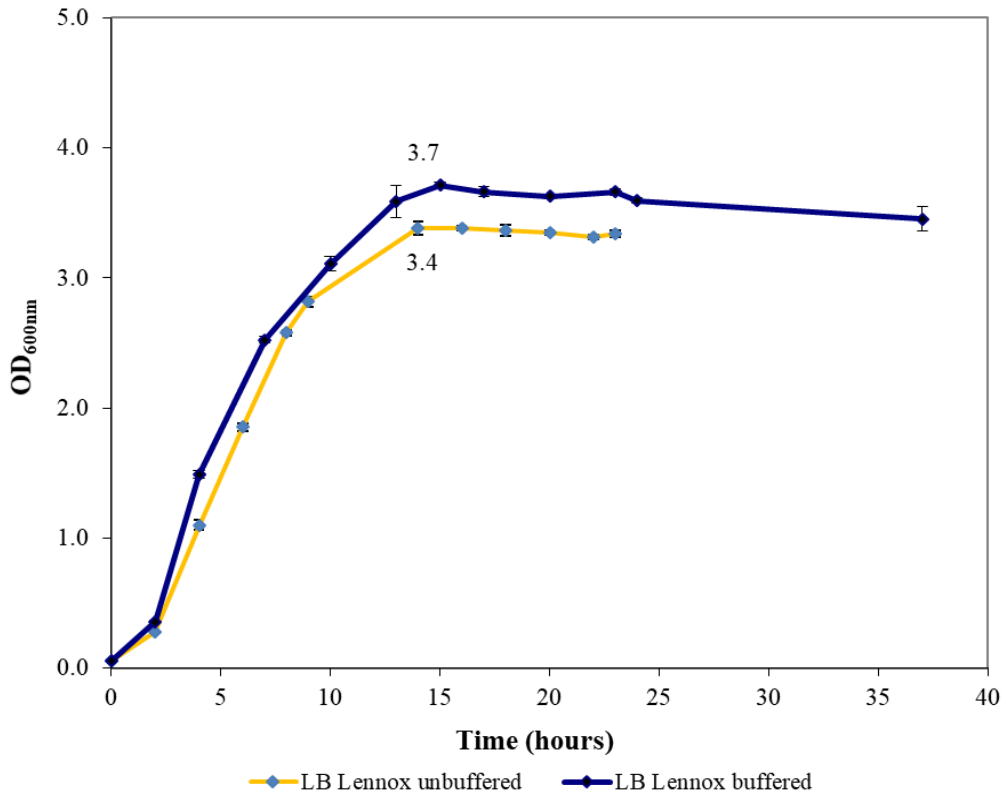


Figure 2a: Growth of *E. coli* DH5α at 37 °C in LB Lennox medium with and without a phosphate buffer of 89 mM phosphate.

Given that low pH could reduce biomass formation, addition of a phosphate buffer system using the salts K_2HPO_4 and KH_2PO_4 would provide LB Lennox medium with a buffering capacity suitable for coping with the pH fluctuations that arise during growth of *E. coli* DH5α in the medium. Experiment results revealed that growth of the bacterium in LB Lennox medium with a buffer system generated slightly higher maximal optical density of 3.7 at about the same time point compared to optical density of 3.4 in LB Lennox medium without a buffer system (Figure 2a). This indicated that pH had some effect on biomass formation, but its effect might not be as critical as that of the amount of acetate accumulation in the culture broth.

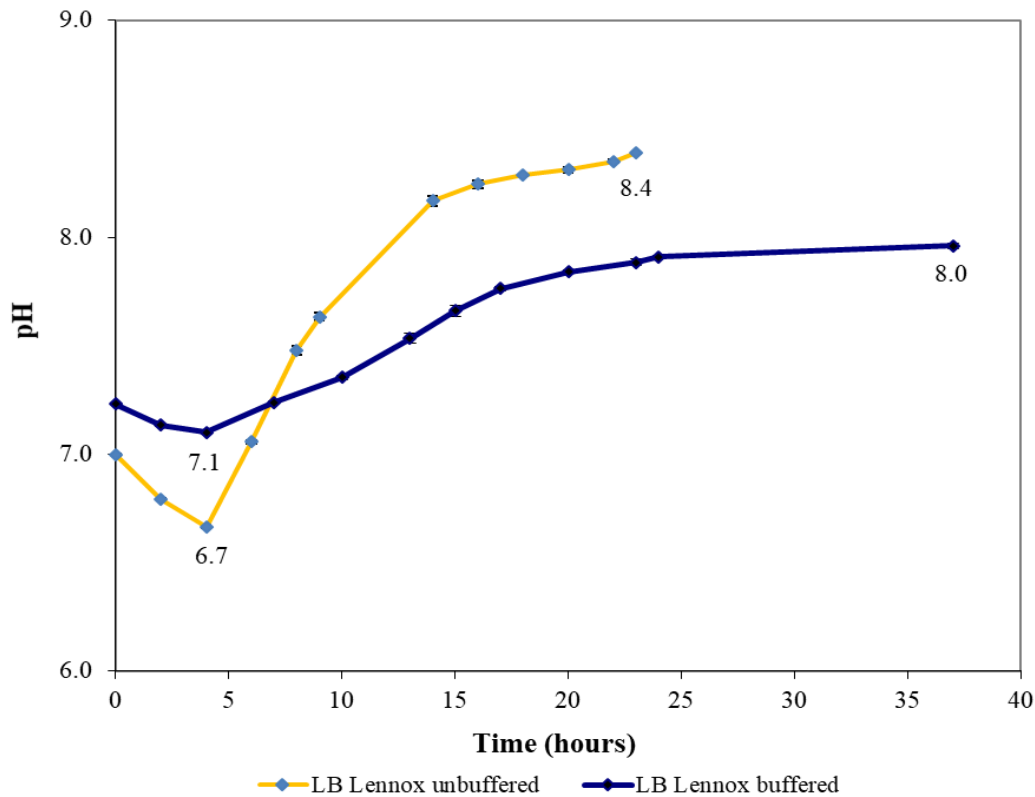


Figure 2b: Variation of culture broth's pH during growth of *E. coli* DH5 α at 37 °C in LB Lennox medium with and without a phosphate buffer system. Note the reduced pH fluctuation that accompanied growth in LB Lennox medium with a buffer system.

Figure 2b revealed that growth of *E. coli* DH5 α in LB Lennox medium with a phosphate buffer system had a smaller pH fluctuation compared to that during growth of the bacterium in unbuffered LB Lennox medium. Specifically, for growth in the unbuffered LB Lennox medium, pH decreased from 7.0 to 6.7, followed by a rise to 8.4 at the end of the culture. On the other hand, pH first decreased from 7.2 to 7.1, followed by a rise to 8.0 during growth of *E. coli* DH5 α in LB Lennox medium with a 89 mM phosphate buffer system. Thus, reduced pH fluctuation did correlate with a slight enhancement in biomass formation.

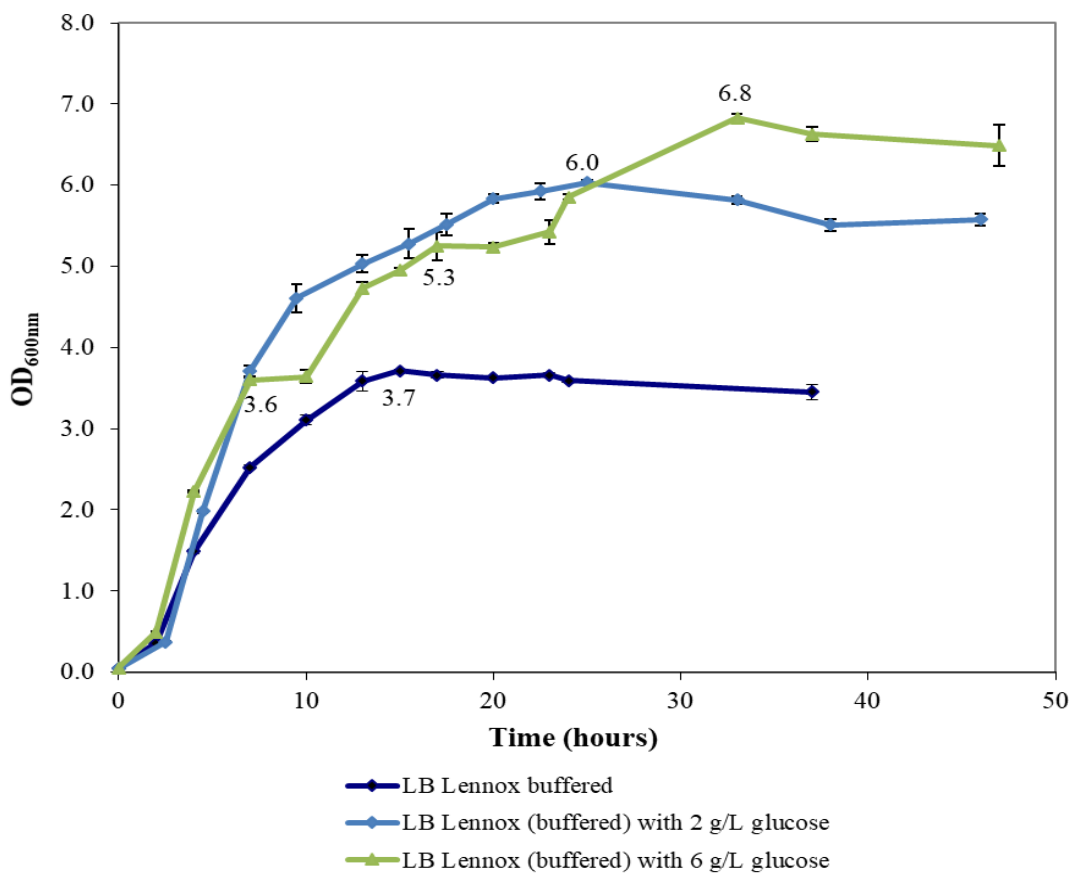


Figure 3a: Growth of *E. coli* DH5 α at 37 °C in LB Lennox (buffered) medium with different concentrations of glucose supplementation. Note that with a phosphate buffer system, 6 g/L of glucose supplementation managed to increase biomass formation; thereby, indicating that reduced pH fluctuation was important for improving biomass formation.

Addition of a high capacity phosphate buffer system into LB Lennox medium improved biomass formation in the case of glucose supplementation of 2 g/L and 6 g/L over an unbuffered LB Lennox medium (Figure 3a). Specifically, growth of *E. coli* DH5 α in buffered LB Lennox medium with 2 g/L of glucose attained a maximal optical density of 6.0 compared to 5.0 with the same glucose supplementation in unbuffered LB Lennox medium. Additionally, there was no diauxic lag phase during the cultivation, which indicated that 2 g/L of glucose supplementation did not significantly alter the carbon and nitrogen balance in buffered LB Lennox medium. Imbalances in carbon and nitrogen content between different nutrient components of a medium has been hypothesized to trigger a re-orientation of metabolic pathways and programmes that manifest as a diauxic lag phase.

On the other hand, compared to the case of reduced biomass formation in unbuffered LB Lennox medium with 6 g/L glucose, buffered LB Lennox medium with the same glucose supplementation increased biomass formation, yielding a maximal optical density of 6.8 at 33 hours of cultivation. However, the cultivation was punctuated by two diauxic lag phases upon the culture attaining optical density of 3.6 and 5.3, respectively. Both lag phases were of 3 hours each. This highlighted that while 6 g/L of glucose could improve biomass formation through the infusion of more nutrients for growth, it also introduced an imbalance in carbon and nitrogen into the medium that triggered diauxic lag phase.

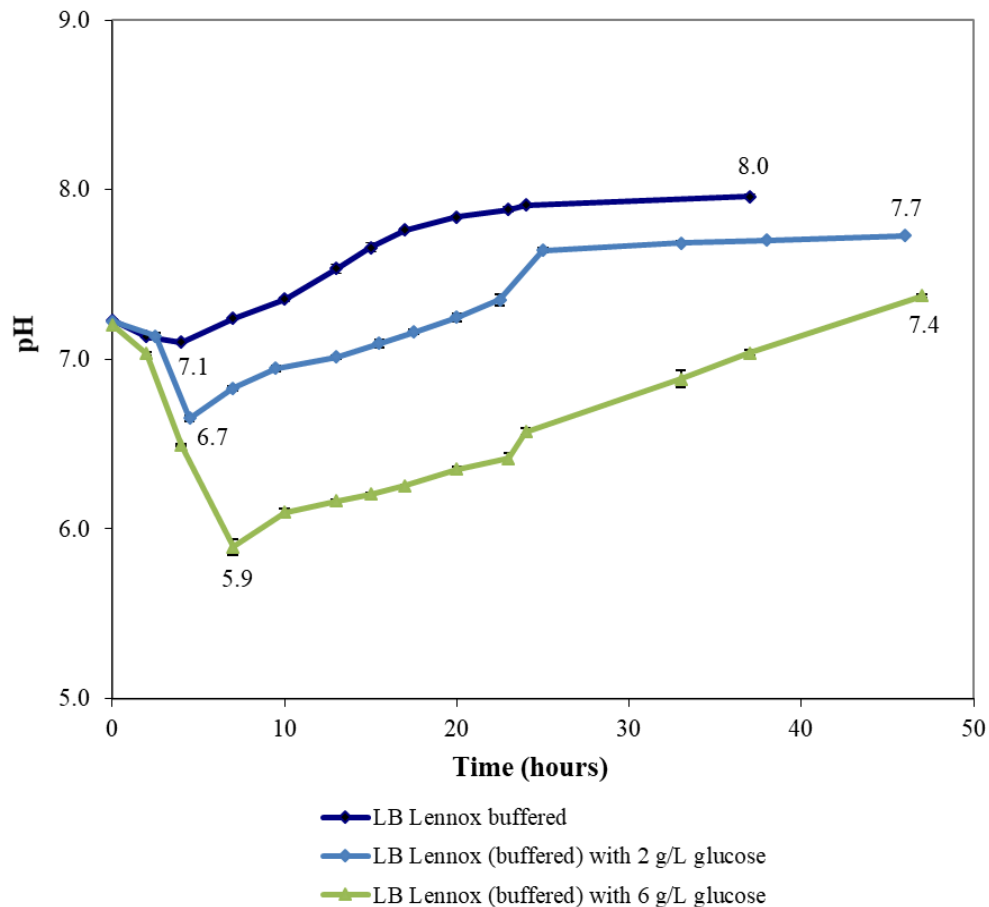


Figure 3b: Variation in pH during growth of *E. coli* DH5 α at 37 °C in buffered LB Lennox medium with different concentrations of glucose supplementation. Note that the greater the amount of glucose supplementation, the greater the initial decrease in pH of the culture broth.

pH profiles of *E. coli* DH5 α grown in buffered LB Lennox medium with different glucose supplementation revealed that the greater the amount of glucose supplementation, the greater would be the initial decrease in pH upon onset of growth (Figure 3b). Specifically, in buffered LB Lennox medium with 2 g/L of glucose, pH declined from 7.2 to 6.7 before a gradual rise to a final pH of 7.7 at the end of the cultivation. On the other hand, in buffered LB Lennox medium with 6

g/L of glucose, growth of *E. coli* DH5 α resulted in a pH drop from 7.2 to 5.9 prior to a slow rise to a final pH of 7.4 at the end of the cultivation. In the case of growth of *E. coli* DH5 α in buffered LB Lennox medium without glucose supplementation, pH decreased slightly from 7.2 to 7.1 before a gradual rise to 8.0 at the end of the culture. Thus, a high capacity phosphate buffer could modulate potentially large pH fluctuation during growth of *E. coli* DH5 α in LB Lennox medium with different glucose supplementation, and provide the environmental conditions favourable to biomass formation. Hence, to increase biomass formation, a high capacity phosphate buffer system must be coupled with suitable amount of glucose supplementation below a threshold value. Given that the metabolism of 6 g/L of glucose was previously associated with activation of overflow metabolism and acetate accumulation that resulted in reduced biomass formation, presence of a high capacity phosphate buffer system likely helped reduced the toxicity of the acidic metabolites secreted; thereby, enabling *E. coli* DH5 α to shift to the biomass formation pathway in more favourable pH and metabolite environment in the culture broth.

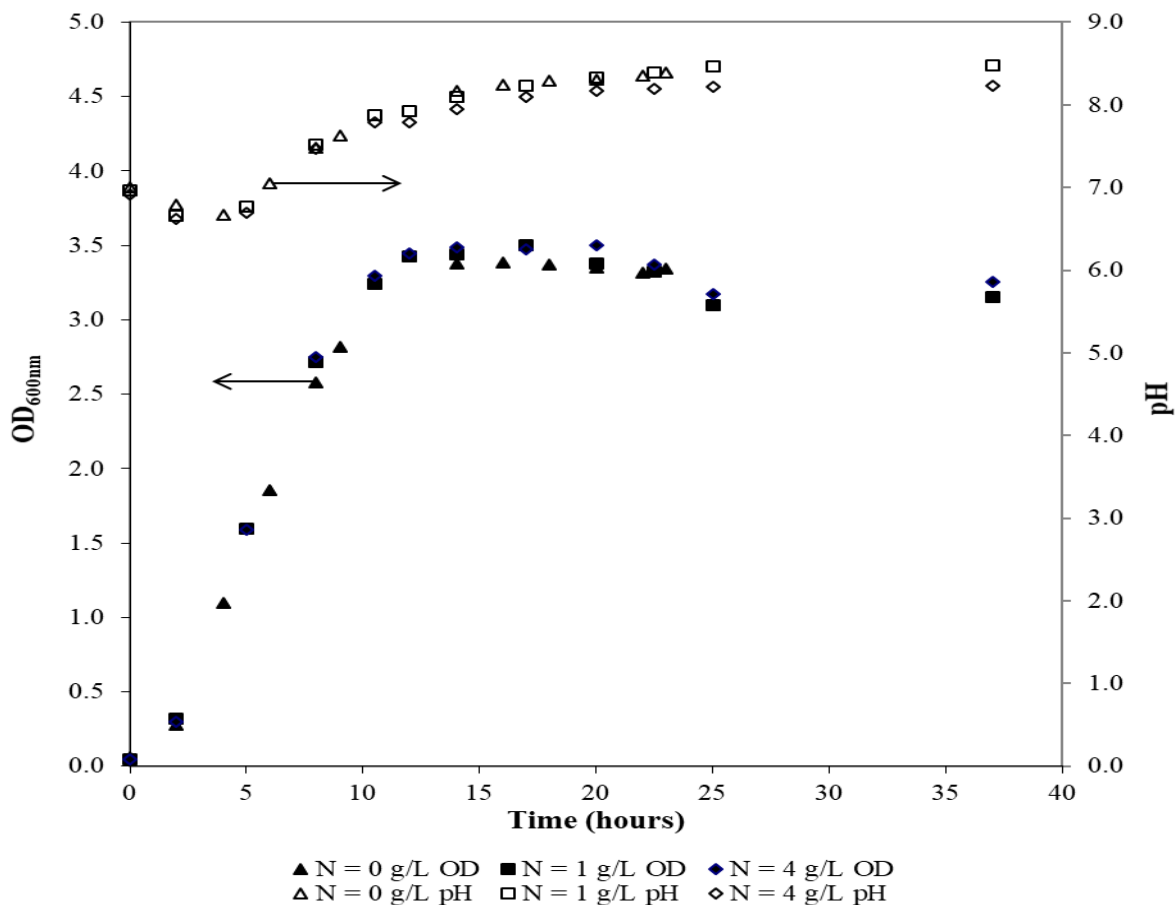


Figure 4: Optical density and pH profile of *E. coli* DH5 α during growth at 37 °C in unbuffered LB Lennox medium with different concentrations of ammonium chloride supplementation. Note that whether for pH or growth, all the data points coalesced into one pH and one growth profile,

which indicated that ammonium chloride supplementation had little effect on growth performance of *E. coli* DH5 α , and nitrogen was not a growth limiting nutrient in LB Lennox medium.

Supplementation of different concentrations of ammonium chloride (1 g/L and 4 g/L) into unbuffered LB Lennox medium did not result in different growth and pH profile (Figure 4), which indicated that ammonium chloride supplementation had little effect on metabolic processes in cells and biomass formation due probably that nitrogen was not a limiting nutrient in LB Lennox medium. Specifically, growth of *E. coli* DH5 α at different concentrations of ammonium chloride supplementation in unbuffered LB Lennox medium resulted in the same optical density and pH profile as that of *E. coli* DH5 α grown in unbuffered LB Lennox medium with no ammonium chloride supplementation. Even at a supplementation level of 4 g/L of ammonium chloride in unbuffered LB Lennox medium, no difference in growth performance or pH profile could be observed. Thus, there was no preference in the uptake of organic nitrogen compounds or ammonium chloride by *E. coli* DH5 α that yielded similar pH and growth profiles in LB Lennox medium with different levels of supplementation of ammonium chloride.

Conclusions

Glucose supplementation is crucial for increasing biomass formation of *E. coli* DH5 α in LB Lennox medium, where without glucose supplementation, the maximal optical density obtained was a relatively low 3.4. However, high concentration of glucose supplementation such as 6 g/L of glucose could trigger overflow metabolism through the central carbon pathway that led to acetate accumulation and low pH, which reduced biomass formation. Thus, glucose supplementation of LB Lennox medium required the use of high capacity phosphate buffer system for modulating the potentially large pH fluctuation that could arise during growth of *E. coli* DH5 α in LB Lennox medium with different glucose supplementations. Specifically, growth of *E. coli* DH5 α in LB Lennox medium with glucose supplementation typically result in a decrease in pH during onset of growth, followed by a gradual rise to a final pH value at the end of the cultivation. If the amount of glucose supplementation was too large (e.g., 6 g/L glucose), there would be a drastic decline in pH that could potentially create a pH environment not favourable to biomass formation. But, a greater problem might be the accumulation of acetate from overflow metabolism induced by the catabolism of excessive amount of glucose supplemented into LB Lennox medium. A high capacity phosphate buffer such as 89 mM phosphate would thus come into play by helping modulate the pH decline during catabolism of glucose, as well as neutralizing potentially toxic acidic metabolites secreted by *E. coli* DH5 α into the medium. Doing so helped improve biomass formation during cultivation of *E. coli* DH5 α in buffered LB Lennox medium with 2 g/L and 6 g/L glucose, where the latter delivered a maximal optical density of 6.8 at 33 hours into the cultivation. Hence, a high capacity phosphate buffer system is necessary for helping modulate potentially large pH fluctuation that arises from the catabolism of relatively large glucose supplementation (e.g., 6 g/L of glucose) and helped provide a more conducive environment for biomass formation. Additionally, supplementation of LB Lennox medium with 2 g/L glucose, with or without a buffer

system did not result in diauxic lag phase, which implied that the 2 g/L glucose supplementation did not significantly alter the carbon and nitrogen balance of the medium. Finally, supplementation of different concentrations of ammonium chloride (1 g/L and 4 g/L) in unbuffered LB Lennox medium did not result in different growth and pH profiles, which indicated that LB Lennox medium is deficient in sugars, not nitrogen compounds.

References

1. Christensen, D. G., Orr, J. S., Rao, C. V. & Wolfe, A. J. Increasing growth yield and decreasing acetylation in *Escherichia coli* by optimizing the carbon-to-magnesium ratio in peptide-based media. *Appl. Environ. Microbiol.* **83**, (2017).
2. Xu Bo, Jahic Mehmedalija & Enfors Sven-Olof. Modeling of overflow metabolism in batch and fed-batch cultures of *Escherichia coli*. *Biotechnol. Prog.* **15**, 81–90 (2008).
3. Vemuri, G. N., Altman, E., Sangurdekar, D. P., Khodursky, A. B. & Eiteman, M. A. Overflow metabolism in *Escherichia coli* during steady-state growth: transcriptional regulation and effect of the redox ratio. *Appl. Environ. Microbiol.* **72**, 3653–3661 (2006).
4. Valgepea, K. *et al.* Systems biology approach reveals that overflow metabolism of acetate in *Escherichia coli* is triggered by carbon catabolite repression of acetyl-CoA synthetase. *BMC Syst. Biol.* **4**, 166 (2010).
5. Enjalbert, B., Millard, P., Dinclaux, M., Portais, J.-C. & Létisse, F. Acetate fluxes in *Escherichia coli* are determined by the thermodynamic control of the Pta-AckA pathway. *Sci. Rep.* **7**, 42135 (2017).

Conflicts of interest

The author declares no conflicts of interest.

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