

**Optimization of Fermentation Conditions for
the Production of Legionaminic Acid in
Recombinant *Escherichia coli***

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Thesis submitted to the
Faculty of Graduate and Postdoctoral Studies
in partial fulfillment of the requirements for the degree of
Master of Chemical Engineering



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July 2017

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Abstract

Legionaminic acid (Leg5,7Ac₂) is a nonulosonic acid similar to sialic acid (Neu5Ac), which can be found in the extracellular glycoconjugates of several bacterial pathogens [1]. Due to the similarity in stereochemistry of the two compounds, legionaminic acid has great potential in the production of pharmaceutical drugs. A novel biosynthetic pathway to produce legionaminic acid was created to overcome the limitations of organic synthesis. This is the first study involving the scale-up of legionaminic acid production by high cell density fermentation processes. In this work, fed-batch cultivations of recombinant *Escherichia coli* BRL04 were carried out in shake flasks and 5-L bioreactors. The final process was optimized by determining the effects of different carbon sources, induction temperatures, pH, dissolved oxygen (DO) content, induction optical density and N-acetylglucosamine (GlcNAc) feed rate on the production of legionaminic acid. Overall, results showed that the titer, yield and productivity for legionaminic acid production achieved relatively high levels, which were 5.53 g/L, 73.29% and 0.092 g/(Lh), respectively. It is hoped that this study accelerates research into the production of legionaminic acid for therapeutic treatments as well as for further study in glycobiology.

Résumé

L'acide légionaminique (Leg5,7Ac₂) est un acide nonulosonique semblable à l'acide sialique (Neu5Ac), qui peut être trouvé dans les glycoconjugués extracellulaires de plusieurs agents pathogènes bactériens [1]. En raison de la similitude de la stéréochimie des deux composés, l'acide légionaminique présente un grand potentiel dans la production de médicaments pharmaceutiques. Une nouvelle voie biosynthétique pour produire de l'acide légionaminique a été créée pour surmonter les limites de la synthèse organique. Il s'agit de la première étude impliquant l'augmentation de la production d'acide légionaminique par des processus de fermentation à haute densité cellulaire. Dans ce travail, des cultures fed-batch d'*Escherichia coli* BRL04 recombinante ont été réalisées dans des flacons à agitation et des bioréacteurs de 5 L. Le processus final a été optimisé en déterminant les effets de différentes sources de carbone, les températures d'induction, du pH, de la teneur en oxygène dissous (DO), du point d'induction et du taux d'alimentation en GlcNAc sur la production d'acide légionaminique. Dans l'ensemble, les résultats ont montré que le titre, le rendement et la productivité de la production d'acide légionaminique atteignaient des niveaux relativement élevés, respectivement 5.53 g / L, 73.29% et 0.092 g / (Lh). On espère que cette étude accélère la recherche sur la production d'acide légionaminique pour des traitements thérapeutiques ainsi que pour une étude approfondie en glycobiologie.

Acknowledgements

I would first like to thank my supervisor, Dr. Jason Zhang, for giving me the opportunity to work in the area of *Escherichia coli* fermentation. His professional guidance was critical to me when I ran into problems or had questions about my research throughout my years of study and through the process of researching and writing my thesis.

I would also like to thank Dr. Christopher N. Boddy from the Department of Chemistry at the University of Ottawa for supplying the bacterial strain, as well as for kindly sharing the biosynthetic method and other useful information on shake flask experiments.

Additionally, I would like to extend thanks to Zhiliang Yang who is a fellow colleague from Dr. Zhang's group for his assistance and guidance during the period of my experiments. Without his theoretical guidance, this research would not have been successful. I am also grateful to Xiangchao Meng, Scott Proulx and Yaqing Liu for their help they provided for my research.

I would like to thank the departmental staff, Louis Tremblay, Gérard Nina and Franco Zirolto for their technical support, as well as Francine Pétrin and Sylvie Saindon for administrative assistance.

I would also like to thank the financial support of the Canadian Government through a NSERC scholarships, and the University of Ottawa through the Excellence scholarship.

Finally, I must express my very profound gratitude to my parents and to my boyfriend for providing me with unfailing support and continuous encouragement. This accomplishment would not have been possible without them.

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Nomenclature

Acronym	Definition
CMP	cytidine monophosphate
DCW	dry cell weight
DNA	Deoxyribonucleic acid
DO	Dissolved oxygen
F(t)	volume flow rate of the feed at time t (Lh^{-1})
GlcNAc	N-acetylglucosamine
HCDC	high cell density cultivation
HPLC	high-performance liquid chromatography
IPTG	isopropyl- β -D-1-thiogalactopyranoside
LB	Luria-Bertani
Leg5,7Ac	5,7-diamino-3,5,7,9-tetradeoxy-D-glycero-D-galacto-non-2-ulosonic acid
LPS	lipopolysaccharide
m	specific maintenance coefficient ($gg(DCW)^{-1}h^{-1}$)
$M_s(t)$	mass flow rate of the feed at time t (gh^{-1})
NADH	Nicotinamide adenine dinucleotide hydrogen
Neu5Ac	N-acetylneuraminic acid
OD	optical density
$S_F(t)$	concentration of the growth limiting nutrient in the feed at time t (gL^{-1})
TCA	tricarboxylic acid
V(t)	volume of culture in the bioreactor at time t(L)
X(t)	biomass density in the bioreactor at time t ($g(DCW)L^{-1}$)
$Y_{X/S}$	biomass density on substrate ($g(DCW)g^{-1}$)
μ	specific growth rate (h^{-1})

Chapter 1: Introduction

Cell-surface glycoconjugates are important to pathogenic bacterial cells, and are connected to the host's immune response and the determination of immunospecificity [2]. A nonulosonic acid family is a group of negatively charged 9-carbon α -keto sugars that are primarily used in cell-to-cell communication [3], [4]. They are generally expressed post-translationally as glycoconjugates such as peptidoglycans and glycolipids [3], [5]. Sialic acid is the most abundant species in this family and is often found on the extracellular surface [3], [5]. Legionaminic acid, a sialic acid-like nonulosonic acid, is found on the extracellular surfaces of several bacterial pathogens including *Acinetobacter baumannii*, *Legionella pneumophila*, *Enterobacter cloacae* and *Campylobacter jejun* [1]. Its virulence in humans is expressed through damaging the host's immune system or through cell-to-cell communication [6]. Because the stereochemistries are similar in both legionaminic acid and sialic acid, it has been suspected that they may have similar effects in promoting host-microbe interactions.

Legionaminic acid was first discovered in 1994 as a component of the lipopolysaccharide in *L. pneumophila*, which causes Legionnaire's disease [7],[8]. However, literature concerning legionaminic acid is limited to its function and occurrence. Previous research has shown that total organic synthesis may be used to produce legionaminic acid [9]. However, this approach has the disadvantages of high technical demand and low yield. To overcome the drawbacks of total organic synthesis, a novel biosynthetic pathway that uses a combination of multiple metabolic modules from three bacteria was created by Dr. Christopher N. Boddy's group from the Department of Chemistry at the University of Ottawa [6].

In this study, several fermentation conditions were tested to increase legionaminic acid

production in a modified *E. coli* BRL04 strain. The comparisons of different carbon sources and various induction temperatures were determined in shake flask cultures. High cell density fermentation experiments were carried out in a 5-L bioreactor fed-batch culture at constant pH and DO to optimize crucial operating parameters, including induction temperature, pH, dissolved oxygen content, induction time and the GlcNAc feed rate. Finally, all optimized conditions were combined to achieve a high titer, yield and productivity. This study is hoped to accelerate research into the production of legionaminic acid for medical and pharmaceutical purposes and for further study in glycobiology.

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Chapter 2: Literature review

2.1 Legionaminic acid

2.1.1 Background

Legionaminic acid, Leg5,7Ac₂, a nonulosonic acid similar to sialic acid (5-acetamido neuraminic acid, Neu5Ac), is the major constituent of the lipopolysaccharide (LPS) of *Legionella pneumophila* [1],[2]. *Legionella pneumophila* promotes Legionnaire's disease as well as a damaging form of human pneumonia. The role of legionaminic acid is often virulence-associated in cell-surface glycoconjugates, causing damage to the immune response or cell-to-cell interactions [3]. Legionaminic acid can also be found in the LPS of *Vibrio alginolyticus*, *Vibrio salmonicida*, *Pseudomonas fluorescens* and other pathogens in humans such as *Campylobacter jejuni*, *Enterobacter cloacae* and *Acinetobacter baumannii* [4]. The existence of legionaminic acid within glycoconjugates of bacterial pathogens makes them feasible targets for pathogen recognition by the host's immune system. This could be manipulated to modify bacterial immune specificity. [5],[2].

Derivatives of 5,7-diamino-3,5,7,9-tetra-deoxy-non-2-ulosonic acids were first identified in the lipopolysaccharide of *Pseudomonas aeruginosa* and *Shigella boydii* in 1984 and in *Legionella* species in 1994. Legionaminic acid derivatives were also found to be O-linked to flagellae of *Campylobacter jejuni* in 2007 [6],[1]. To date, three isomers of legionaminic acids with D-glycero-D-galacto (Leg), D-glycero-D-talo (4eLeg), and L-glycero-D-galacto (8eLeg) structures have been recognized [5]. Furthermore, di-N-acetyl derivative of 8-epilegionaminic acid has been discovered within *E. coli* O-antigens [4].

2.1.2 Structure

Legionaminic acid has a molecular formula of $C_{13}H_{22}N_2O_8$, and a molecular mass of 334.1376 [7]. It is structurally related to sialic acid; however, Leg elements are not hydrolyzed by sialidases. Some sialyltransferases have been determined by Watson et al. to use CMP-Leg5Ac7Ac as a donor [8]. Figure 2 shows that both legionaminic acid and sialic acid have similar stereo structure but different substituents. The hydroxyl groups at positions 7 and 9 are replaced by an acetamido group and hydrogen, respectively. The biosynthetic incorporation of both legionaminic acids and sialic acid may be similar due to their stereochemistries [9].

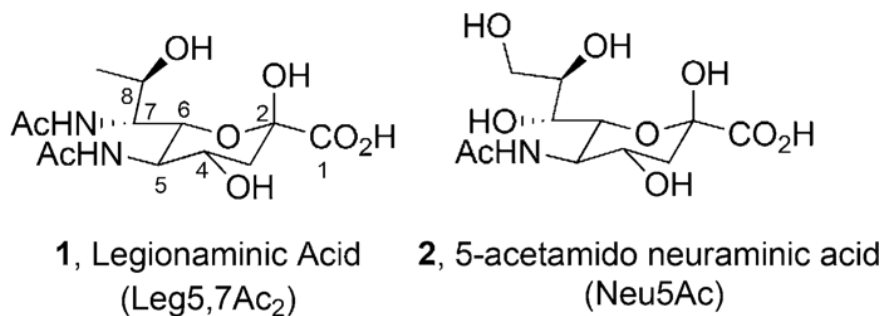


Figure 2.1 Structures of legionaminic acid (Leg5,7Ac₂) and sialic acid (Neu5Ac) [6]

2.1.3 Synthesis

Legionaminic acid was synthesized by condensation of 2,4-diacetamido-2,4,6-trideoxy-D-mannose with oxalacetic acid in 2000 as shown in Figure 2.2 [10],[11]. The synthesis routes are highly demanding and low yielding. Legionaminic acid may be isolated by anion-exchange chromatography and the isomers are separated by reversed-phase HPLC after chemical synthesis [12]. A stereoselective total organic synthesis shown in Figure 2.3 that yielded a legionaminic acid building block and linker-equipped conjugation-ready legionaminic acid starting from cheap D-threonine was reported in 2015 [2]. But the technical challenge and low yield of this route still limit the glycobiology study of legionaminic acid. Due to its complex structure, there is great difficulty in producing legionaminic acid by total

organic synthesis [6]. The biosynthetic pathway for legionaminic acid in *Campylobacter jejuni* was described in 2009. Eleven candidate biosynthetic enzymes from *C. jejuni* have been purified and biochemically characterized, thereby fully reconstituting the biosynthesis of legionaminic acid and its CMP-activated form, starting from fructose-6-P [13]. Unpredictably this pathway used GDP-GlcNAc (Figure 2.4) as the key building block, unlike related novel pathways, which use UDP-GlcNAc.

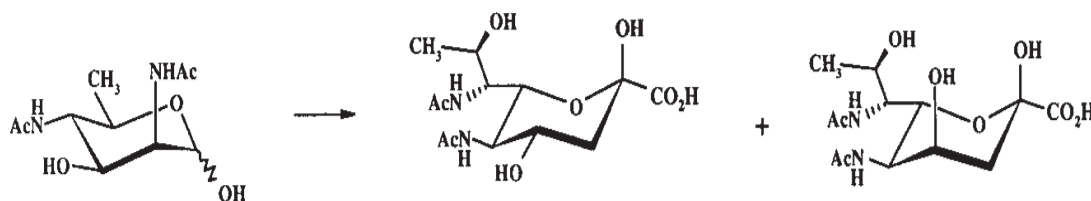


Figure 2.2 Synthesis of legionaminic acid from 2,4-Diacetamido-2,4,6-trideoxy-D-mannose [10]

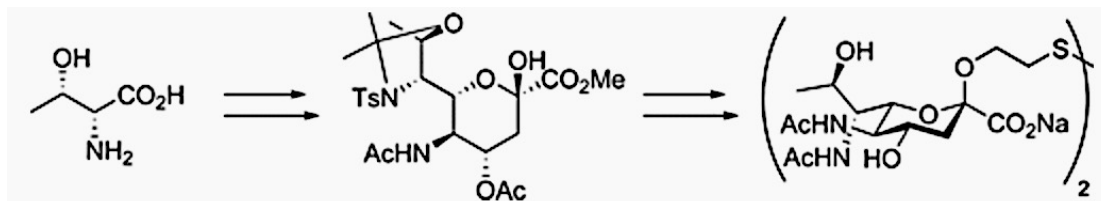


Figure 2.3 Synthesis of legionaminic acid building block from D-threonine [2]

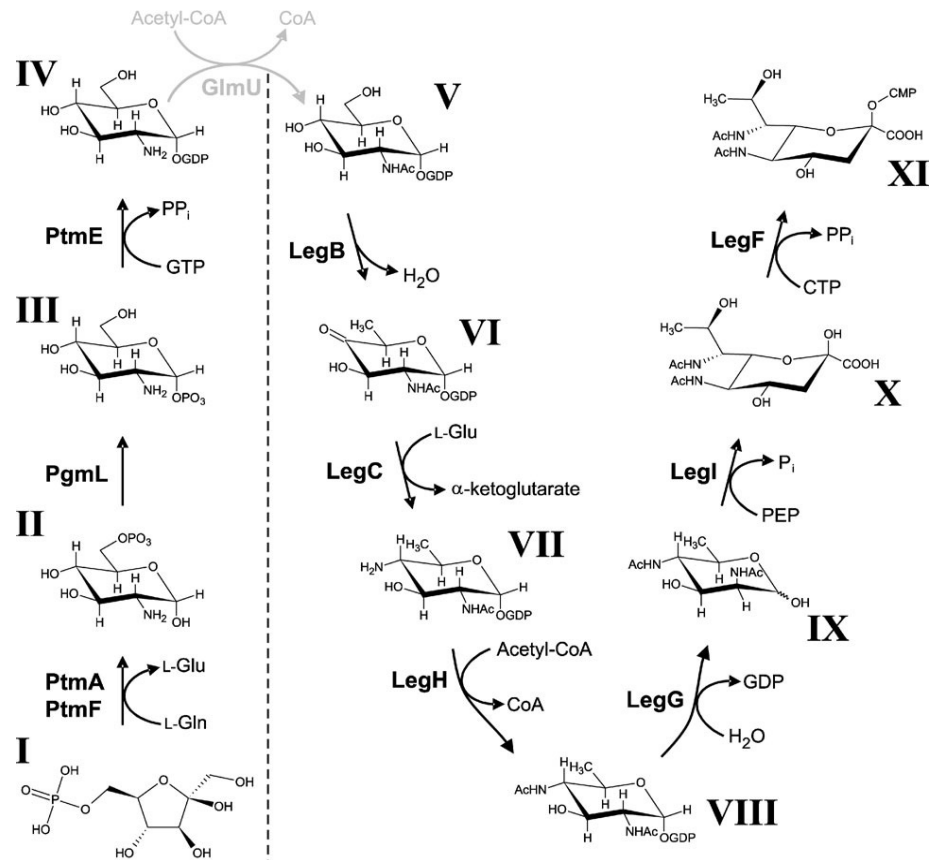


Figure 2.4 The CMP-legionaminic acid biosynthetic pathway in *C. jejuni*. This biosynthetic pathway involves two segments: (1) synthesis of a GDP-sugar building block (left of the dashed line) and (2) synthesis of the final CMP-nonulosonate (right of the dashed line), which are linked by the enzymatic step shown in gray [14].

As shown in Figure 2.5, this is the first *in vivo* total biosynthesis production system used to produce a sufficient source of Leg5,7Ac₂. Hassan et al. designed the *de novo* biosynthetic pathway to produce legionaminic acid by using a combination of metabolic units. These modules were obtained from three microorganisms: *C. jejuni*, *L. pneumophila* and *Saccharomyces cerevisiae*. [3]. The synthetic process is achieved through seven steps, beginning from GlcNAc as the initial substrate. There are seven enzymes which are expressed by combining multiple vectors. The mutase Agm1 and uridyltransferase Uap1 were obtained

from *Saccharomyces cerevisiae*. The dehydratase PglF, the aminotransferase PglE and the acetyltransferase PglD were obtained from *C. jejuni*. In addition, a hydrolyzing 2-epimerase LegG and the synthase LegI come from *L. pneumophila*. In order to improve the quantities of Leg5,7Ac₂ in *E. coli*, the *de novo* pathway was created to convert GDP-GlcNAc to UDP-GlcNAc [3]. It is therefore hoped that it will play an essential role in prokaryotic and eukaryotic glycobiology [3].

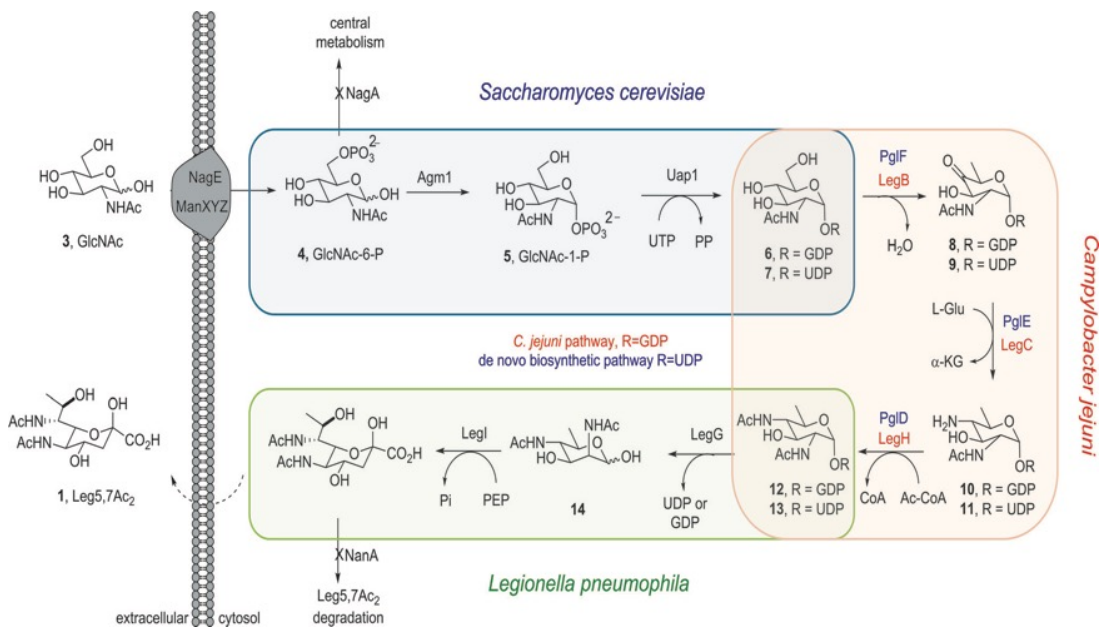


Figure 2.5 De novo biosynthetic pathway for Leg5,7Ac₂ production in *E. coli*. Enzymes listed in blue are from the engineered UDP-linked pathway and those in red from the native *C. jejuni* GDP-linked biosynthetic pathway [3].

2.2 High cell density cultivation

High cell density cultivation (HCDC) of recombinant *Escherichia coli* is an attractive biotechnological method for industrial bioprocesses to produce at high yields and high volumetric productivities. High cell density cultures have the benefits of improving the cost-

effectiveness of a process while also decreasing the working volume of a culture and its wastewater [15]. Various techniques for high cell density cultures have been developed including host design consideration, tuning recombinant protein expression, medium composition, growth methodologies, and even control and analysis of the process to increase the cell densities of heterologous strains in fed-batch cultures to over 100 grams (dry cell weight) per liter [16].

High cell densities are achieved in a defined medium that includes a considerable amount of the carbon source. The formulation of the media should ideally meet the demands for both cell growth and production [17]. It has been reported that the composition and feeding strategies of nutrients and fermentation parameters such as temperature, pH and dissolved oxygen, which should be optimized, may influence the rate of transcription and translation, as well as proteolytic activity [17], [18]. Effectively, the level and stability of production may be compromised in suboptimal conditions. There are some disadvantages of HCDC, including the limitation of dissolved oxygen availability, the inhibition of growth rate as well as an accumulation of acetate and other by-products formed due to an excess of carbon dioxide, a decreased mixing efficiency in the bioreactor and the generation of excess heat.

Acetate accumulation, one of the main issues in high cell density cultivation of *E. coli*, may be avoided by maintaining the specific growth rate at less than 0.2 h^{-1} [19]. The presence of acetic acid reduces productivity since its production acts as a drain on the carbon source, preventing complete conversion to biomass and product. Under insufficient aerobic conditions with excess glucose, *E. coli* may produce acetate, an unexpected by-product that influences cell growth, physiology and performance. An optimal feeding schedule as well as sufficient control of dissolved oxygen by agitation and air sparging are strategies which are commonly

used to avoid acetate formation in the culture. Even when sufficient oxygen is present, acetate can still be produced when the concentration of cells exceeds a threshold amount for the specific glucose consumption rate. Supplying glucose to the culture at a slow speed to lower the specific glucose consumption rate under the threshold value avoids metabolic overflow, which would normally be redirected to acetate production [17]. Glycerol can be used as a carbon source to control uptake rates and to restrict metabolic overflow effects. However, levels of glycerol that are too high may lead to acetate accumulation, while those that are too low may lead to cell famine, and even cell stress that selects for plasmid-free propagation, diminishing enzyme expression. Therefore, it is necessary to find an equilibrium state between reaching high cell density and controlling acetate production to successfully produce a product [20].

Fed-batch fermentation is a preferred strategy which may be successfully applied at different production scales for large concentrations of cells and products. A medium with defined chemical compounds is often used in this kind of production strategy since the culture conditions may be easily managed and inhibiting elements such as an accumulation of carbon source are uncommon [21]. Fed-batch cultures can produce more than 50 g/L of *E. coli* [16]. Cell concentrations of over 100 g/L cell dry mass can be achieved when using exponential feeding strategies, which is ideal to sustain the specific rate of glucose consumption at a stable and determined value [16]. However, this strategy suffers from its own drawbacks. Due to a low specific rate of glucose consumption, the specific growth rate is maintained at a low level and the fermentation period becomes longer, resulting in a reduction of overall productivity. Additional control, supplementary equipment and extra provisions required for longer runs need to therefore be available to run desirable fed-batch cultures at industrial fermentation

levels. Additionally, glucose gradients resulting from improper mixing often occur at large scales, which may cause some physiological responses that have harmful influences on the process. These issues can be mitigated by changing to a simpler batch mode to culture cells when excessive metabolism is prevented [17]. High biomass concentrations are usually obtained in fed-batch fermentations with a severe control of growth conditions, inhibiting the formation of by-products such as acetic acid.

High levels of biomass must be guaranteed before induction so that a sufficient number of cells are available to produce large quantities of product. Because the induction process tends to slow cell growth when at a low temperature, it makes sense to produce a product after a dedicated period of growth. Furthermore, the type and quantity of the inducer as well as the induction time must be optimized. IPTG is one of the most commonly used chemical inducers. Due to metabolic burden, high concentrations of inducer do not always contribute to the greatest protein expression, so a suitable inducer concentration is essential to balance the biomass reduction with increased expression levels. Normally, IPTG concentrations should be below 1.0 mM, and a second inducer may be used to further enhance induction [20].

High levels of gene expression early in a fermentation experiment may cause plasmid instability and inhibit cell growth. Employing an inducible expression system can effectively solve this problem. Some of the most frequently used promoters in *E. coli* are not tightly regulated and show high levels of 'leaky' expression. For instance, plasmid instability and loss often appear in cultures when the tac promoter is used. Heat-inducible promoters such as the PL promoter require rapid changes in temperature, while the T7 promoter requires expression of T7 RNA-polymerase in the host cell [19]. Some examples of production of different recombinant proteins by high cell density cultures of *E. coli* are show in Table 2.1.

Table 2.1 Production of recombinant proteins by high cell density cultures of *E. coli*

Products	Host	Culture condition and carbon source	Productivity and characteristics	References
6-O-sulfotransferase isoform-3 (6-OST-3)	<i>E. coli</i> BL21(DE3)	pH-stat and DO-stat, 5-L, IPTG R medium, glycerol	21.2 mg/g _{cdw} , transmembrane protein	[14]
6-O-sulfotransferase-1 (6-OST-1)	<i>E. coli</i> K5	pH-stat and DO-stat, 7-L, IPTG and galactose, coupled inducer, R medium, glycerol	482mg/L, maltose-binding protein	[19]
rhIFN-β	<i>E. coli</i> BL21(DE3)	pH-stat and DO-stat, IPTG, R medium, glycerol	4.8g/L	[21]
Human kringle domains 548 (KD548)	<i>E. coli</i> BL21(DE3)	pH-stat, 6.6-L, defined R/2 medium, IPTG, glucose,	5.4g/L, soluble proteins	[22]
capsular polysaccharide	<i>E. coli</i> K4	DO-stat, R medium, glycerol	4.73g/L Microfiltration bioreactor	[23]
Heterologous alcohol dehydrogenase (ADH)	<i>E. coli</i> BL21(DE3)	Do-stat, defined culture, glucose	13g/L, Rocking-motion-type bioreactor	[24]
Green fluorescense protein (GFP)	<i>E. coli</i> K12	pH stat, DO stat, glucose	24.95+_1.8g/L,	[16]
Recombinant PvII	<i>E. coli</i> K12	pH-stat, defined medium, glucose	0.7mg/g dry wt, refolded	[17]
L-N-Carbamoylase	<i>E. coli</i> BL21	pH-stat, DO-stat, defined media, glucose, rhaBAD promoter	3.8g/L,	[18]
Ribulose 1,5-bisphosphate carboxylase/oxygenase(RubisCO)	<i>E. coli</i> K12	R media, glucose	1g/L	[25]

2.3 Factors affecting cell growth and recombinant protein expression

2.3.1 Medium and carbon sources

The medium formulation is a fundamental step in the successful design of laboratory experiments, as well as pilot-scale and industrial processes. All microbes require water as well as sources of energy, carbon, nitrogen, mineral constituents and additional vitamins and oxygen if aerobic. The elements of a medium must meet the needs of cellular biomass and the production of metabolites. Furthermore, there must be a sufficient supply of the energy source to achieve biosynthesis and cell maintenance [27].

The quantities of elements used to solve the elemental balance may not be available, thus the data displayed in Table 2.2 may be used as a guide to determine approximate minimum quantities of N, S, P, Mg and K in a medium element recipe. Trace metals such as Fe, Zn, Cu, Mn, Co, Mo and B may also be required at lower concentrations [27].

Table 2.2 Element composition of bacteria, yeasts and fungi (%by dry weight)[27]

Element	Bacteria	Yeasts	Fungi
Carbon	50-53	45-50	40-63
Hydrogen	7	7	
Nitrogen	12-15	7.5-1.1	7-10
Phosphorus	2.0-3.0	0.8-2.6	0.4-4.5
Sulphur	0.2-1.0	0.01-0.24	0.1-0.5
Potassium	1.0-4.5	1.0-4.0	0.2-2.5
Sodium	0.5-1.0	0.01-0.1	0.02-0.5
Calcium	0.01-1.1	0.1-0.3	0.1-1.4
Magnesium	0.1-0.5	0.1-0.5	0.1-0.5
Chloride	0.5	—	—
Iron	0.02-0.2	0.01-0.5	0.1-0.2

The classic medium for *E. coli* growth in shake flasks or test tubes is the Luria-Bertani broth (LB), which is a complex medium with 1% (w/v) peptone, 0.5% (w/v) yeast extract and 0.5% (w/v) NaCl. All reported strains can propagate on LB, but their cell densities are limited

to an OD₆₀₀ of 2-4 in the medium without any additional carbon sources. In order to increase cell density and in turn the volumetric productivity in LB cultures, carbon sources and minerals should be supplied. Supplementation of minerals and complex constituents can be added to the initial culture or fed at later stages of fermentation.

Abandoning the use of complex compounds can cut down process costs and simplify the chemical composition of a culture to satisfy the needs of high-purity production [28]. Variable concentrations of elements and impurities in the media may cause unexpected biomass or product concentrations at harvest. Furthermore, undefined media may result in difficulties with recovering product and effluent treatment, since not all components are consumed by the bacteria. Excess N and P sources as well as trace metals may be fed to the culture using complex strategies during the period of fermentation [29].

It has been reported that various carbon sources may be used for recombinant protein expression in *E. coli*, such as glucose, glycerol, galactose, xylose, sucrose, fructose, mannitol, acetate, and succinate [29], [30].

Glucose is the most widely used and preferred carbon source for *E. coli*. However, its utilization likely leads to high levels of acetate production, which may negatively affect not only cell growth, but also protein production. The use of glycerol as a carbon source can reduce acetate concentrations and improve production activity [21].

Glycerol has become an inexpensive and promising carbon source for industrial microbiology because it is generated as an inevitable by-product from biodiesel production. Every 100 lbs of biodiesel produced by transesterification generates 10 lbs of crude glycerol [31]. Development of the biodiesel industry has produced tremendous amounts of glycerol residue, leading to a reduction in the price of crude glycerol [31]. A variety of microorganisms

can metabolize glycerol in the presence of external electron acceptors; however, few can do so by fermentation (i.e. in the absence of electron acceptors). Nowadays, the fermentative metabolism of glycerol has been studied in some species of the *Enterobacteriaceae* family, such as in *Citrobacter freundii* and *Klebsiella pneumoniae* [32].

2.3.2 Temperature

Temperature variation is a potential stress factor for *E. coli*. In order to protect against such stress, *E. coli* has developed sophisticated intracellular systems which may be characterized by physiological and genetic changes [29]. For example, a temperature rise induces the bacterial heat shock response allowing bacteria to adapt and survive under thermal stress conditions [29].

Cell growth as well as the production and activities of recombinant proteins in *E. coli* are affected by temperature [33]. Normally, *E. coli* will grow in a temperature range from 23°C to 40°C [34]. The optimal temperature for maximal cell growth is 37°C [35],[36]. Unlike the growth rate which increases up to a temperature of 37°C, the rate of protein production increases up to 44°C [33]. However, lower expression levels may result following induction at temperatures of up to 42°C because proteins accumulate as inclusion bodies under conditions of overexpression [37]. High culture temperatures may increase the growth rate, but also results in a higher probability of plasmid loss [38]. The growth of *E. coli* is typically inhibited at 45°C, at which phenotypic colony features are not available, as cells may be viable but noncultureable (VBNC) under these conditions [35]. In addition, some issues may arise when at high temperatures such as cell wall alterations, host toxicity, formation of inclusion bodies and incorrect folding of recombinant proteins. When induction temperatures are increased up to 42°C, the levels of product which are obtained are lower, while cell viability

and productivity may also be negatively affected [37].

Compared to rapid growth cultures, cultures grown slowly at lower temperatures are better for control of gene expression and heterologous protein production. It has been reported that a temperature range of 16-23°C is preferred for inducible protein production [39]. At low temperatures (15°C), cell growth stops, as protein production is severely inhibited [37].

2.3.3 pH

The pH of a culture affects the expression levels of certain genes, uptake rates, cell structure and the activities of enzymes. The pH regulates the expression of catabolic enzymes and periplasmic proteins under aerobiosis in *E. coli* [40]. Therefore, cellular growth and product synthesis are influenced by pH levels. Because fermentation is a multi-enzyme system and the optimal pH of various enzymes are not the same, the optimal pH for growth and product formation are also different [39].

The concentration of H^+ in a culture has an indirect impact on cells. At first, the extracellular H^+ reacts with extracellular weak acids, forming molecular weak acids which can easily pass through the cell membrane. The molecular weak acids may affect the intracellular neutral state when they enter the cell, thereby influencing the structures and activities of enzymes. A similar phenomenon occurs with OH^- and weak bases [41].

A feasible growth pH for *E. coli* is approximately neutral. Due to the constitutive homeostatic mechanisms of *E. coli*, the cells maintain their internal pH between 7.4-7.8 [42]. Thanks to these mechanisms, *E. coli* may survive in a wide range of environmental pH from 4.5-9, which relates to environments such as the human gastrointestinal tract, stomach and pancreatic secretions [40]. Compared to a high pH, *E. coli* cells tend to better tolerate a low pH [43]. *E. coli* has three distinct systems for acid resistance (a glucose-catabolite-repressed

system and two amino acid decarboxylase-dependent systems), so the organism can also survive under strongly acidic conditions at a pH range of 2-3 for several hours [44]. At a high pH, the Na⁺/H⁺ antiporter helps to maintain a stable internal pH and protects cells from excess sodium. A total of 18 proteins induced by acidic and alkali conditions in *E. coli* have been studied using two-dimensional gel electrophoresis. Tryptophan deaminase (TnaA), glutamate decarboxylase (GadA) and MalE are overexpressed at an alkaline pH. On the acidic end, the gad system (GadA/GadBC), YfiD alkyl hydroperoxide reductase (AhpC), the galactitol fermentation enzyme GatY and the phosphotransferase system components ManX and PtsH are overexpressed at low pH [45]. At a highly alkaline pH, *E. coli* O157: H7 can survive and some related proteins are expressed well [46].

2.3.4 Dissolved oxygen

As an essential parameter, dissolved oxygen (DO) content may significantly impact the aerobic growth of *E. coli*, as well as the formation of the recombinant products. The optimal level of aseptic air should be supplied to support cellular propagation, while also supporting the accumulation of metabolic production during the fermentation process [47].

The supply of oxygen is difficult to maintain due to its low solubility in water. The actual level of dissolved oxygen depends on temperature, pressure and salinity. Normally, decreasing the temperature and salt level, as well as increasing pressure will improve the solubility of oxygen [48]. Furthermore, the DO level may be controlled by improving the rate of oxygen transfer by means such as increased sparge rates, agitation and vessel pressure, or by diminishing the uptake rate of oxygen in the broth by reducing the temperature for growth or the substrate concentration. However, each of these approaches has physical limits [49]. The changes in agitation and aeration must meet the oxygen demand, while also promoting even

mixing. Improved agitation therefore contributes to the DO content in order to overcome the resistance to oxygen transfer into the culture medium and into the bacterial cells [50].

The demand for oxygen levels varies with different species and at different stages of the process. More than 200 genes may be expressed by recombinant *E. coli* at 10 to 50% saturation [50]. Increasing dissolved oxygen levels from 30% to 300% does not affect the growth parameters of *E. coli*, but does result in a temporary decrease in respiration and acetate accumulation profiles [51]. In order to prevent irreversible damage from intracellular reactive oxidative species that are produced from dissolved oxygen, the SoxRS regulon may be activated [51]. The growth of *E. coli* and the production of intracellular enzymes may be inhibited in oxygen-limiting conditions.

The most production of xylanase was gained at the DO level of 20% saturation [50]. In this study, four recombinant strains of *E. coli* were grown at stable DO levels of 0, 50, and 100% air saturation. It was found that biomass and plasmid contents under aeration were greater under aerobic conditions, although recombinant protein activities vary with strain [49]. Due to the demands for oxygen by a culture, an available method to maintain proper aeration is to mix pure oxygen with air in order to attain a high level of oxygen [51].

High DO levels have been correlated with the formation of free radical species. As with other aerobic organisms, *E. coli* has a complicated defense system to deal with these challenges that includes superoxide dismutase and catalase, which reduce free radicals. However, if the concentration of oxidizing species rises above the capabilities of the cellular defense system, oxidative stress may damage the structures of proteins, lipids and DNA. The expression of recombinant proteins, especially those that produce oxygen-sensitive products under excessive aerated conditions may be influenced by oxidative damage. Therefore in a

proper fermentation process, dissolved oxygen should be controlled at levels high enough to support cell growth and metabolism, but not so high as to result in oxidative damage [52].

2.3.5 Induction parameters

A prevalent method to express foreign genes in bacteria is through chemical induction [53]. Induction parameters involve a specific promoter-inducer combination, inducer concentration and induction time, which are optimized so as to maximize production of recombinant proteins in *E. coli* [57].

High-level expression of foreign proteins in *E. coli* relies on a strong, regulated promoter that permits a cell growth phase followed by a separate production and induction phase [55]. Without this control, overly expressed genes may lead to plasmid instability and growth limitations, even if the gene product is nontoxic [56]. Most of the popular promoters which are used for recombinant expression in *E. coli* are not tightly regulated and even perform at high levels of expression during the growth phase. The most commonly used promoters include Trp, Tac, pL, lac and T7. For example, the hybrid trp-lac (tac) promoter may lead to instability and plasmid loss in the media. The lac promoter may be expressed as a fusion product with other promoters, including tac, pac and rat. The PL and PR promoters from lambda, which are heat-inducible, need to be induced at a lower temperature after shifting for native and basal folding. Even stronger promoters can be made by combining the lac promoter with those from T5 or T7 phages. The T7 system is very useful since the T7 RNA polymerase system is an exceptionally fast and powerful enzyme [56],[19],[57],[55].

The decisions of a desirable inducer concentration and induction time are vital elements to take into account to increase both cell growth and protein production. Upon induction, the host cell physiology and metabolism are affected directly by recombinant protein expression

in *E. coli*. These effects make it necessary to find an optimal induction time and inducer concentration which would impart positive effects [58].

Shitu et al. found that induction early in the exponential growth phase was preferred when expressing recombinant interleukin-13, although this resulted in a lower total cell density and a lower maximum specific growth rate. However, by expressing during the growth phase, the final quantity and quality of the heterologous protein were considerably influenced [53]. The production of protein was found to be higher at lower growth rates [58]. Induction during the mid-exponential growth phase was found to be the least beneficial to cell quality measured by cytoplasmic membrane depolarization [53]. The findings of Yildir and coworkers revealed that the late exponential growth phase was the ideal period for induction of restriction enzyme EcoRI from recombinant *E. coli* 294 [57]. Reports from Norsyahida et al showed that inducing during the late lag phase achieved a high yield of proteins [59],[58].

IPTG (Isopropyl β -D-1-thiogalactopyranoside) is a commonly used gratuitous inducer for expression of protein, which cannot be metabolized by the bacteria. The preferred concentration of IPTG differs from system to system. Wood and Peretti carried out the study of various IPTG concentrations from 0.01 to 7.5 mmol/L for expression of β -galactosidase mRNA [58]. The results showed that increasing the concentration of IPTG under 1 mmol/L led to a linear increase in the rate of synthesis of β -galactosidase mRNA [53]. However, adding additional IPTG did not contribute to stoichiometric growth in LacZ transcription. It was found by Yazdani et al. that a recombinant malarial antigen in *E. coli* may be totally induced by 1 mmol⁻¹ of IPTG. Comparing 1 mmol/L to 5 mmol/L of IPTG by A. Norsyahida et al, the former had a better effect towards the yield of BmR1 [58]. Therefore, higher concentrations of inducer were found to hamper the expression of a target protein, resulting in low production.

This may be due to a higher level of toxicity at increasing concentrations of IPTG that influence physiological activity and inhibit protein production [58]. On the other hand, a low IPTG concentration of 0.1 mmol/L contributed to a higher production of restriction enzyme EcoRI [54].

2.3.6 Acetate and other by-products

Media that contains a sufficient concentration of glucose is generally used for high-density cultivations that will produce large amounts of recombinant protein. However, growing *E. coli* under aerobic conditions with excess glucose results in the production of acidic by-products [60]. The major fermentation acids created by *E. coli* include acetate, formate, D-lactate and succinate. At neutral pH, the most highly produced fermentation acids are acetate and formate [61]. In a liquid culture, the acetate anion (Ac^-) and undissociated acetic acid (HAc) coexist, with their relative amounts based on pH. According to the Henderson-Hasselbalch equation, the concentration of HAc is approximately 0.56 mM per 100 mM of total acetate at pH 7.0. Additionally, the neutral HAc molecule can move freely through the cell membrane of *E. coli*, and may dissociate to Ac^- and H^+ intracellularly [62]. The production of acetate by *E. coli* under aerobic conditions is a prevalent phenomenon with reports depicting that acetate concentrations over 40 mM impart a negative impact on growth and the expression of foreign proteins. However, the maximum concentration of acetate appears in the absence of oxygen at a high growth rate [63].

The probable causes of acetate accumulation include a disequilibrium between metabolism and respiration with glucose, an overflow of carbon fed to the cell for excess biosynthesis, the presence of excess nicotinamide adenine dinucleotide hydrogen (NADH), the restriction of tricarboxylic acid cycle enzymes or an uncoupled metabolism [60]. There

are generally three different scenarios that lead to acetate formation during *E. coli* fermentations. Firstly, a restricted capacity in the oxygen uptake rate makes a great contribution to the accumulated acetate. In a continuous culture, acetic acid may be detected when the dilution rate approaches a specific threshold value that depends on the *E. coli* strain, growth conditions and the actual glucose concentration in the medium, as well as the overall substituents of the fermentation medium. For example, *E. coli* K12D1 excretes acetate at a lower growth rate in a complicated medium than in a chemically determined medium [64]. Secondly, the presence of excess NADH switches carbon flow to acetate. The third case is that both restricted capacities of the tricarboxylic acid (TCA) cycle and electron transport chain are the rate limiting steps to affect the produce of acetate [64],[63].

Acetate formation has some drawbacks. A concentration of acetate over approximately 1 g/L negatively affects cell growth and recombinant protein expression [65]. In addition, it may also destabilize intracellular proteins. The non-dissociated form of acetate can pass through the cell membrane easily to further accumulate in the medium. Therefore, acetate acts as a proton conductor that results in a decrease in the proton motive force. The medium also becomes acidic due to acetate accumulation. If the pH is lower than 5.0, proteins and DNA become irreversibly denatured, leading to cell lysis [65].

Many studies have been done to find strategies to decrease acetate accumulation. These methods mainly include optimization of fermentation conditions and alteration of the host organism. Approaches at the bioprocess level mostly involve culture composition and conditions optimized by managing parameters such as temperature, pH, DO content, agitation regimes, and volume, among others. It was suggested by Eiteman and Altman that the concentration of glucose in a culture should be limited, which is considered to be a viable

approach to reduce acetate formation [66]. Akesson and coworkers designed a glucose feeding strategy that automatically supplies glucose by controlling the dissolved oxygen content by altering the stirrer rate, decreasing acetate concentrations to below 60 mg/L (1.0 mM) [65]. By using alternative feedstocks such as glycerol, mannose or fructose, acetate accumulation may also be minimized. Any acetate that does accumulate may be separated from the culture by performing a dialysis fermentation. However, this strategy may also remove crucial nutrients along with acetate. This strategy does not involve the transfer of carbon to by-products, thus minimizing economic sinking. Although these approaches are widely used in industry, they are not the preferred solutions since they weaken maximum growth and production capacity [65].

Genetic modification that minimizes acetate formation should be emphasized. Strategies such as reducing the uptake rate of glucose and improving *E. coli* tolerance towards acetate on the genetic level have been reported. These methods depend on the modification of the central metabolism of *E. coli* [62].

2.3.7 GlcNAc

GlcNAc (N-acetylglucosamine) is a monosaccharide derivative of glucose. It serves as an essential constituent of bacterial cell wall peptidoglycan, fungal cell wall polymer chitin and the extracellular matrix of animal cells [67]. In humans, GlcNAc is also known as a precursor to the disaccharide units found in glycosaminoglycans such as chondroitin sulfate, hyaluronic acid and keratan sulfate, which is essential for maintaining and repairing cartilage and joint function [68]. Due to its diverse functional structure, GlcNAc is expressed on the cell surface and used for cell-to-cell interactions [69]. In addition, GlcNAc is a potential source of both carbon and nitrogen, but can only be used with other carbon sources. GlcNAc

has been shown to have an inhibitory effect on the cell growth of *Streptococcus sobrinus* and *Streptococcus mutans*, while also increasing the lag period and doubling time [70]. Many reports describe the enzymatic synthesis of less complex Neu5Ac from GlcNAc in *E. coli* [71][72][19][74].

2.3.8 Feeding strategies

The approach of carbon source feeding is vital to successfully obtain a high cell density culture, as it not only influences the maximum cell density, but also productivity and yield of the product. A HCDC is commonly performed under carbon-limiting conditions, as both overfeeding and underfeeding of carbon source impart negative effects. Feeding approaches can be classified into two kinds: feeding without any feedback control and feeding involving feedback control [75].

The feeding strategies that do not involve feedback control do not depend on any online equipment but include some simple feeding strategies such as a constant feed rate, a stepwise increase in feed rate and exponential feeding. These have been applied to achieve high-efficiency fed-batch fermentations of *E. coli* [76].

A constant feed rate involves adding concentrated carbon sources at a preset rate to the bioreactor. Because both the culture volume and cell population increase with time in the fermenter, the specific growth rate drops gradually, while the increase in cell density also deteriorates with time. Constant feeding is therefore an easy way to carry out protein production [75].

The increased feed rate method involves feeding the carbon source at a growing (stepwise, gradual or linear) rate that can boost cell growth by compensating for deficient nutrition when cell density is higher. This strategy implements a variety of feed rates at different stages of the

process [75].

If the feed rate of a carbon source increases with cell growth, there will be an exponential growth curve of *E. coli* during the culture's operational period. The exponential feed rate approach involves feeding the carbon source at an exponential rate that is designed to allow cell density to increase at a stable specific growth rate. It also has the benefit of lower rates of acetate formation by controlling the specific growth rate. If the constant specific growth rate is known, the feed rate can be calculated with equation (2-1) as follows [16]:

$$M_s(t) = F(t)S_F(t) \quad (2-1)$$
$$M_s(t) = \left(\frac{\mu}{Y_{X/S}} + m \right) X(t)V(t)$$
$$M_s(t) = \left(\frac{\mu}{Y_{X/S}} + m \right) X(t_0)V(t_0)\exp [\mu(t - t_0)]$$

$M_s(t)$: mass flow rate of the feed at time t (gh^{-1}),

$F(t)$: volume flow rate of the feed at time t (Lh^{-1}),

$S_F(t)$: concentration of the growth limiting nutrient in the feed at time t (gL^{-1}),

$Y_{X/S}$: biomass density on substrate (g(DCW)g^{-1}),

$X(t)$: biomass density in the bioreactor at time t (g(DCW)L^{-1}),

$V(t)$: volume of culture in the bioreactor at time t (L),

$X(t_0)$: initial biomass density of the biomass (g(DCW)L^{-1}),

$V(t_0)$: initial volume in the bioreactor (L),

μ : specific growth rate (h^{-1}),

m : specific maintenance coefficient ($\text{g(DCW)}^{-1}\text{h}^{-1}$)

t_0 : initial feeding time (h)

The equation is derived from a simple mass balance with an assumed constant cell density in the culture. The specific growth rate is commonly used from 0.1 to 0.3 h^{-1} in order to minimize acetate accumulation [16]. Due to an increasing cell concentration, mass transfer

limitations and other unexpected factors may result in the failure of this control strategy. The value of the maintenance coefficient, m , is normally too small to be considered for calculations. The yield coefficient, $Y_{x/s}$, gained from experimental batch cultures, is also a constant value.

In feedback-controlled feeding, the nutrient feed rate can be indirectly or directly controlled by online measuring equipment. Feeding can be managed by the analysis of pH, dissolved oxygen, agitation rate, the rate of carbon dioxide evolution, the respiratory quotient, the biomass density and the rate of metabolic heat generation [16].

Other advanced feeding strategies with feedback control schemes have been studied. DO-stat operations rely on the online analysis of DO levels in a fermentation culture [77]. Due to substrate exhaustion, metabolic activity slows and oxygen is consumed slowly. Finally, DO levels increase dramatically. The DO-stat method is used to control the addition of a carbon source within a feasible range by feeding automatically when DO levels increase above a set value. An ideal balanced DO-stat can guarantee adequate oxygen supplementation and prevent excess carbon source feeding. However, DO-stat responds more quickly to a depleted carbon source in a defined medium than in a complex medium because after consumption of the carbohydrate nutrient, other complex substrates may support further cell growth [78]. The use of pH-stat operation is based on findings that the consumption of substrate causes a rise in pH, which is the result of increasing concentrations of ammonium ions released by cells [79]. The rate of carbon dioxide evolution is approximately proportional to the rate at which the carbon source is consumed. In order to control feeding, the concentration of CO_2 in the gas effluent can be estimated online from a mass spectrometer. The respiratory quotient can be calculated with similar online measurements. The cell concentration is used to determine the nutrient feed rate with an online laser turbidimeter [75].

Direct feedback control has been developed by online estimation of substrate concentration, automatically modifying the concentration to approach a set point. For example, an online glucose analyzer may be used to adjust the concentration of glucose to 0.2 g/L [80]. However, it is difficult to estimate the critical concentration of glucose ahead of time, as it differs in various strains. Furthermore, the concentration may change over time, so the expected set point of the controller is difficult to predict [81]. A temperature-limited fed-batch strategy is described as follows. The concentration of glucose should be overloaded in the fermenter. The dissolved oxygen concentration in the culture is controlled by the temperature, which in this case is the limiting parameter of fermentation. One benefit of this technique is the formation a low level of endotoxins. The accumulation of endotoxins has been reported to avoid a strict limitation of glucose [82]. To overcome the lack of online sensors used for analysis, some mathematical models are used to estimate concentrations in real time.

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Chapter 3: Effect of cultivation conditions in shake flask experiments

Abstract

Shake flask experiments were performed to investigate the carbon sources and induction temperatures that may be suitable for legionaminic acid production in *Escherichia coli* BRL04. Three different carbon sources, including glycerol, glucose and fructose were tested to observe the effect on legionaminic acid production. Results indicate that the highest titer was obtained using glycerol. Furthermore, the induction temperature was tested at 32°C, 34°C and 37°C. The results showed that when *E. coli* was induced at 34°C, the highest concentration of legionaminic acid was produced. Both of these parameters were tested in triplicate and the results for each were averaged. Shake flask experiments were studied to screen for suitable conditions for bioreactor experiments.

Keywords: legionaminic acid, *Escherichia coli*, shake flask cultivation

3.1 Introduction

When designing an industrial fermentation, the increasing requirement for cost and time efficient process development has resulted in a strong interest in shake flasks [1]. Probably more than 90% of sub-merged culture experiments in biotechnology are performed in these lab-scale culture vessels [2]. Shake flasks have been frequently used for microbial research purpose such as recombinant protein production due to their simple set-up and operation, low cost as well as their availability to conducted many experiments simultaneously [3], [4], [5]. Some tasks such as media optimization, screening of strains, strain development, elucidation of metabolic pathways, investigations of fundamental growth conditions and basic growth kinetics are often performed in shake flasks [3], [6]. The experimental studies in these vessels are usually the first step in basic investigations and in developing a large-scale process of fermentation [6]. The effects of momentum and heat transfer in shake flasks are affected by the contact area between the liquid and the friction zone of the rotating bulk liquid. Furthermore, mass transfer is affected by the mass exchange area which is the wet wall exposed to the surrounding air [7]. Shake flasks are sealed with various plugs to prevent contamination as well as modified with baffles to offer adequate aeration and shear stress [3], [6].

The purpose of this study was to evaluate the culture of legionaminic acid using a novel biosynthetic pathway by recombinant *E. coli*. In this work, 168 h-induction shake flask fermentation experiments were carried out to select for suitable fermentation conditions aimed for further study in bioreactor experiments. Different carbon sources and various induction temperatures were investigated in shake flask cultures for scale-up study. The effects of three different carbon sources on legionaminic acid production and cell growth were tested. In

addition, the impact of induction temperature on the final concentration of legionaminic acid was also investigated.

3.2 Materials and Methods

3.2.1 Bacterial strain

A strain of recombinant *E. coli* BRL04 capable of producing legionaminic acid was provided by the lab group of Dr. Christopher N. Boddy from the Department of Chemistry at the University of Ottawa. Modifications of this strain include knock-outs of two native legionaminic acid catabolic pathways, as well as the addition of an exogenous synthetic pathway by means of an ampicillin-resistant plasmid and a chloramphenicol-resistant plasmid, using the T7 promoter [8]. This strain was sub-cultured on LB agar (10 g/L tryptone, 5 g/L yeast extract, 10 g/L sodium chloride, 15 g/L agar) plates with 100 µg/mL ampicillin and 30 µg/mL chloramphenicol, and cultured at 37°C in an incubator for 12 h, then stored at 4°C for no longer than two weeks. For long-term storage, the strain was stored frozen at -80°C in 25% glycerol.

3.2.2 Media preparation

All media components, fermentation reagents, as well as chemicals and standards used in HPLC analysis were purchased from Fisher Scientific (Pittsburgh, PA, USA). Antifoam (contains 100% active components and is a mixture of non-silicone organic defoamers in a polyol dispersion) and N-acetyl-D-glucosamine were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Pre-cultures were prepared in 1 mL of LB media in 50-ml centrifuge tubes, supplemented with 100 µg/mL of ampicillin and 30 µg/mL of chloramphenicol. The cultures were shaken at

250 rpm and 37°C on a rotary shaker for 18 h to propagate prior to fermentation.

Flask media contained 12.24 g/L K₂HPO₄, 6.00 g/L KH₂PO₄, 4.00 g/L (NH₄)₂HPO₄, 1.077 g/L MgSO₄·7H₂O, 0.25% (w/v) casitone and 0.5% (v/v) glycerol (or 2% (w/v) carbon sources including glycerol, glucose and fructose) [8]. The solution was autoclaved for 20 min at 121°C. Next, 100 µg/mL ampicillin, and 30.0 µg/mL chloramphenicol were added separately by filtration through a 25-mm syringe filter with a 0.22 µm pore size membrane. After cooling, all solutions were combined prior to inoculation. All constituents for the feeding, including 0.3% (v/v) glycerol (unless otherwise specified) and 0.1% (w/v) GlcNAc were autoclaved individually for 20 min at 121°C [9].

3.2.3 Cultivation conditions

Production cultures were grown at 37°C, 250 rpm, until an OD₆₀₀ of 0.5 (middle exponential phase) was reached. At this point, 0.2 mM IPTG (final concentration) and 0.1% GlcNAc were added to induce legionaminic acid production. The incubation temperature was decreased to 30°C (unless otherwise specified) for production. Production cultures were grown for 168 h and supplemented with 0.3% (v/v) glycerol (unless otherwise specified), 0.1% (w/v) GlcNAc as well as the necessary antibiotics at 0, 24, and 48 h post-induction. After 72 h following induction, GlcNAc and antibiotics were added again to the culture. At 96 and 120 h post-induction, only antibiotics were added to the culture. The production experiment was arrested after 168 hours. During the course of each experiment, 1-mL aliquots were drawn, their OD₆₀₀ was measured, and the remainder was then centrifuged at 13,000 rpm for 10 min.

Shake flask experiments were carried out to investigate the effect of induction temperatures on the expression of legionaminic acid in *E. coli*. Cultures of 20 mL volumes were in 250-mL flasks with breathable foam stoppers at an agitation rate of 250 rpm. The

temperature at batch and fed-batch phases prior to induction was maintained at 37°C. Carbon sources including glycerol, glucose and fructose were each tested. Induction temperatures of 32°C, 34°C and 37°C were each evaluated. All experiments were conducted in triplicate.

3.2.4 Analytical methods

Cell growth was monitored by measuring the optical density of samples at a wavelength of 600 nm with a spectrophotometer (UItraspec 60 DBL BEAM 2NM, Biochrom Ltd, England). Samples were serially diluted and referenced using blank media.

The concentrations of legionaminic acid and GlcNAc in the medium throughout the fermentation experiments were measured by HPLC analysis (Dionex with RI and UV-VIS detectors, Agilent Technologies 1200 Agilent 1200, Santa Clara, CA, USA). The system used an ion exclusion column (Aminex HPX-87H, 300mm ×7.8 mm, I.D, London, UK) with a cationic micro-guard column purchased from BioRad Labs (Richmond, CA, USA). The mobile phase was composed of 3 mM sulphuric acid at a flowrate of 0.6 mL/min. Prior to analysis, samples were diluted 10-fold. Legionaminic acid and GlcNAc were detected by UV absorbance at a wavelength of 202 nm and a temperature of 60°C. The peaks were integrated using HPLC solution software (Agilent ChemStation).

3.3 Results and discussion

3.3.1 Effects of various carbon sources

In order to find the optimal carbon source for legionaminic acid production in *E. coli* BRL04, three different carbon sources including glycerol, glucose and fructose were tested in triplicate, the results for each of which were averaged. The initial culture was established at 20 g/L of each respective carbon source. The induction temperature was 32°C. During the

fermentations, a solution of the carbon source (0.3% (v/v)) was fed three times to support cell growth. All experiments were stopped 168 h after induction. For the activity assays, cell samples were saved at the end of the process.

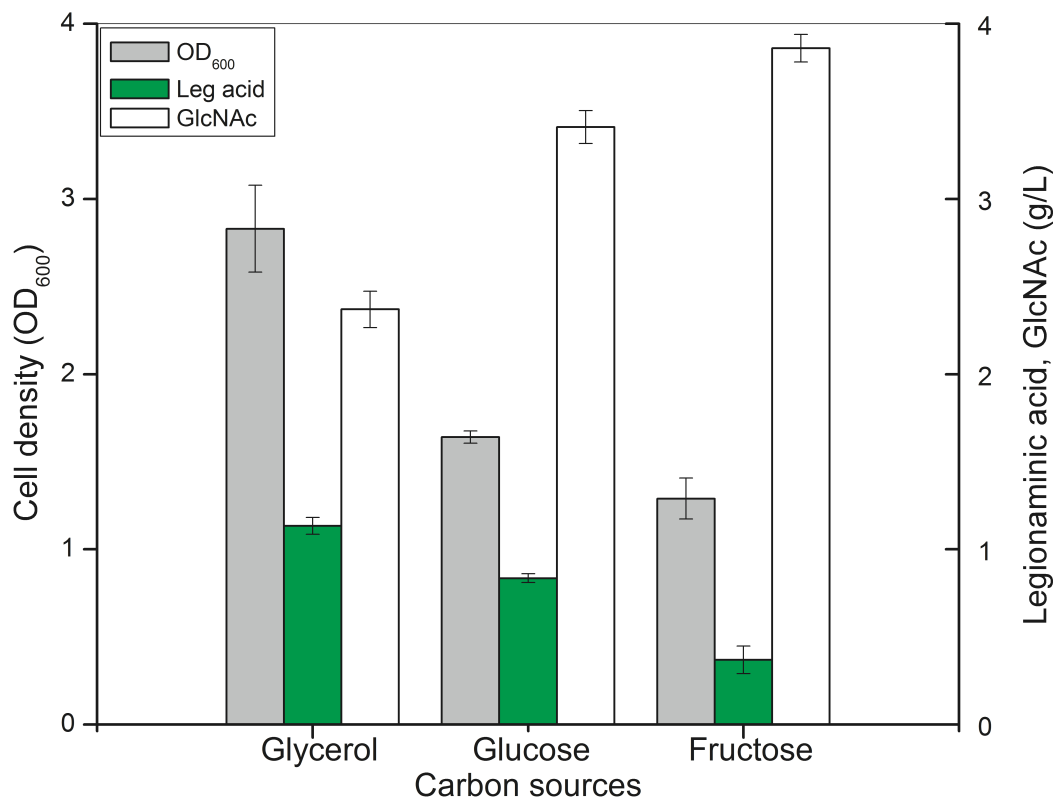


Figure 3.1 Effect of carbon source on cell growth, legionaminic acid production and residual GlcNAc in shake flasks. (average \pm std dev, n=3)

The results from Figure 3.1 show that glycerol cultures performed better than the glucose and fructose cultures tested in terms of both optical density and legionaminic acid production, which were 2.83 ± 0.25 and 1.13 ± 0.05 g/L, respectively. The legionaminic acid production of glucose and fructose were 0.83 ± 0.02 g/L and 0.37 ± 0.08 g/L, separately. Higher concentrations of GlcNAc remained when *E. coli* grew on fructose (3.86 ± 0.08 g/L) and glucose (3.40 ± 0.09 g/L) than when it grew on glycerol (2.24 ± 0.10 g/L) as shown in Table

3.1. It can be seen that a lower residual concentration of GlcNAc would be better in terms of utilization efficiency. The effects of carbon source on cell growth and legionaminic acid production appear to be similar, both of which are maintained at low levels by fructose.

Table 3.1 Effects of carbon sources on legionaminic acid production (average \pm std dev, n=3)

Parameters	Carbon sources		
	Glycerol	Glucose	Fructose
Induction duration time (h)	168	168	168
Maximum cell density (OD ₆₀₀)	2.83 \pm 0.25	1.64 \pm 0.03	1.29 \pm 0.12
GlcNAc residue (g/L)	2.24 \pm 0.10	3.40 \pm 0.09	3.86 \pm 0.08
Maximum Legionaminic acid concentration (g/L)	1.13 \pm 0.05	0.83 \pm 0.02	0.37 \pm 0.08

It is generally known that *E. coli* can grow on a wide range of carbon sources. The formation of acidic by-products such as acetate produced through oxidative phosphorylation and the tricarboxylic acid cycle (TCA cycle) would suppress the growth of *E. coli* and decrease the production of legionaminic acid [10]. As a glucose structural isomer, fructose may offer a reasonable alternative for glucose, since its uptake and utilization are more tightly regulated [12]. Glycerol, as an energy-poor carbon source, is an ideal replacement of glucose as a feedstock due to its availability, low price and a high degree of reduction. Specifically, when *E. coli* grows aerobically on glycerol, the feedstock is integrated into the central metabolism as dihydroxyacetone phosphate which can take part in both gluconeogenic and glycolytic processes. The specific growth rate decreased and low level or no acetate production is measured when *E. coli* is cultured on glycerol [11].

3.3.2 Effect of induction temperature

In order to determine a suitable induction temperature for legionaminic acid production in *E. coli* BRL04, three different temperatures (32°C, 34°C, 37°C) were tested in triplicate with glycerol as carbon source. Production cultures were grown at 37°C to propagate the cell concentration. When the optical density of each culture approached approximately equal levels, cultures were induced. All experiments were arrested 168 h after induction. For the activity assays, cell samples were saved at the end of the process.

The effect of induction temperature on cell density, the concentration of legionaminic acid and the residual concentration of GlcNAc in each case are shown in Figure 3.2. At an induction temperature of 34°C, the highest titer of legionaminic acid was achieved at 1.80 ± 0.21 g/L. The concentration of product at 32°C (1.08 ± 0.14 g/L) was found to be similar with that at 37°C (0.85 ± 0.03). As a substrate for the production of legionaminic acid, GlcNAc concentrations are expected to decrease when more product is produced [13]. More GlcNAc should be found to be consumed with the reduction of temperature. Higher concentrations of GlcNAc remained at 37°C (3.23 ± 0.35 g/L) and 34°C (2.16 ± 0.28 g/L) than at 32°C (0.44 ± 0.03 g/L) as shown in Table 3.2.

It is interesting that although the substrate was consumed at a greater rate, the production of legionaminic acid was limited at 32°C. The concentration of legionaminic acid was found to display a different trend, possibly because GlcNAc is also a potential source of both carbon and nitrogen [14]. While in this study, the *nagA* gene of *E. coli* BRL04 encoding catabolic pathways for GlcNAc consumption was successfully knocked out. Due to the inability to control pH and dissolved oxygen tension in shake flasks, the consequences of more overflow metabolism at 32°C probably a drift of pH or by-product inhibition on legionaminic acid

formation [4], [15].

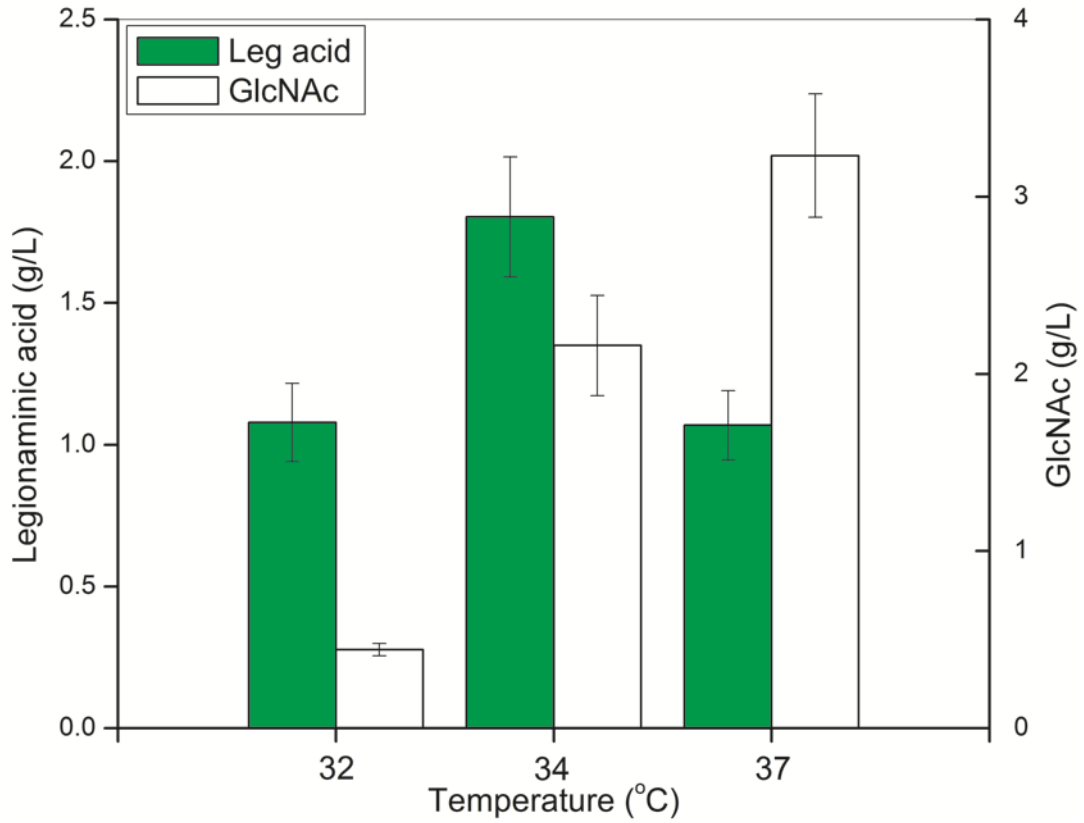


Figure 3.2 Effect of induction temperature on legionaminic acid production and residual concentration of GlcNAc in shake flasks. (average \pm std dev, n=3)

Table 3.2 Effects of the induction temperature on legionaminic acid production (average \pm std dev, n= 3)

Parameters	Induction temperature (°C)		
	32	34	37
Induction duration time (h)	168	168	168
GlcNAc residue (g/L)	0.44 \pm 0.03	2.16 \pm 0.28	3.23 \pm 0.35
Maximum Legionaminic acid concentration (g/L)	1.08 \pm 0.14	1.80 \pm 0.21	0.85 \pm 0.03

3.4 Conclusions

This is the first study indicating how culture conditions can affect the degree in legionaminic acid production by recombinant *E. coli*. Here, shake flask culture experiments have been performed to select the most suitable fermentation conditions including carbon source and induction temperature. Both of these parameters were tested in triplicate. These results showed that both the highest optical density (2.83 ± 0.05) and concentration of legionaminic acid (1.13 ± 0.10 g/L) were obtained as well as the lowest concentration of GlcNAc that was retained in a glycerol media compared to glucose and fructose. In addition, the induction temperature was tested at 32°C, 34°C and 37°C. At 34 °C, the highest concentration of legionaminic acid was produced, which is 1.80 ± 0.21 g/L. A better understanding of how carbon source and induction temperature effect the ability of *E. coli* to produce legionaminic acid. Then, shake flask experiments were studied to screen for suitable conditions for bioreactor experiments.

3.5 References

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Chapter 4: Optimization of bioreactor fermentation conditions

Abstract

In this study, legionaminic acid was produced by recombinant *Escherichia coli* BRL04 in a 5-L bioreactor using fed-batch cultivations with pH-stat and DO-stat control. Various fermentation conditions were investigated to identify the optimal induction temperature, pH, dissolved oxygen content (DO), induction optical density and GlcNAc feed rate for the production of legionaminic acid. The highest-performing results were obtained at an induction temperature of 32°C, a pH 7.2, a dissolved oxygen content of 40%, a GlcNAc feed rate of 0.3 ml/min and an induction optical density of 23. With all parameters fully optimized, the final titer of legionaminic acid was found to be 5.54 g/L while the yield and productivity were 73.29% and 0.092 g/L, respectively.

Keywords: legionaminic acid, recombinant *Escherichia coli*, fed-batch cultivation, fermentation conditions

4.1 Introduction

High cell density cultivation of recombinant *Escherichia coli* is an attractive biotechnological technique for industrial bioprocesses to produce heterologous proteins and metabolic products at high yields and titers [1]. It carries the benefits of improving the cost-effectiveness of a process by decreasing the volumes of culture and wastewater [2]. High cell densities are achieved with defined medium that contains a considerable amount of carbon source. The formulation of a medium should be desirable for both cell growth and protein expression [3]. Glycerol-based media are often used in high cell density cultures to fine-tune the cellular uptake of carbon and to restrict excessive acetate formation [4][5]. Fed-batch fermentation is a preferred strategy successfully applied to different production scales for large concentrations of cells and products, achieving relatively high cell densities and high productivities of recombinant proteins. A high cell concentration is generally obtained with fed-batch fermentation under strictly controlled culture conditions. These conditions typically avoid a relatively high (10 g/L) accumulation of acetate, which is known to have an inhibitory impact on cell growth and protein expression [6]. It has been reported that composition and feeding strategies of nutrients and fermentation parameters such as temperature, pH and dissolved oxygen should be optimized [7][8].

This is the first study involving the scale-up production of legionaminic acid by high cell density fermentation processes. In this work, high cell density fermentation using recombinant *E. coli* BRL04 was carried out in a 5-L bioreactor under fed-batch culture with pH-stat and DO-stat controls. The final process was optimized by determining the effects of induction temperature, pH, dissolved oxygen content, induction optical density and the feed rate of GlcNAc on the production of legionaminic acid. Finally, high titer, yield and productivity of

legionaminic acid were achieved after optimization. This work is hoped to accelerate research into the production of legionaminic acid for medical and pharmaceutical purposes and for further study of glycobiology.

4.2 Materials and Methods

4.2.1 Bacterial strain

A strain of recombinant *E. coli* BRL04 capable of producing legionaminic acid was provided by the lab group of Dr. Christopher N. Boddy from the Department of Chemistry at the University of Ottawa. Modifications of this strain include knock-outs of two native legionaminic acid catabolic pathways, as well as the addition of an exogenous synthetic pathway by means of an ampicillin-resistant plasmid and a chloramphenicol-resistant plasmid, using the T7 promoter [8]. This strain was sub-cultured on LB agar (10 g/L tryptone, 5 g/L yeast extract, 10 g/L sodium chloride, 15 g/L agar) plates with 100 µg/mL ampicillin and 30 µg/mL chloramphenicol, and cultured at 37°C in an incubator for 12 h, then stored at 4°C for no longer than two weeks. For long-term storage, the strain was stored frozen at -80°C in 25% glycerol.

4.2.2 Media preparation

All media components, fermentation reagents, as well as chemicals and standards used in HPLC analysis were purchased from Fisher Scientific (Pittsburgh, PA, USA). Antifoam (contains 100% active components and is a mixture of non-silicone organic defoamers in a polyol dispersion) and N-acetyl-D-glucosamine were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Pre-cultures were prepared by inoculating a single colony in a 1000-ml shake flask

containing 300 ml of LB media, supplemented with 100 µg/mL of ampicillin and 30 µg/mL of chloramphenicol. The cultures were shaken at 250 rpm and 37°C on a rotary shaker for 18 h.

Fed-batch experiments were performed in a glycerol-based medium which contained 20.00 g/L glycerol, 12.24 g/L K₂HPO₄, 6.00 g/L KH₂PO₄, 4.00 g/L (NH₄)₂HPO₄, and 1.077 g/L MgSO₄·7H₂O [9]. The solution was autoclaved for 20 min at 121°C. Next, 4.5 mg/L thiamine, 100.0 µg/mL ampicillin, 30.0 µg/mL chloramphenicol, and 10.0 mL/L of a trace metal solution were added by filtration through a 25-mm syringe filter with 0.22 µm pore size membrane. The trace metal composition was 15.0 g/L FeCl₃·6H₂O, 2.0 g/L ZnCl₂·4H₂O, 2.0 g/L CaCl₂·6H₂O, 2.2 g/L ZnSO₄·7H₂O, 0.5 g/L MnSO₄·4H₂O, 1.9 g/L CuSO₄·5H₂O, 2.0 g/L Na₂MoO₄·2H₂O, 4.0 g/L CoCl₂·6H₂O, 0.5 g/L H₃BO₃ and 100 mL/L concentrated HCl [10]. After cooling, 0.1 mL/L of antifoam reagent was added to the media prior to inoculation. All separately fed constituents including 40% (v/v) glycerol and 100 g/L GlcNAc were autoclaved individually for 20 min at 121°C before feeding [11].

4.2.3 Fed-batch bioreactor cultivation conditions

A 5-L fermentor (BioFlo 320, Eppendorf, Hamburg, Germany), which is fitted with a 7.04 cm-diameter Rushton turbine for mixing, equipped with pH and DO probes (Mettler Toledo, Switzerland) and three peristaltic pumps for addition of feeding solutions was used for fed-batch experiments. The schematic diagram of the fermenter system is shown in Figure 4.1. The fermenter was also equipped with a stable state temperature controller. This was coupled with a fast response thermocouple that provided a range of $\pm 0.4^\circ\text{C}$ to the controller. The media temperature was monitored by the thermistor sensor inserted into the stainless steel thermowell in the culture, providing constant feedback to the controller to automatically

respond to temperature fluctuations. Changes in temperature were achieved via water recirculation. The pH was controlled by addition of ammonium hydroxide based on changing conditions in a culture. Proper calibration of the pH electrode is necessary prior to being autoclaved. The pH controller can within a control range of 7.0 to 7.6. The “dead band” is used to control the addition of base, maintaining a pH fluctuation of only 0.01 [16]. The DO content was controlled in the range of 20% to 40%. DO control was maintained by a controller along with a variety of cascades. During growth, the fermentation parameters were controlled and data was collected by New Brunswick BioCommand software (Eppendorf, Hamburg, Germany). An example of a controlled culture condition summary is shown in Figure 4.2.

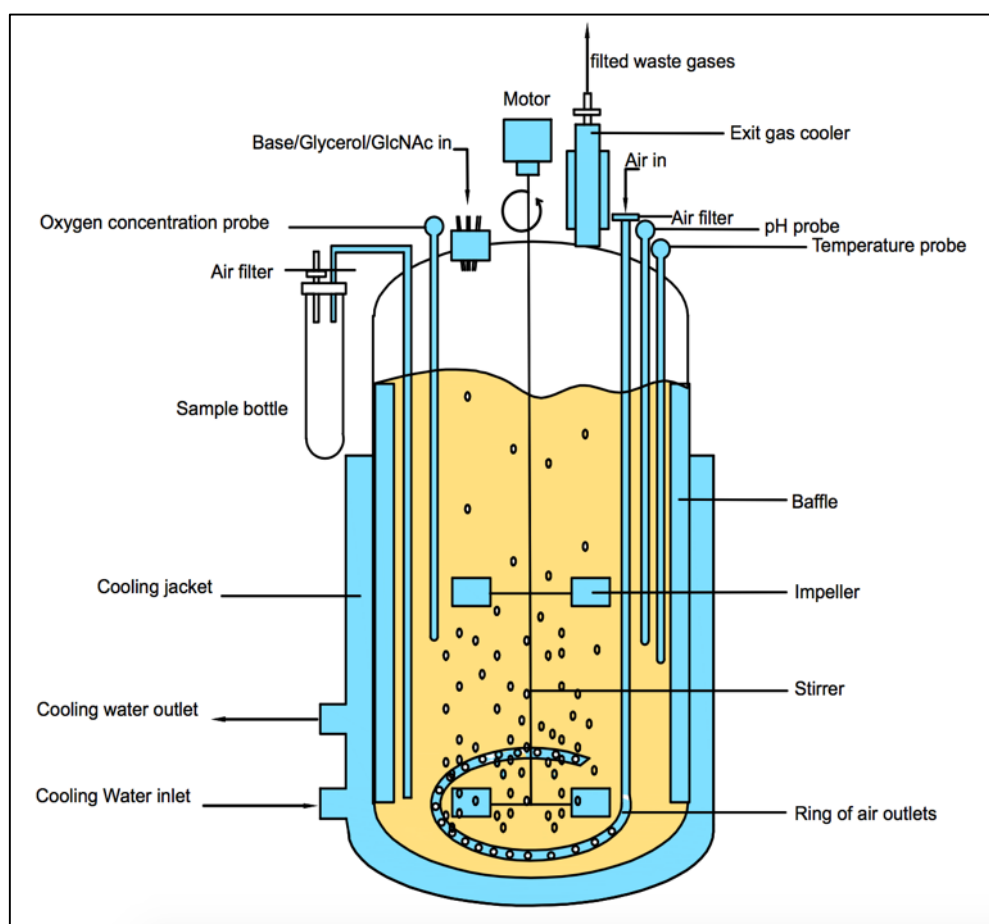


Figure 4.1 A schematic diagram of the fermenter system

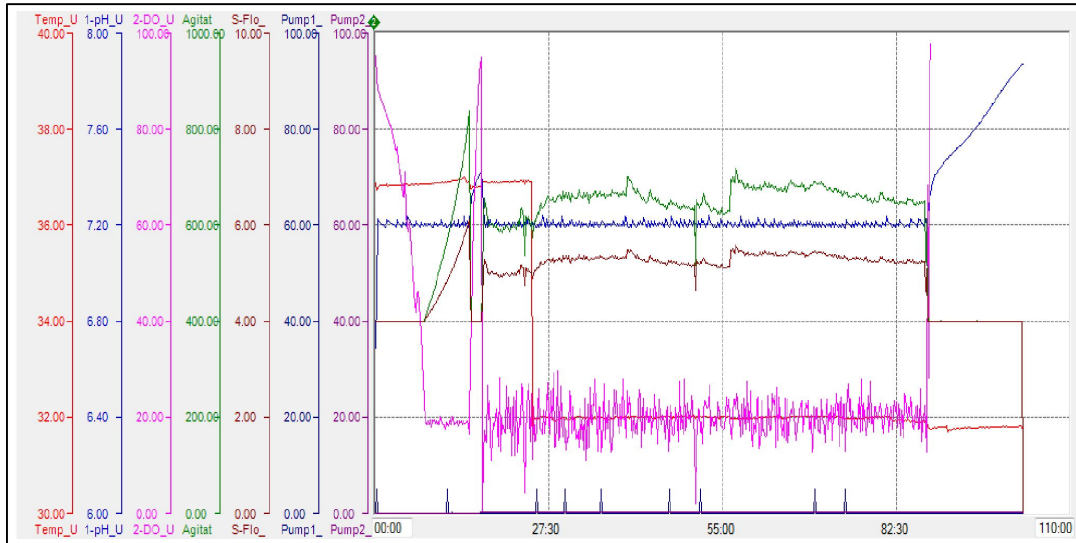


Figure 4.2 Typical culture conditions summary during fermentation experiment

The fermenter containing 3 L of media was inoculated with 300 mL of exponential-phase pre-culture cells. Fed-batch experiments were performed at 37°C, a pH of 7.4 (unless otherwise specified) by adding 30% (v/v) ammonium hydroxide to the culture when necessary, and a DO content of 30% (unless otherwise specified) by cascading the agitation rate (from 400 to 900 rpm) and the air flowrate (from 4 to 7 L/min). After approximately 16 h of batch operation, the initial glycerol was usually completely consumed, as shown by a sharp increase in DO (Figure 4.3). At this point, 40% (v/v) glycerol was pumped to the culture. During the fed-batch phase, glycerol was supplied at a constant rate until the OD_{600} reached the desired value. Induction using 0.2 mM IPTG (final concentration) was done after decreasing the temperature to 30°C (unless otherwise specified). At the same time, 150 mL of 100 g/L GlcNAc was added to the culture at a rate of 0.16 mL/min (unless otherwise specified). The summary of bioreactor fermentation conditions is shown in Table 4.1. Culture samples (10 mL) were regularly withdrawn to monitor cell growth by measuring the absorbance at 600 nm (OD_{600}). The samples were then centrifuged at 13,000 rpm for 10 min (Sorvall Legend Micro

21, Thermo Fisher Scientific, Germany). The biomass pellets were separated from the sample and the supernatants kept frozen at -20°C for long-term storage.

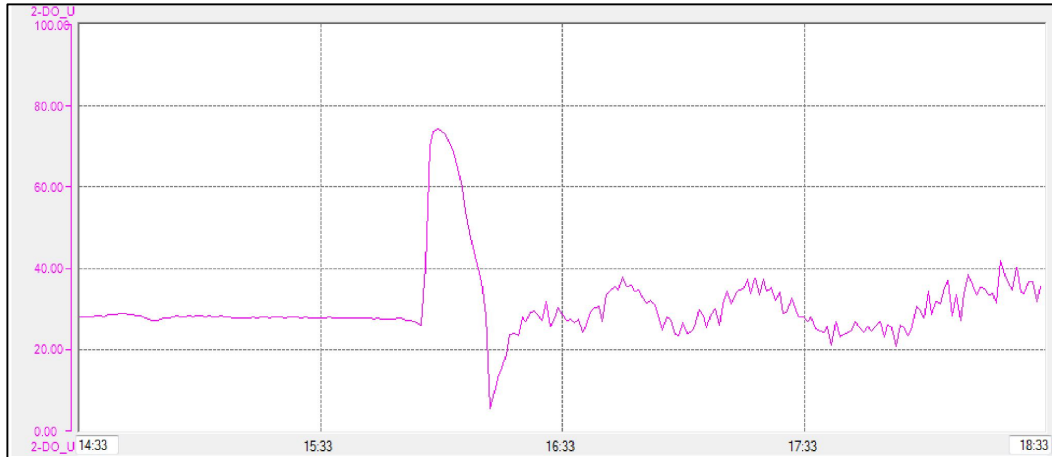


Figure 4.3 Sharp increase of dissolved oxygen upon glycerol depletion

Table 4. 1 Summary of bioreactor fermentation conditions

Parameter	Induction			Induction optical density	GlcNAc feed rate (ml/min)
	temperature ($^{\circ}\text{C}$)	pH	DO		
Induction temperature($^{\circ}\text{C}$)	31-37	32	32	32	32
pH	7.4	7.0-7.6	7.2	7.2	7.2
DO	30%	30%	20-40	30%	40%
Induction optical density	20-25	20-25	20-25	19-40	20-25
GlcNAc feed rate (ml/min)	0.16	0.16	0.16	0.16	0.16-0.50
Glycerol feed rate (ml/min)	0.2	0.2	0.3	0.3	0.3

4.2.4 Analytical methods

Cell growth was monitored by measuring the optical density of samples at a wavelength of 600 nm with a spectrophotometer (Ultraspac 60 DBL BEAM 2NM, Biochrom Ltd, England). Samples were serially diluted and referenced using blank media.

The concentrations of legionaminic acid and GlcNAc in the medium throughout the fermentation experiments were measured by HPLC analysis (Dionex with RI and UV-VIS detectors, Agilent Technologies 1200 Agilent 1200, Santa Clara, CA, USA). The system used an ion exclusion column (Aminex HPX-87H, 300mm ×7.8 mm, I.D, London, UK) with a cationic micro-guard column purchased from BioRad Labs (Richmond, CA, USA). The mobile phase was composed of 3 mM sulphuric acid at a flowrate of 0.6 mL/min. Prior to analysis, samples were diluted 10-fold. Legionaminic acid and GlcNAc were detected by UV absorbance at a wavelength of 202 nm and a temperature of 60°C. The peaks were integrated using HPLC solution software (Agilent ChemStation).

The concentration of glycerol in the culture during fermentation was determined by an offline biochemistry analyzer (YSI 2900, Xylem Inc, USA).

4.3 Results and discussion

4.3.1 Effect of induction temperature

To determine an appropriate induction temperature to achieve both a high cell density and a high titer of legionaminic acid, fermentation was carried out at induction temperatures of 31°C, 32°C, 33°C, 34°C and 37°C. Production cultures were grown during the period prior to induction at 37°C to gain sufficient biomass, as 37 °C is the optimal moderate temperature for maximal growth of *E. coli* in many media [12],[13]. Other conditions were unchanged,

with pH at 7.4, a DO level of 30%, a glycerol feed rate of 0.2 mL/min, and a substrate feed rate of 0.16 mL/min, with mid-exponential phase.

Figure 4.4 to Figure 4.8 display the concentration and optical density profiles of fed-batch fermentations over time carried out at different induction temperatures (31°C, 32°C, 33°C, 34°C and 37°C, respectively). Trends in cell growth were similar at each temperature. In the early exponential phase of cultivation (before approximately 16 h), the cell density was found to increase slightly, before growing exponentially following feeding with glycerol. During the post-induction period, the cell density gradually rose. The highest final OD₆₀₀ of 42.4 was gained at an induction temperature of 33°C. Final optical densities at other induction temperatures reached approximately 35.

The concentration profiles for GlcNAc during the fermentation process are presented in these figures (Figure 4.4 – Figure 4.8). There was typically a rapid upward trend of the concentration of GlcNAc at the beginning of the induction phase, which then gradually decreased after peaking at approximately 2.5 g/L at approximately 40 h. Under these conditions, the substrate was never completely depleted, except at 37°C. But the legionaminic acid concentration are not the highest at 37°C, maybe the substrate was mainly used for high level of cell growth. At 32°C, the lowest concentration of the residual GlcNAc was nearly zero (Figure 4.5). These results revealed that GlcNAc may be consumed most efficiently at an induction temperature of 32°C.

From these five figures, the concentration profiles of legionaminic acid are also shown. At all temperatures, the production of legionaminic acid typically rose, although to differing degrees. The highest amount of legionaminic acid produced (4.22g/L) was obtained at an induction temperature of 32°C. Only 1.71 g/L of legionaminic acid was produced at 31°C.

In terms of the concentration of legionaminic acid and optical density, the overall results for induction in a temperature range of 31-37°C are shown in Figures 4.4 to 4.8. The highest production of legionaminic acid was found to be 4.22 g/L, obtained at 32°C, while the maximum optical density obtained was 42.4 at 33°C. The pronounced temperature optimum (32°C) for legionaminic acid production was particularly striking. However, the optimal temperature for cell growth was found to be higher at 33°C.

Comparison of the overall results in Figure 4.9 shows that the optimal induction temperature for highest level of legionaminic acid production is 32°C, where 55.89% of yield was obtained after 99h production with a productivity of 0.056 g/L (Table 4.2). The replicates were performed at induction temperature of 32°C, 33°C and 34°C. These results suggest that the induction temperature not only affects the cell's naturally expressed proteins, but also those that are recombinant. A lower temperature is better for controlling gene expression and heterologous proteins production [14]. The biosynthesis of legionaminic acid was controlled by multiple enzymes; hence, the optimal induction temperature is a comprehensive result.

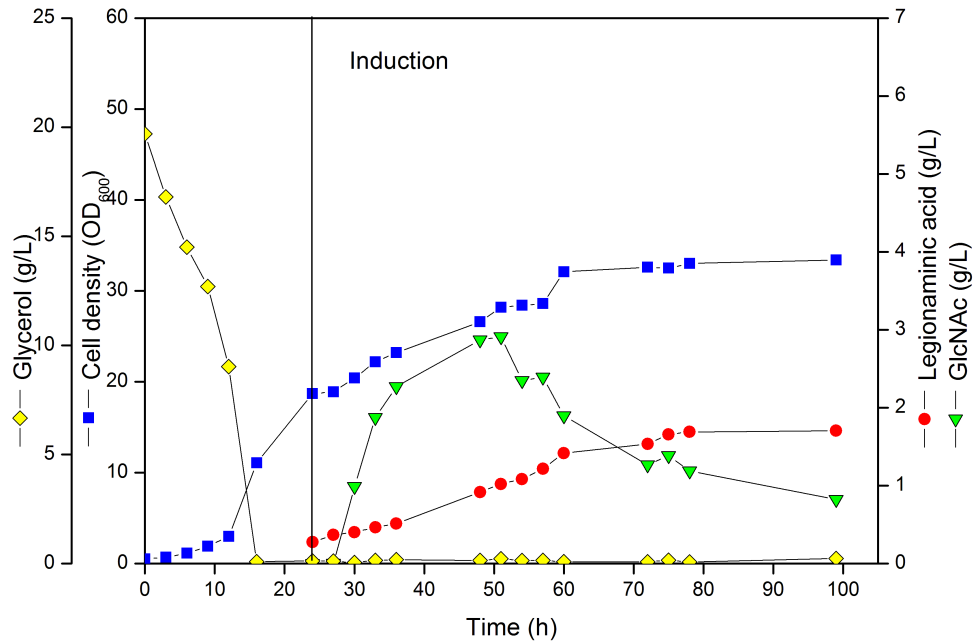


Figure 4.4 Curves for cell growth and concentrations of glycerol, legionaminic acid and GlcNAc at an induction temperature of 31°C

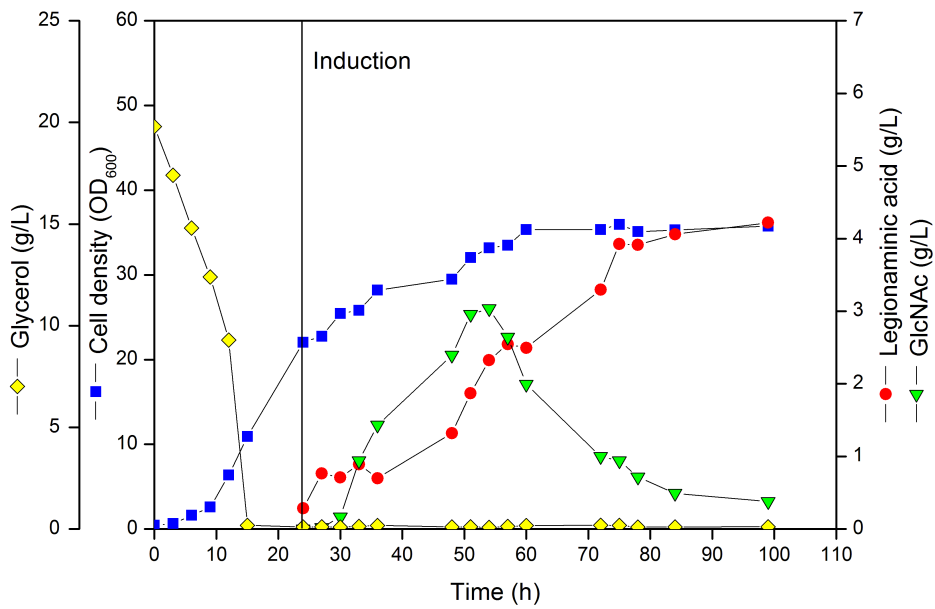


Figure 4.5 Curves for cell growth and concentrations of glycerol, legionaminic acid and GlcNAc at induction temperature of 32°C

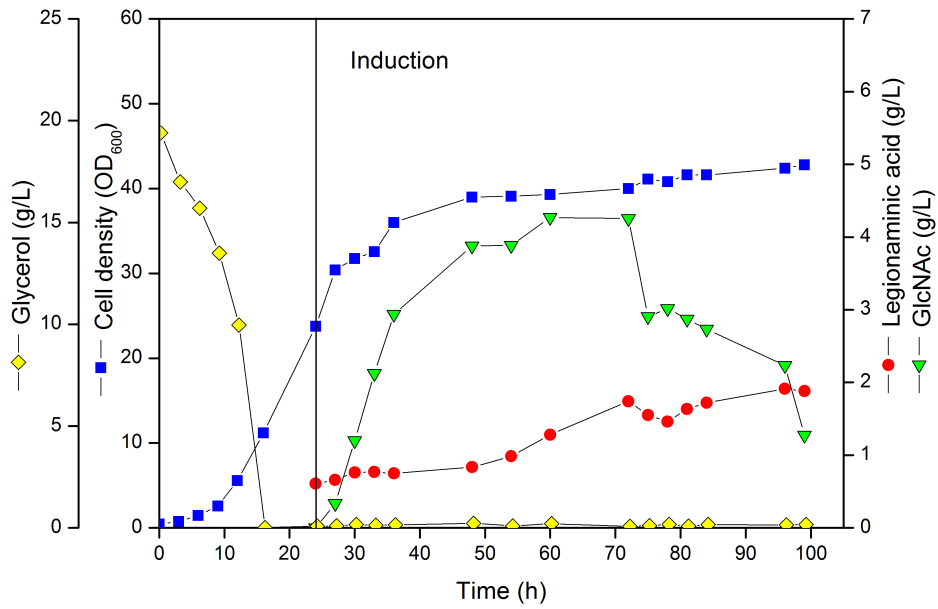


Figure 4.6 Curves for cell growth and concentrations of glycerol, legionaminic acid and GlcNAc at induction temperature of 33°C

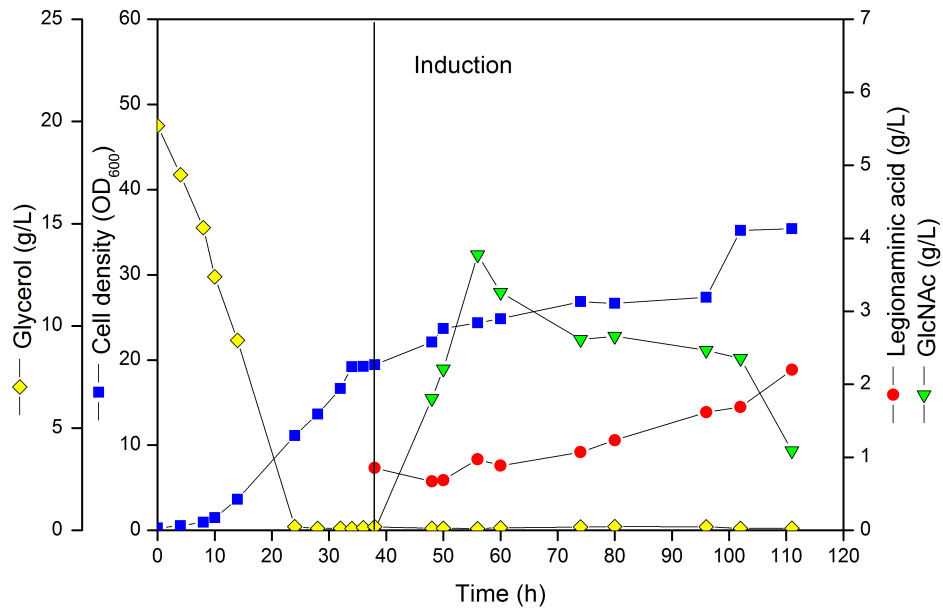


Figure 4.7 Curves for cell growth and concentrations of glycerol, legionaminic acid and GlcNAc at induction temperature of 34°C

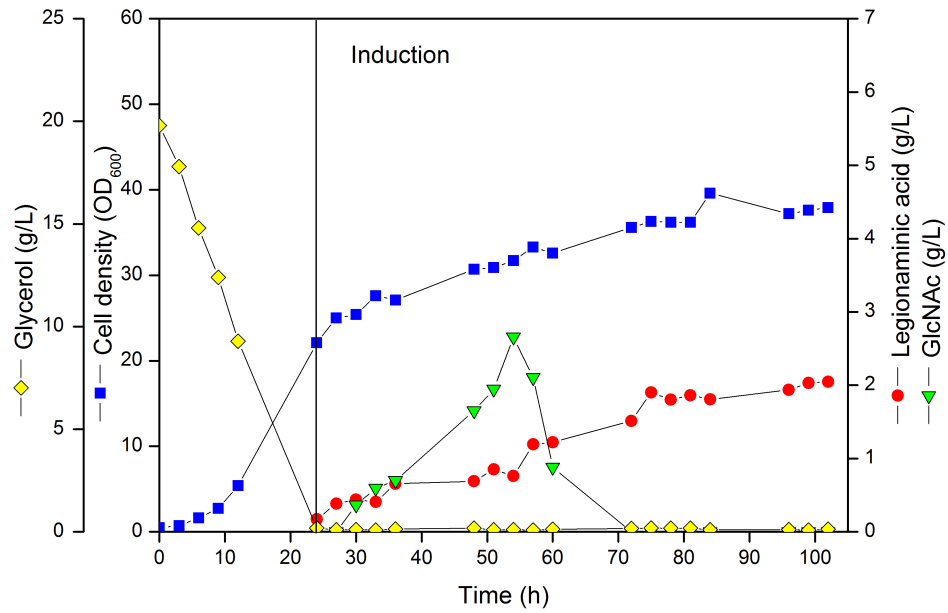


Figure 4.8 Curves for cell growth and concentrations of glycerol, legionaminic acid and GlcNAc at induction temperature of 37°C

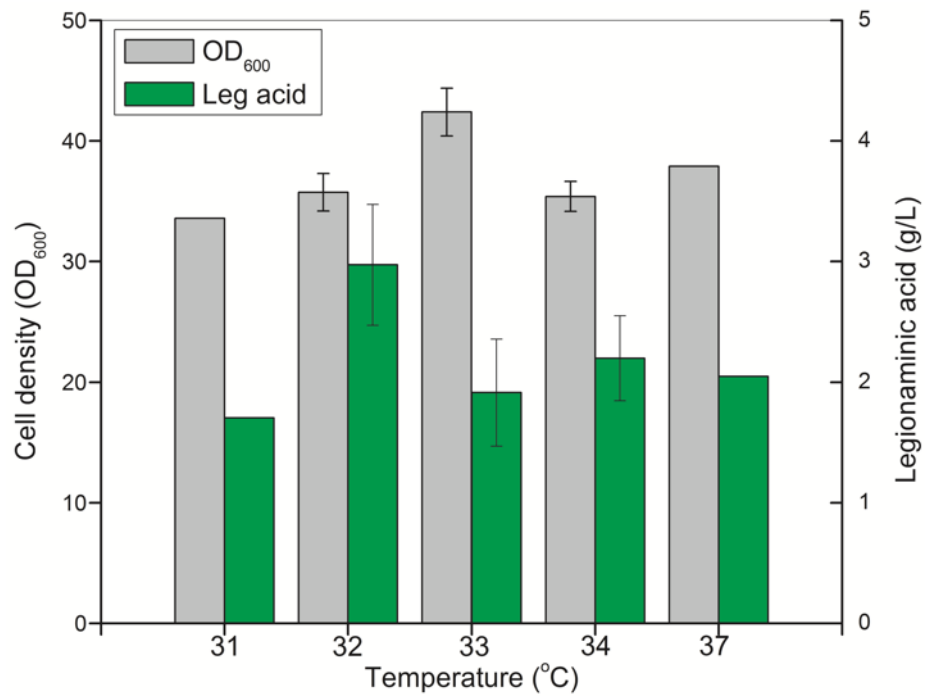


Figure 4.9 Effect of induction temperature on cell growth and legionaminic acid production. (average ± std dev, n=1 to 2)

Table 4.2 Effect of induction temperature on cell growth and legionaminic acid production (average \pm std dev, n=1 to 2)

Parameters	Induction temperature ($^{\circ}$ C)				
	31	32	33	34	37
Maximum cell density (OD ₆₀₀)	33.60	35.75 \pm 1.56	42.40 \pm 1.98	35.40 \pm 1.24	37.90
Maximum DCW (g/L)	13.39	14.19 \pm 0.58	16.67 \pm 0.74	14.06 \pm 0.46	14.99
Maximum Legionaminic acid concentration (g/L)	1.71	4.22 \pm 0.50	1.91 \pm 0.45	2.20 \pm 0.35	2.05
Culture time (h)	99	99	99	102	102
Induction duration time (h)	75	75	75	75	75
Cell productivity (g/L/h)	0.14	0.14 \pm 0.0059	0.17 \pm 0.0072	0.14 \pm 0.0047	0.15
Legionaminic acid productivity (g/L)	0.023	0.056 \pm 0.0024	0.026 \pm 0.0059	0.029 \pm 0.0047	0.027
Legionaminic acid yield on substrate (g/g)	0.34	0.84 \pm 0.353	0.38 \pm 0.089	0.44 \pm 0.070	0.41
Legionaminic acid percent yield (%)	22.59	55.89 \pm 7.35	25.34 \pm 5.90	29.12 \pm 4.66	27.14

4.3.2 Effect of pH

The most commonly used pH for cell cultivation of *E. coli* and heterologous protein expression in a medium without acetate is approximately 7.0, ranging from 6.5 to 7.5 [18]. Because the optimal pH for producing sialic acid in the similar strain is 7.4, in order to investigate the optimal pH value that improves both cell density and product yield, the pH of fermentation was varied from 7.0 to 7.6. Cells were grown in glycerol media at 37 $^{\circ}$ C during the pre-induction period to gain sufficient biomass. Other conditions which were maintained were an induction temperature of 32 $^{\circ}$ C, 30% dissolved oxygen, a glycerol feed rate of 0.2

mL/min, a GlcNAc feed rate of 0.16 mL/min and induction during the mid-exponential phase.

From Figure 4.10 to Figure 4.13, the optical density can be seen to maintain a nearly identical increasing trend for the duration of the fermentation experiments. After being induced in the mid-exponential phase, the OD₆₀₀ at pH values of 7.0 and 7.2 increased steadily, reaching values of 52.0 and 53.4 respectively. After 60 h of batch operation, the OD₆₀₀ at a pH of 7.4 typically remained at approximately 35.75. Finally, the OD₆₀₀ at a pH of 7.6 plateaued at a value of 36.0 after 40 h of batch operation.

Induction was commenced by addition of 0.2 mM IPTG, followed by feeding with GlcNAc at 0.16 mL/min after changing the temperature to 32°C. Feeding of GlcNAc was completed 15.6 h post-induction. The concentration of substrate peaked after approximately 50 h of batch operation (approximately 27 h post-induction), after which it steadily decreased, as shown in Figure 4.10 to Figure 4.13. However, this decrease in substrate concentration differed at different pH values. At a pH of 7.2, the decrease of GlcNAc concentration was the fastest. The substrate was depleted early after 57 h of batch operation (31 h post-induction). The substrate was found to be completely consumed after 60 h of batch operation (36 h post-induction). However, residual GlcNAc concentrations of 0.38 g/L and even 3.44 g/L at pH values of 7.4 and 7.6 were observed, respectively. These results reveal that GlcNAc is most efficiently utilized at pH values of 7.0 and 7.2, but is not fully consumed at a pH of 7.4 or 7.6. In terms of substrate utilization, a pH of 7.2 is most optimal.

In the four figures, it can be seen that the concentration of legionaminic acid generally tends to increase, albeit at different rates. The highest titer (5.09 g/L) of legionaminic acid was observed at a pH of 7.2, as the concentration rapidly increased prior to the 50 h post-induction time point, after which the increase was more gradual. The yield followed the same trend,

with a rapid rise followed by a gradual tapering. A titer of only 1.66 g/L legionaminic acid was obtained at a pH of 7.6.

Figure 4.14 shows a summary of the effects of pH on cell growth and legionaminic acid production. The replicates were performed at pH of 7.2, 7.4 and 7.6. At a pH of 7.4-7.6 the cell density was lower compared to that at a pH of 7.0-7.2. These results indicate that the optimal pH is 7.2, where both the optical density and concentration of legionaminic acid were achieved at the highest levels of 53.4 and 5.09 g/L, respectively. The most optimal percent yield and productivity at a pH of 7.2 were 67.45% and 0.068 g/L shown in Table 4.3, respectively.

Fermentation is a multi-enzymatic system and the optimal pH values of various enzymes are not the same. Furthermore, the optimal pH for growth and for product formation are also likely different [42]. However, the effect of pH to legionaminic acid production and cell growth shows similar trend in this study. The optimal pH for growth of *E. coli* is approximately neutral [17], so is that for legionaminic acid production.

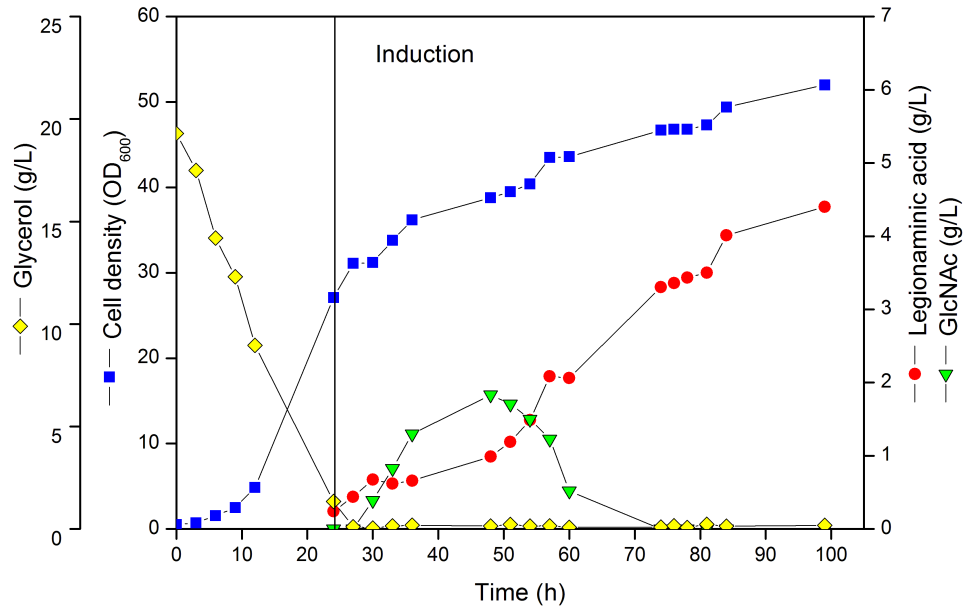


Figure 4.10 Curves for cell growth and concentrations of glycerol, legionaminic acid and GlcNAc at a pH of 7.0

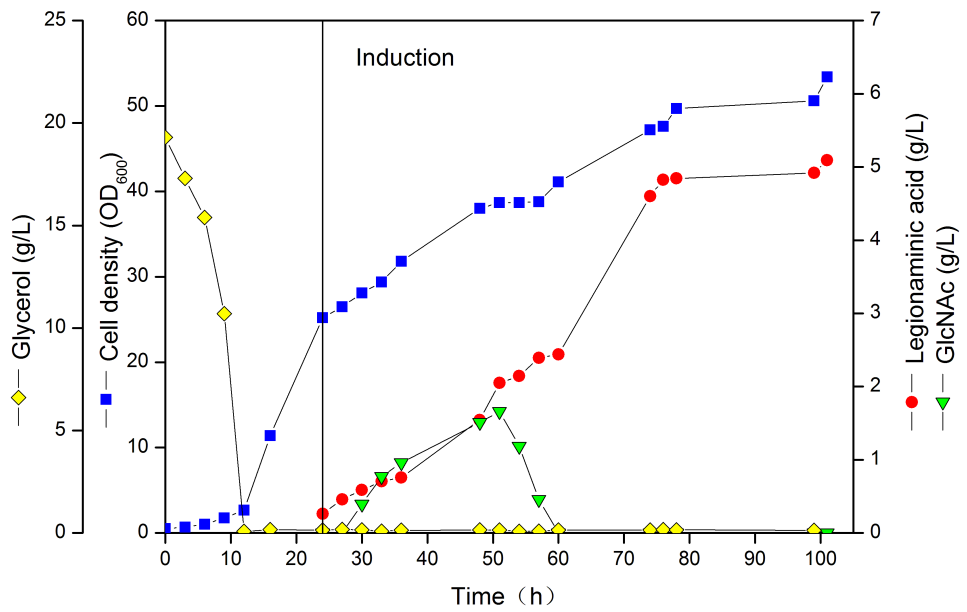


Figure 4.11 Curves for cell growth and concentrations of glycerol, legionaminic acid and GlcNAc at a pH of 7.2

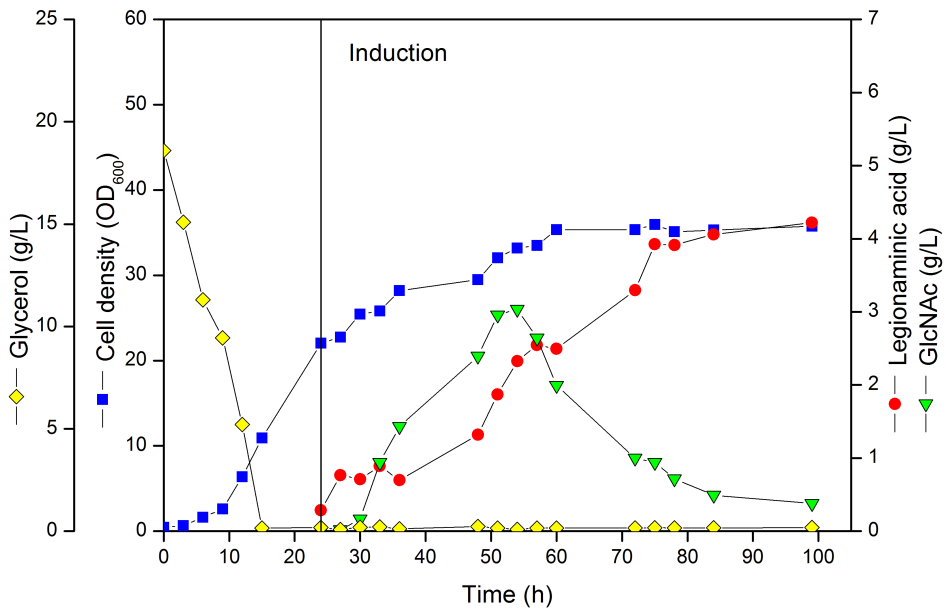


Figure 4.12 Curves for cell growth and concentrations of glycerol, legionaminic acid and GlcNAc at a pH of 7.4

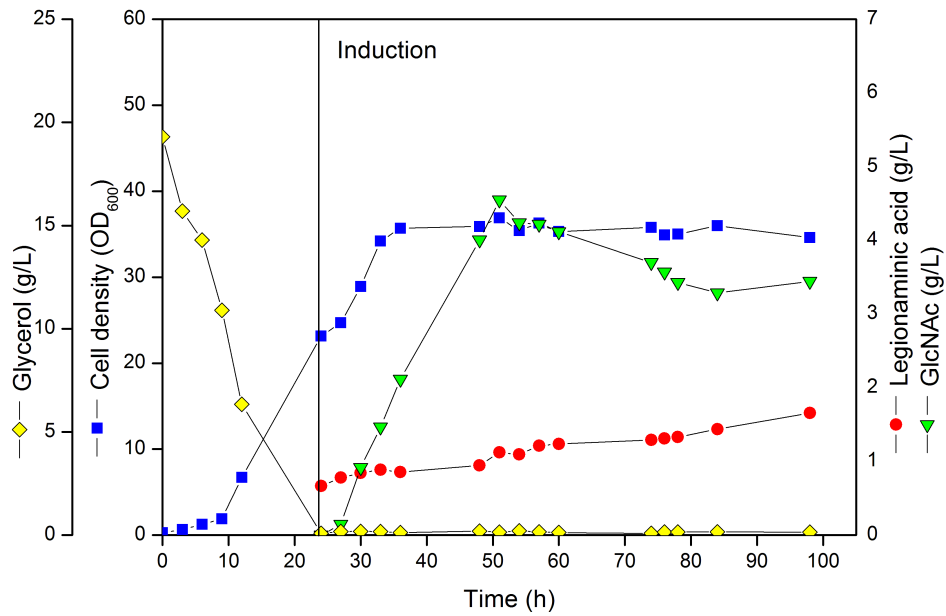


Figure 4.13 Curves for cell growth and concentrations of glycerol, legionaminic acid and GlcNAc at a pH of 7.6

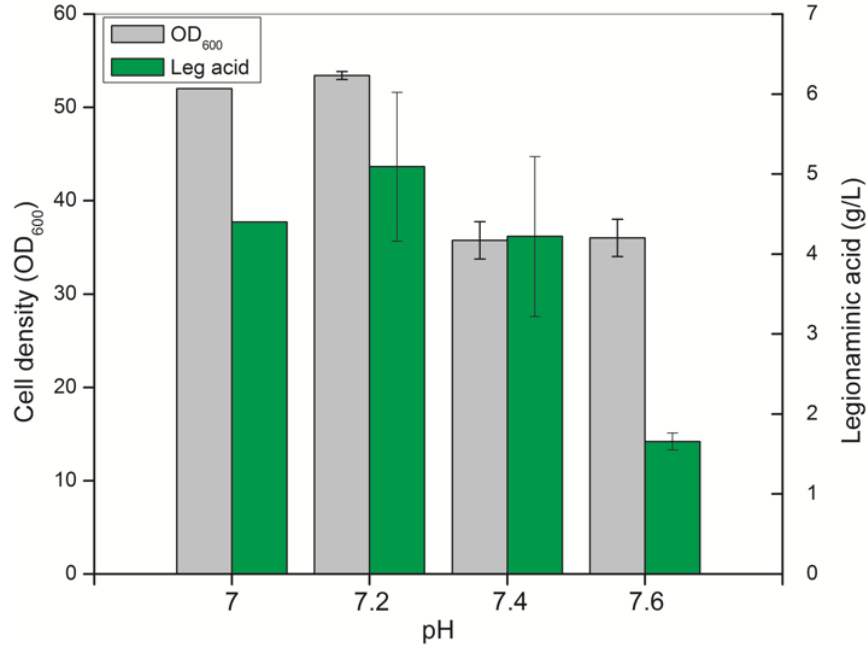


Figure 4.14 Effect of pH on cell growth and legionaminic acid production (n=1 to 2)

Table 4.3 Effect of pH on legionaminic acid production (average ± std dev, n=1 to 2)

Parameters	pH			
	7.0	7.2	7.4	7.6
Maximum cell density (OD ₆₀₀)	52.00	53.40±0.42	35.75±2.97	36.00±8.13
Maximum DCW (g/L)	20.24	20.76±0.16	14.19±1.11	14.28±3.03
Maximum Legionaminic acid concentration (g/L)	4.40	5.09±0.93	4.22±1.76	1.66±0.11
Culture time (h)	101	99	99	98
Induction duration time (h)	75	75	75	72
Cell productivity (g/L/h)	0.52	0.54±0.004	0.36±0.030	0.37±0.086
Legionaminic acid productivity (g/L)	0.059	0.068±0.012	0.056±0.0024	0.023±0.001
Legionaminic acid yield on substrate (g/g)	0.88	1.02±0.19	0.84±0.35	0.33±0.021
Legionaminic acid percent yield (%)	58.30	67.45±12.33	55.89±23.35	21.93±1.39

4.3.3 Effect of DO

In order to find the preferred dissolved oxygen level that optimizes both cell growth and legionaminic acid production, fermentations were performed at different dissolved oxygen concentrations (20%, 30% and 40%). Cells were grown at 37°C during the pre-induction period. Following initial glycerol depletion, 60% (v/v) glycerol was fed at a constant rate of 0.3 mL/min. At this point, 0.2 mM IPTG was added and 100 g/L GlcNAc was supplied at a feed rate of 0.16 mL/min. Other post-induction conditions which were maintained are an induction temperature of 32°C and a pH of 7.2. Control of the dissolved oxygen content throughout the duration of the batch process was achieved by agitation and airflow cascades.

Figure 4.15 to Figure 4.17 illustrate the profile of cell growth, as well as concentration profiles for legionaminic acid, glycerol and GlcNAc at different dissolved oxygen concentrations (20%, 30% and 40%). The cell growth trends were similar for all DO levels. In the early phase of cultivation (before approximately 16 h), the optical density was observed to increase slightly, after which it grew exponentially following feeding with glycerol. During the post-induction period, the cell density rose gradually. The highest final cell density OD₆₀₀ of 50.8 was observed at a dissolved oxygen level of 40%.

When the DO was 40%, the concentration of GlcNAc reached the highest level seen and decreased faster than under other conditions. The substrate was depleted after 60 h of batch operation when the DO level was 40%. All GlcNAc was nearly completely consumed at 30% DO by the end of the fermentation process. A residual concentration of 0.55 g/L of substrate was observed at a DO of 20%. These results show that increasing the DO improves the usage efficiency of GlcNAc. This is probably because under lower DO the rate of GlcNAc utilization became slower.

Evidently, these four figures present a trend of increasing legionaminic acid concentration with the rise of DO, reaching 4.32 g/L at a DO of 40%. At a DO of 20% however, only 1.14 g/L of product was produced.

Results from Figure 4.18 showed that a dissolved oxygen concentration of 40% gives better results than those at other DO levels, as determined in terms of the maximum concentration of legionaminic acid, which is 4.32 g/L. Based on the maximum OD₆₀₀ reached, these results show that a batch at 20% oxygen gives better results than at 30% or 40%. This does not correspond to expected results as higher percentages of dissolved oxygen should allow increased cell growth [19]. The highest level of optical density was found to be 50.8, which occurred at a DO of 20%. However, the concentration of legionaminic acid climbed with increasing DO. When the DO was raised to 40%, the largest titer of legionaminic acid of 4.32 g/L was obtained (Figure 4.17). The yield and productivity of legionaminic acid were found to be 68.80% and 0.069g/L shown in Table 4.4, respectively. Therefore, these results demonstrate that a DO level of 40% is optimal for the production of legionaminic acid.

Sufficient agitation and aeration may be used to meet oxygen demands while also promoting even mixing [20]. However, agitation is limited and high aeration may lead to oxidative damage to the heterologous proteinaceous products [21]. Although higher DO levels than what were tested in this study should be investigated to further investigate its effect, the high costs of air supplementation limit this aspect of further study.

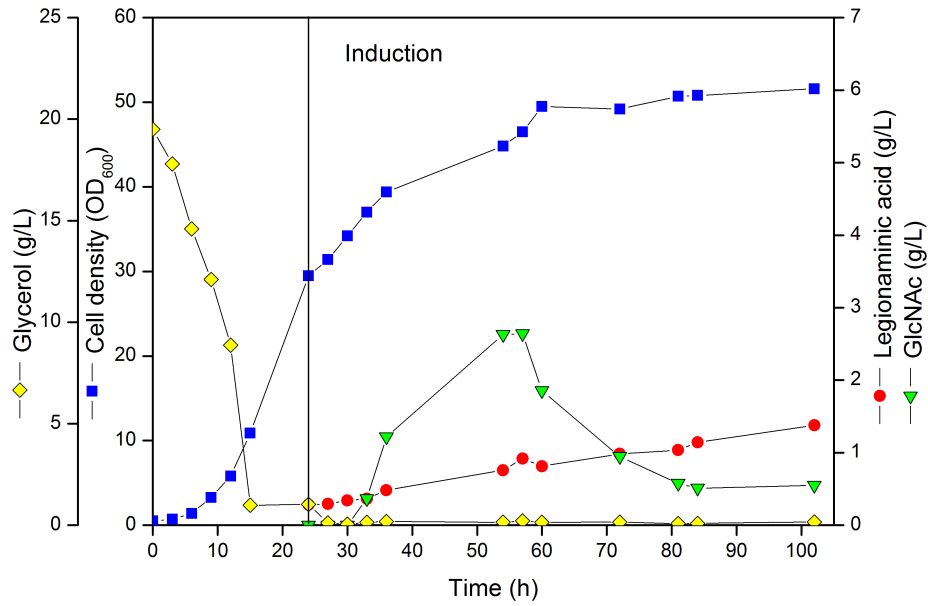


Figure 4.15 Curves for cell growth and concentrations of glycerol, legionaminic acid and GlcNAc with 20% dissolved oxygen

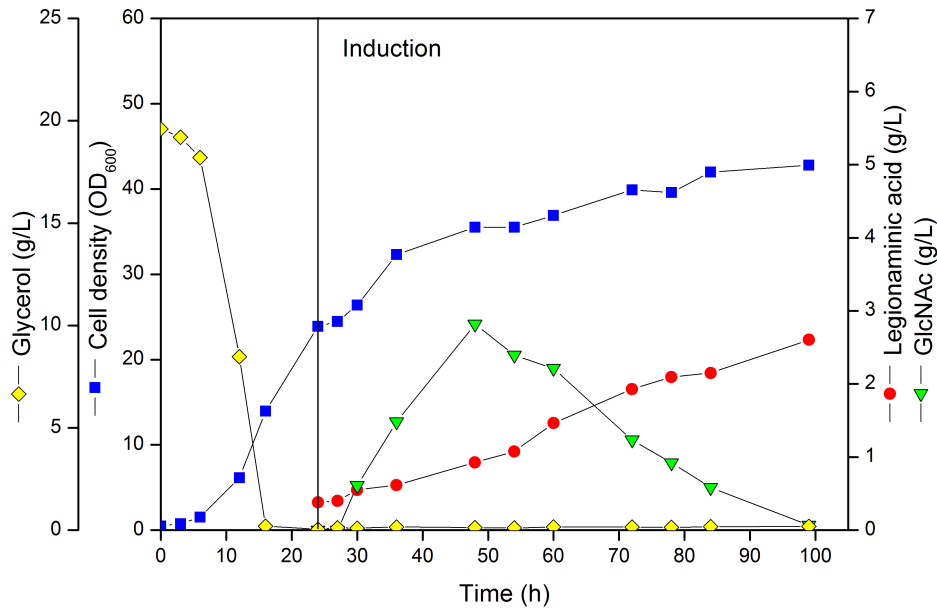


Figure 4.16 Curves for cell growth and concentrations of glycerol, legionaminic acid and GlcNAc with 30% dissolved oxygen

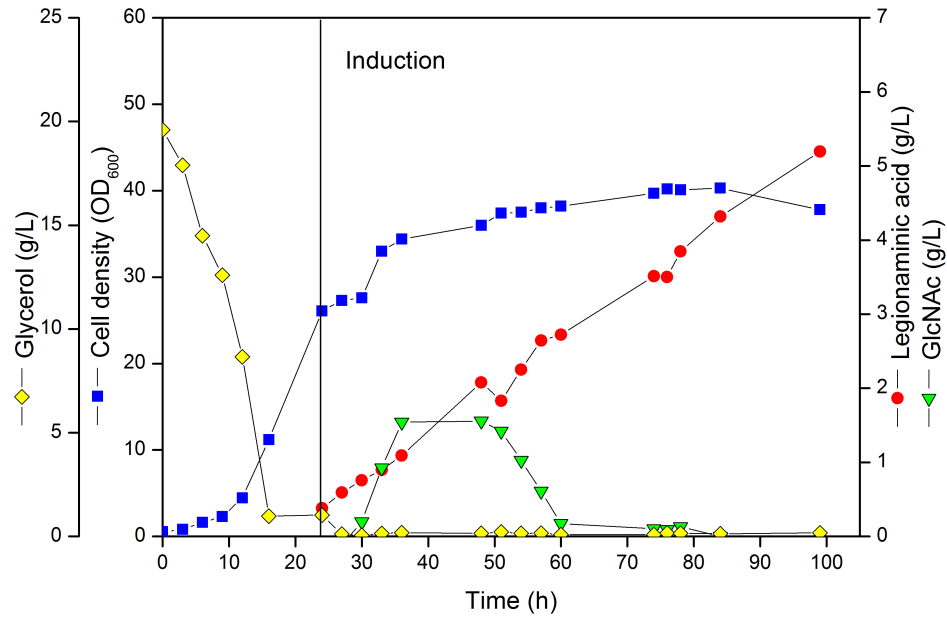


Figure 4.17 Curves for cell growth and concentrations of glycerol, legionaminic acid and GlcNAc with 40% dissolved oxygen

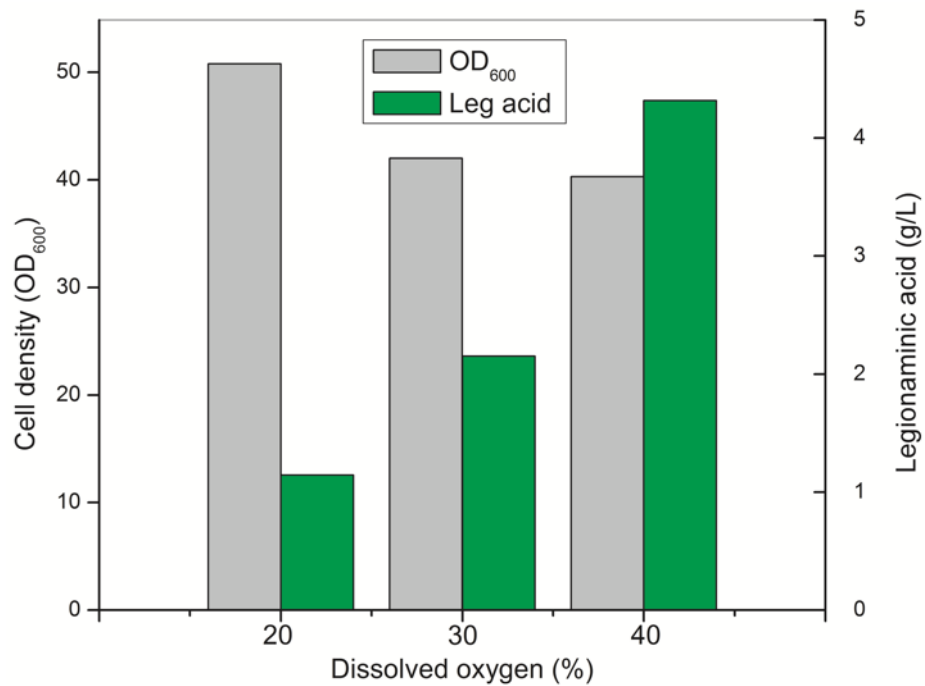


Figure 4.18 Effect of dissolved oxygen on cell growth and legionaminic acid production

Table 4.4 Effect of dissolved oxygen on legionaminic acid production

Parameters	DO (%)		
	20	30	40
Maximum cell density (OD ₆₀₀)	50.8	42.0	40.3
Maximum DCW (g/L)	19.79	16.52	15.89
Maximum Legionaminic acid concentration (g/L)	1.14	2.15	4.32
Culture time (h)	102	99	99
Induction duration time (h)	78	75	75
Cell productivity (g/L/h)	1.38	2.60	5.19
Legionaminic acid productivity (g/L)	0.018	0.035	0.069
Legionaminic acid yield on substrate (g/g)	0.28	0.52	1.04
Legionaminic acid percent yield (%)	18.24	34.50	68.80

4.3.4 Effect of induction optical density

Upon induction, the characteristics of growth and metabolic activity of the host cells are often influenced by the expression of recombinant proteins [22]. The growth curve for the current recombinant *E. coli* was determined by plotting optical density (600 nm) over time at intervals of 3 h or more. It is important to induce a culture during a physiological state which is favorable to the host cell to commence expression of heterologous proteins [23]. This study was designed to determine the phase of growth most appropriate for induction to gain maximum product and cell density. The cells were induced at various values of OD₆₀₀, which were 19, 23, 30, 35, and 40. Cells were grown at 37°C during the pre-induction period. Other conditions which were maintained include an induction temperature of 32°C, a pH of 7.2, a

DO level of 30%, a glycerol feed rate of 0.3 mL/min, and a GlcNAc feed rate of 0.16 mL/min.

It can be seen from Figure 4.19 to Figure 4.23 that the trends for cell growth were similar at all induction optical densities. In the early phase of cultivation (before approximately 16 h), the optical density was observed to increase slightly, then grow exponentially following feeding with glycerol. During the post-induction period, the cell density rose gradually. At an OD₆₀₀ of 40 prior to induction, the highest optical density of 55 was achieved. Likely due to the inhibition of GlcNAc at an induction optical density of 19, the post-induction optical density increased only minutely.

Figure 4.19 to Figure 4.23 show that for induction at an OD₆₀₀ of 23, 30, 35 or 40, all GlcNAc may be completely consumed by the end of the fermentation. The substrate was depleted more rapidly at an induction OD₆₀₀ of 30 and 35. However, it is interesting that at an OD₆₀₀ of 40, the concentration of GlcNAc exhibited an unobvious trend (Figure 4.23). This may be due to the utilization of GlcNAc for cell growth instead of production of legionaminic acid. The residual concentration of GlcNAc was less than 1 g/L when induced at an OD₆₀₀ of 19 (Figure 4.19).

In each of the mentioned figures, the concentration of legionaminic acid climbs gradually. The maximum concentration of legionaminic acid achieved was 2.613 g/L when induced at an OD₆₀₀ of 23.

From Figure 4.24, a summary of the effect of induction optical density is shown. With an increase in induction time, final cell density maintains an upward trend, reaching 55 when cells were induced at an OD₆₀₀ of 40. However, the concentration of legionaminic acid was greatest at an OD₆₀₀ of 23, at a value of 2.613 g/L. The yield and productivity of legionaminic acid at optimal OD₆₀₀ of 23 were found to be 34.61% and 0.035g/L shown in Table 4.5,

respectively. Higher induction optical densities exhibited reduced legionaminic acid production. The higher cell density suggests that more cells were available for recombinant protein expression; however, the activities of cells were unknown. A possible explanation is that the cell activity was not feasible, or that GlcNAc offers more to rapid cell growth instead of production.

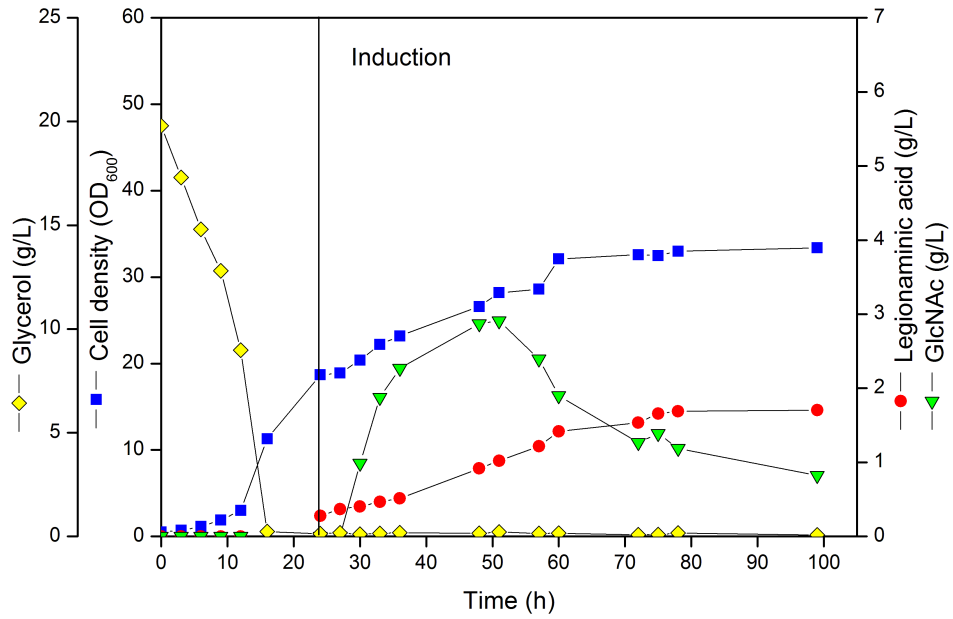


Figure 4.19 Curves for cell growth and concentrations of glycerol, legionaminic acid and GlcNAc at an induction OD₆₀₀ of 19

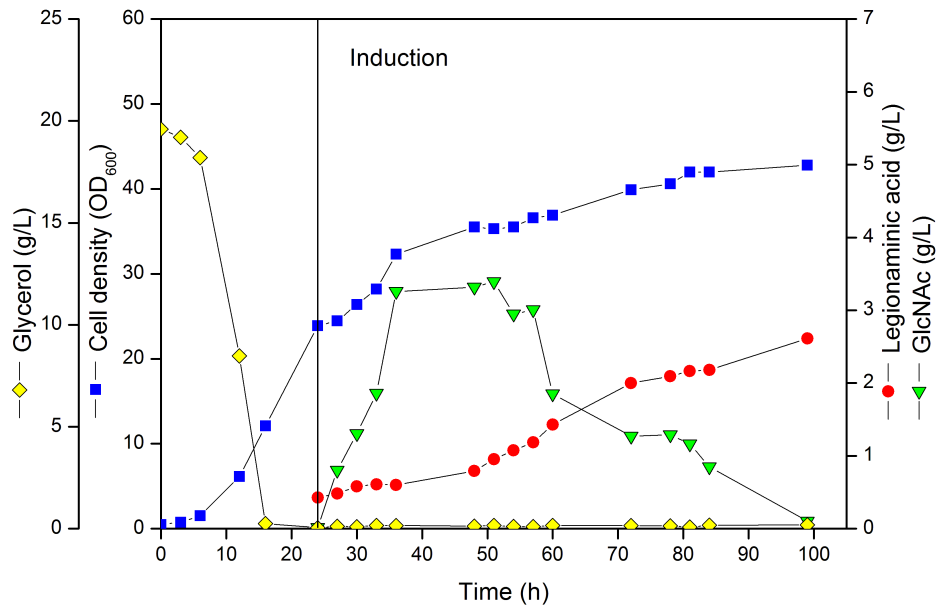


Figure 4.20 Curves for cell growth and concentrations of glycerol, legionaminic acid and GlcNAc at an induction OD₆₀₀ of 23

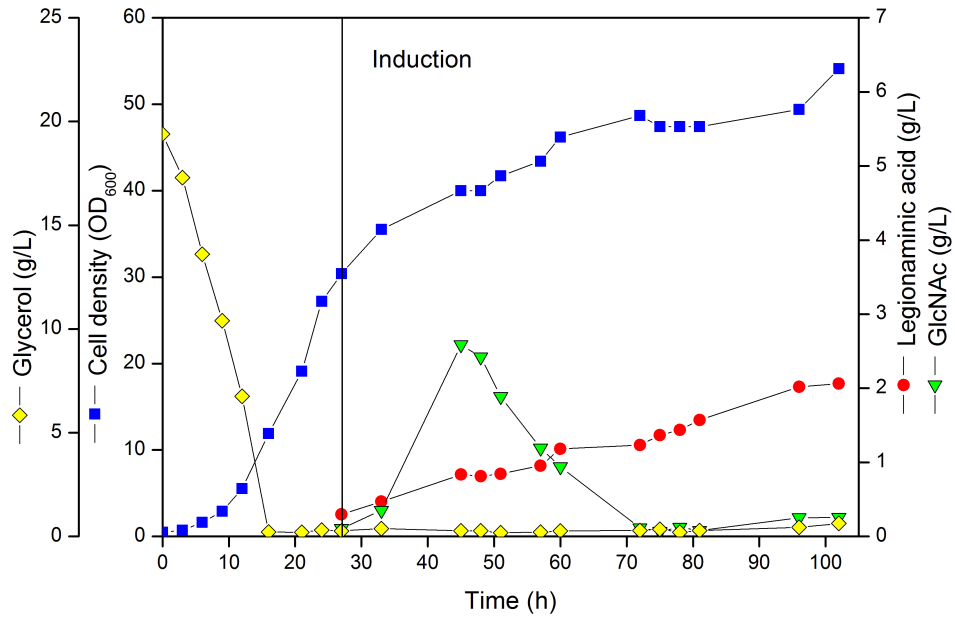


Figure 4.21 Curves gorcell growth and concentrations of glycerol, legionaminic acid and GlcNAc at an induction OD₆₀₀ of 30

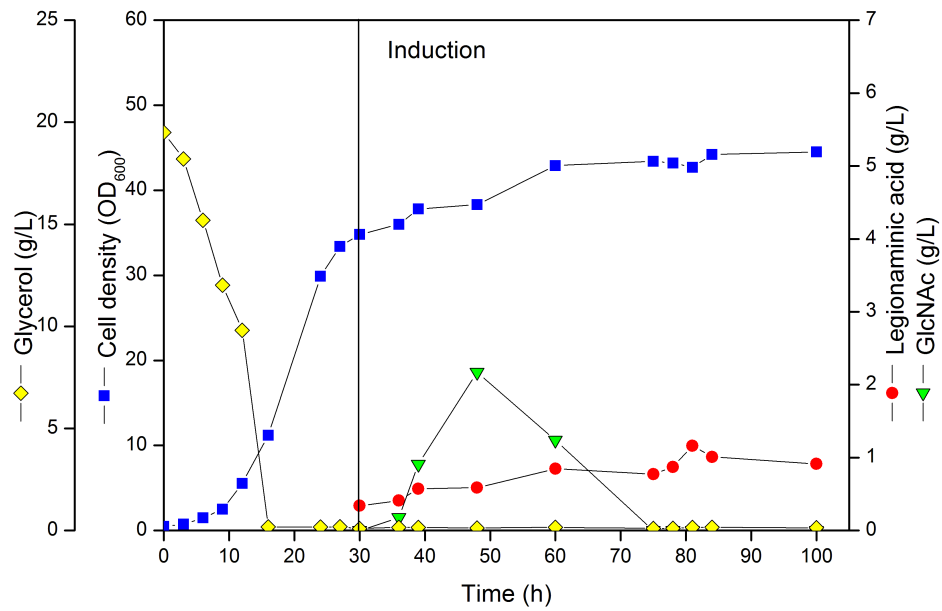


Figure 4.22 Curves for cell growth and concentrations of glycerol, legionaminic acid and GlcNAc at an induction time of OD₆₀₀ of 35

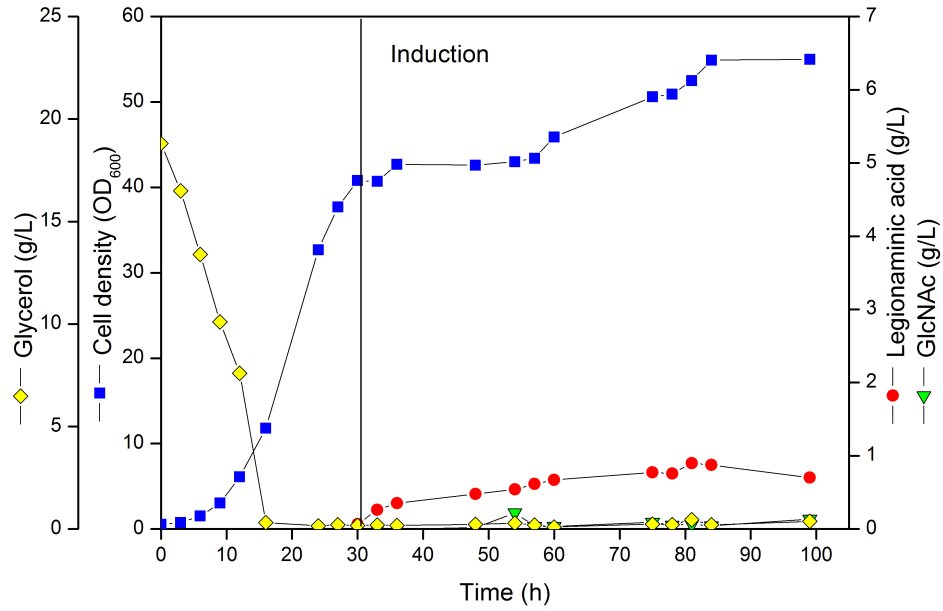


Figure 4.23 Curves for cell growth and concentrations of glycerol, legionaminic acid and GlcNAc at an induction OD₆₀₀ of 40

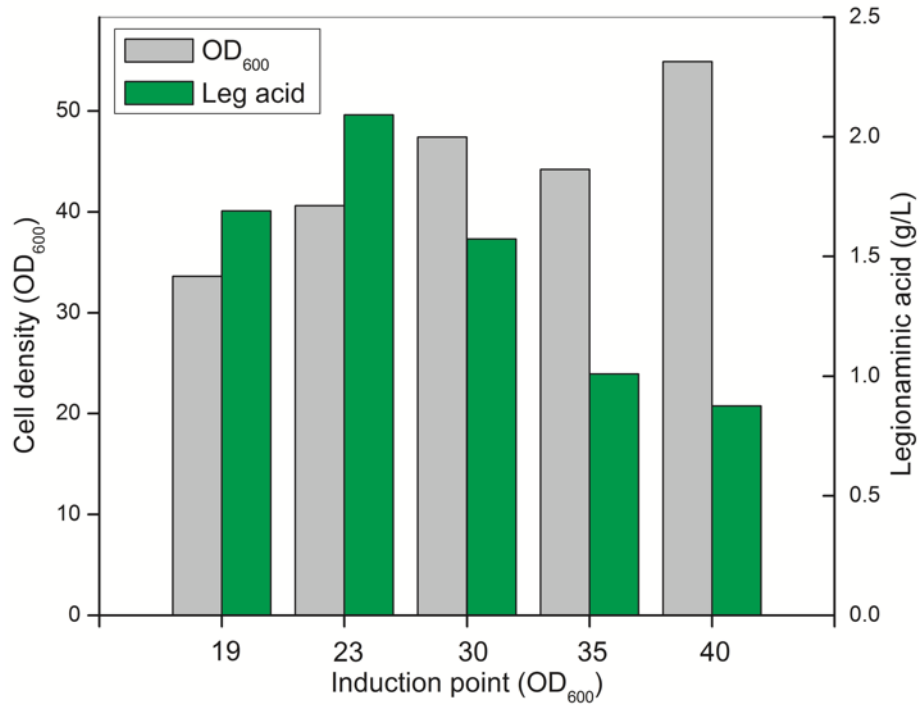


Figure 4.24 Effect of induction OD₆₀₀ on cell growth and legionaminic acid production

Table 4.5 Effect of induction OD₆₀₀ on legionaminic acid production

Parameters	Induction point (OD 600)				
	19	23	30	35	40
Maximum cell density (OD ₆₀₀)	33.6	42.8	51.4	44.5	55.0
Maximum DCW (g/L)	13.39	16.82	20.02	17.45	21.36
Maximum Legionaminic acid concentration (g/L)	1.71	2.61	2.06	1.16	0.90
Culture time (h)	99	99	102	100	99
Induction duration time (h)	75	75	75	70	69
Cell productivity (g/L/h)	0.34	0.43	0.51	0.45	0.56
Legionaminic acid productivity (g/L)	0.023	0.035	0.028	0.017	0.013
Legionaminic acid yield on substrate (g/g)	0.34	0.52	0.41	0.23	0.18
Legionaminic acid percent yield (%)	22.59	34.61	27.33	15.42	11.92

4.3.5 Effect of GlcNAc feed rate

In order to determine the optimal feed rate of GlcNAc for both cell growth and production of legionaminic acid, three different rates were examined: 0.16 mL/min, 0.30 mL/min and 0.50 mL/min. During the pre-induction period, cells were grown at 37°C. Glycerol (60% (v/v)) was pumped at a constant rate of 0.30 mL/min after complete consumption of glycerol. Following this, the temperature was decreased to 32°C, and the culture was supplied with 0.2 mM IPTG. Other conditions which were maintained include a pH of 7.2, and a DO level of 40%.

In the following three figures (Figure 4.25, Figure 4.26 and Figure 4.27), profiles for optical density and concentration are shown at different feed rates (0.16 mL/min, 0.30 mL/min and 0.50 mL/min). Cell growth typically increased gradually near the beginning of a run, then exponentially increased after supplying glycerol after approximately 16 h when the carbon

source had been depleted. After induction, a gradual increasing trend of cell growth was shown. This increase was not as rapid as that over the time prior to induction. At the end of the fermentation, the maximal optical density reached was 46.9 at a feed rate of 0.50 mL/min (Figure 4.27). Final optical densities of 40.3 and 45.5 were achieved at feed rates of 0.16 (Figure 4.25) and 0.30 mL/min (Figure 4.26). Thus, it can be said that greater GlcNAc feed rates, increased the rate of cell growth.

The curves for GlcNAc concentration are displayed in Figure 4.25, Figure 4.26 and Figure 4.27. The substrate concentration was shown to reach a maximum point then reduce. The peaks amplitude increased higher GlcNAc feed rates. However, the efficiency of substrate consumption did not follow the same trend. The concentration of GlcNAc decreased to 0 after 50 h of operation with a feed rate of 0.30 mL/min (Figure 4.26). With a feed rate of 0.16 mL/min, this time was increased to 60 h (Figure 4.25), with less than 1 g/L GlcNAc remaining (Figure 4.27). These results illustrate that the substrate was completely and rapidly depleted at a feed rate of 0.3 mL/min, which was the appropriate GlcNAc feed rate in terms of efficiency.

Figure 4.25 to Figure 4.27 also describe the concentration of legionaminic acid. The increasing trend at a feed rate of 0.50 mL/min seemed to be less pronounced than those at feed rates of 0.16 mL/min and 0.30 mL/min. The greatest results were observed at a feed rate of 0.30 mL/min, boasting the highest production of legionaminic acid with a titer of 5.53 g/L. A slightly lower value of 5.19 g/L was observed at a feed rate of 0.16 mL/min.

In terms of the concentration of legionaminic acid and optical density, the overall results for the effects of the GlcNAc feed rate are shown in Figure 4.28. Although the optical density followed an upward trend with rising substrate feed rate, the concentration of legionaminic acid reduced at a feed rate of 0.50 mL/min. The greatest production of legionaminic acid

observed was 5.53 g/L obtained at a feed rate of 0.30 mL/min, while the maximum optical density of 46.9 was obtained at a feed rate of 0.5 mL/min. The optimal GlcNAc feed rate (0.3 mL/min) for legionaminic acid production was particularly striking as the optimal GlcNAc feed rate for cell growth was higher at a feed rate of 0.50 mL/min. Figure 4.28 shows that the optimal induction temperature for highest level of legionaminic acid production is 32°C, where 73.29% of yield was obtained after 84h production with a productivity of 0.092 g/L (Table 4.6).

It was expected that higher substrate concentrations would lead to higher yield and titer of legionaminic acid. In this study, the *nagA* gene of *E. coli* BRL04 encoding catabolic pathways for GlcNAc consumption was successfully knocked out. The novel recombinant *E. coli* strain should be advantageous as a host strain for legionaminic acid production from GlcNAc with few side reactions. The results for the *E. coli* K12 strain whose catabolic gene coding for GlcNAc degradation was knocked out, showed that higher concentrations of GlcNAc did not seriously inhibit enzymatic activities, producing higher final concentrations of Neu5Ac [24]. However, when the feed rate of GlcNAc was increased to 0.50 mL/min, production of legionaminic acid appears to have been inhibited. A high substrate feed rate in this case produced a higher cell concentration but a lower product yield. It is possible that there were other unknown catabolic pathway for GlcNAc consumption and the expression of recombinant proteins was affected by the culture environment [22].

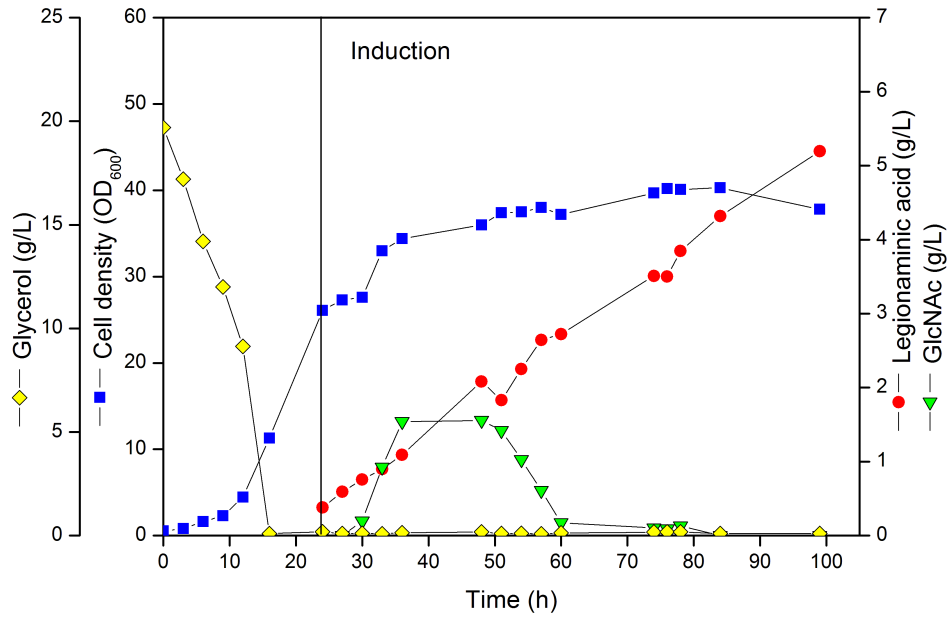


Figure 4.25 Curves for cell growth and concentrations of glycerol, legionaminic acid and GlcNAc at a GlcNAc feed rate of 0.16 mL/min

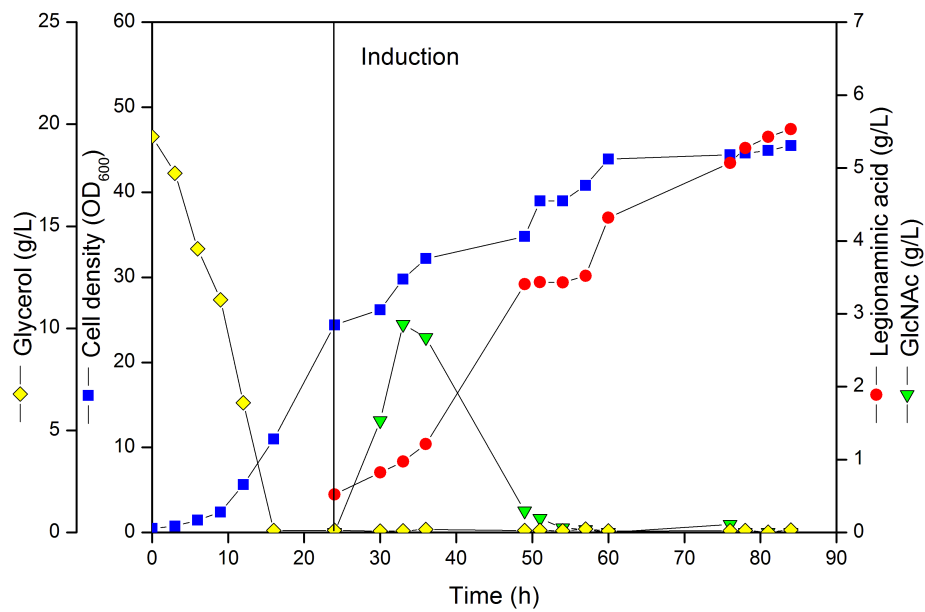


Figure 4.26 Curves for cell growth and concentrations of glycerol, legionaminic acid and GlcNAc at a GlcNAc feed rate of 0.30 mL/min

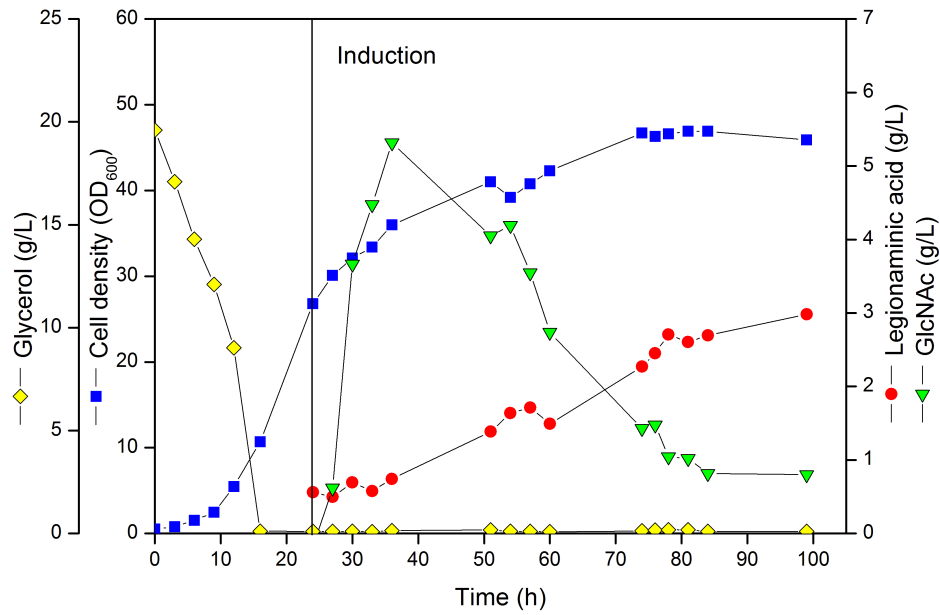


Figure 4.27 Curves for cell growth and concentrations of glycerol, legionaminic acid and GlcNAc at a GlcNAc feed rate of 0.50 mL/min

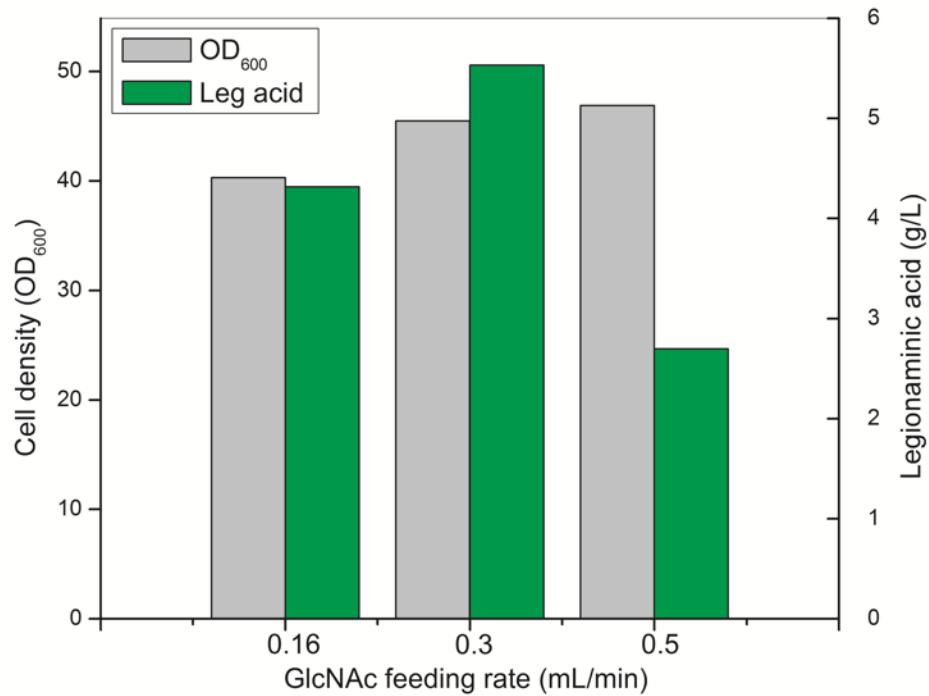


Figure 4.28 Effect of GlcNAc feed rate on cell growth and legionaminic acid production

Table 4.6 Effect of GlcNAc feed rate on legionaminic acid production.

Parameters	GlcNAc feed rate (ml/min)		
	0.16	0.3	0.5
Maximum cell density (OD ₆₀₀)	40.3	45.5	46.9
Maximum DCW (g/L)	15.89	17.82	18.34
Maximum Legionaminic acid concentration (g/L)	5.19	5.53	2.98
Culture time (h)	99	84	99
Induction duration time (h)	75	60	75
Cell productivity (g/L/h)	0.41	0.54	0.47
Legionaminic acid productivity (g/L)	0.069	0.092	0.040
Legionaminic acid yield on substrate (g/g)	1.04	1.11	0.60
Legionaminic acid percent yield (%)	68.80	73.29	39.50

4.3.6 Combination of optimal conditions

At last all the optimal parameters were tested together in triplicates to gain the final results. Glycerol (60% (v/v)) was pumped at a constant rate of 0.30 mL/min after complete consumption of glycerol. In mid exponential phase, the temperature was decreased to 32°C, and the culture was supplied with 0.2 mM IPTG as well as GlcNAc at a constant rate of 0.30 mL/min. Other conditions which were maintained include a pH of 7.2, and a DO level of 40%.

Under optimized culture conditions, the highest legionaminic acid concentration was achieved 5.53 g/L with a percent yield of 73.29%, which was achieved in a fermenter 60 h post-induction with a productivity of 0.092 g/(Lh) shown in Table 4.7.

Table 4.7 Effect of optimized conditions on legionaminic acid production. (average \pm std dev, n=2)

Parameters	Optimized conditions
Maximum cell density (OD ₆₀₀)	45.5 \pm 6.01
Maximum DCW (g/L)	17.82 \pm 2.24
Maximum Legionaminic acid concentration (g/L)	5.53 \pm 0.62
Culture time (h)	84
Induction duration time (h)	60
Cell productivity (g/L/h)	0.54 \pm 0.003
Legionaminic acid productivity (g/L)	0.092 \pm 0.005
Legionaminic acid yield on substrate (g/g)	1.11 \pm 0.13
Legionaminic acid percent yield (%)	73.29 \pm 8.20

4.4 Conclusions

This is the first study indicating how culture conditions can affect the degree in legionaminic acid production by recombinant *E. coli*. In the present study, fermentation conditions including induction temperature, pH, dissolved oxygen (DO), induction optical density and the GlcNAc feed rate for legionaminic acid production in *E. coli* were investigated.

When it comes to the effects of different induction temperatures (31°C, 32°C, 33°C, 34°C and 37°C), the highest production of legionaminic acid was 4.22 g/L obtained at 32°C. In a range of pH values (7.0, 7.4 and 7.6), the preferred pH was 7.2 where the optical density and concentration of legionaminic acid were 53.4 and 5.09 g/L, respectively. Based on a titer of 4.32 g/L legionaminic acid, 40% DO performed greater than 20% and 30%. The optimal induction optical density was found to be 23, producing 2.61 g/L of legionaminic acid. The ideal substrate feed rate was determined to be 0.30 mL/min, resulting in a titer of 5.53 g/L.

The most optimal results were obtained at an induction temperature of 32°C, a pH of 7.2, 40% dissolved oxygen, a GlcNAc feed rate of 0.30 mL/min and an induction OD₆₀₀ of 23. Under optimized culture conditions, the highest legionaminic acid concentration produced was 5.53 g/L at a percent yield of 73.29%, which was achieved in a fermenter 60 h post-induction with a productivity of 0.092 g/(Lh).

4.5 References

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Chapter 5: Conclusions and Recommendations

5.1 Conclusions

In conclusion, this was the first study to enhance legionaminic acid production in *E. coli* by high cell density fermentation. Shake flask cultures were screened for feasible induction temperatures and carbon sources. Other parameters were also measured at the 5-L bioreactor fed-batch fermentation scale. In this study, the concentrations of legionaminic acid and GlcNAc in the culture were measured by ion exclusion chromatography instead of ion exchange chromatography. The most optimal results were obtained with glycerol at an induction temperature of 32°C, a pH of 7.2, 40% dissolved oxygen, a GlcNAc feed rate of 0.3 mL/min and an induction OD₆₀₀ of 23.0. Under optimized culture conditions, the highest legionaminic acid concentration of 5.53 g/L with a percent yield of 73.29% was achieved in a fermenter 60 h post-induction with a productivity of 0.092 g/(Lh).

These results suggest that these fermentation conditions were important factors that significantly affected legionaminic acid production in *E. coli*. This optimization may be used in conjunction to strain improvements made by genetic strategies. Optimization of the fermentation process enhanced the yield of legionaminic acid dramatically, consequentially increasing profitability. Therefore, this work may aid in the development of legionaminic acid production for potential therapeutic purposes and promotion of the industry development.

5.2 Recommendations

All replicate trials in bioreactor fermentation are recommended to be performed in order to do statistics analysis. However, under sophisticated measurement and control, the necessary replicate trials and the final combination test have been performed for the validity of the overall results. In addition, the set-up of the bioreactor fermentation was time-consuming and each fermentation experiment of complicated biosynthetic process lasted for a significant amount of time. The cost of raw materials and operation was also too high to do all the replicates.

Interactions between independent parameters were not considered. Various changes in single parameters were tested while keeping other variables constant. While the experiments only tested the major impacts of producing legionaminic acid based on the literatures, but there may be other impacts with minimal effect. So it was hard to correctly judge and analysis if the small changes come from interaction effects or other minimal effects.

Although the culture conditions produced a relatively high level of titer and yield of legionaminic acid, there were other parameters can be optimized to enhance the productivity, such as feeding strategies for the carbon source and concentration of GlcNAc. To achieve high productivity, effects on both cell growth and product accumulation should be perfectly considered.

Appendix A: Standard curves for legionaminic acid and GlcNAc concentrations

Important chemicals observed in these experiments such as legionaminic acid and GlcNAc were monitored by an Agilent HPLC with an Aminex HPX-87H column. Before starting experiments, a standard curve was developed. Legionaminic acid and GlcNAc were detected by UV at a wavelength of 202 nm and a temperature of 60°C with 3mM sulphuric acid acting as the mobile phase at a rate of 0.6 mL/min.

A standard curve was developed for the HPLC peak area by using standard concentrations of legionaminic acid. A linear regression was performed in Figure A1, resulting in a slope of 37547 L/g. The coefficient of determination (R^2) was determined to be 0.99998, suggesting a good fit to the data.

A standard curve was developed for the HPLC peak area by using standard concentrations of GlcNAc. A linear regression was performed in Figure A2, resulting in a slope of 41771 L/g. The coefficient of determination was determined to be 0.9992, suggesting a good fit to the data.

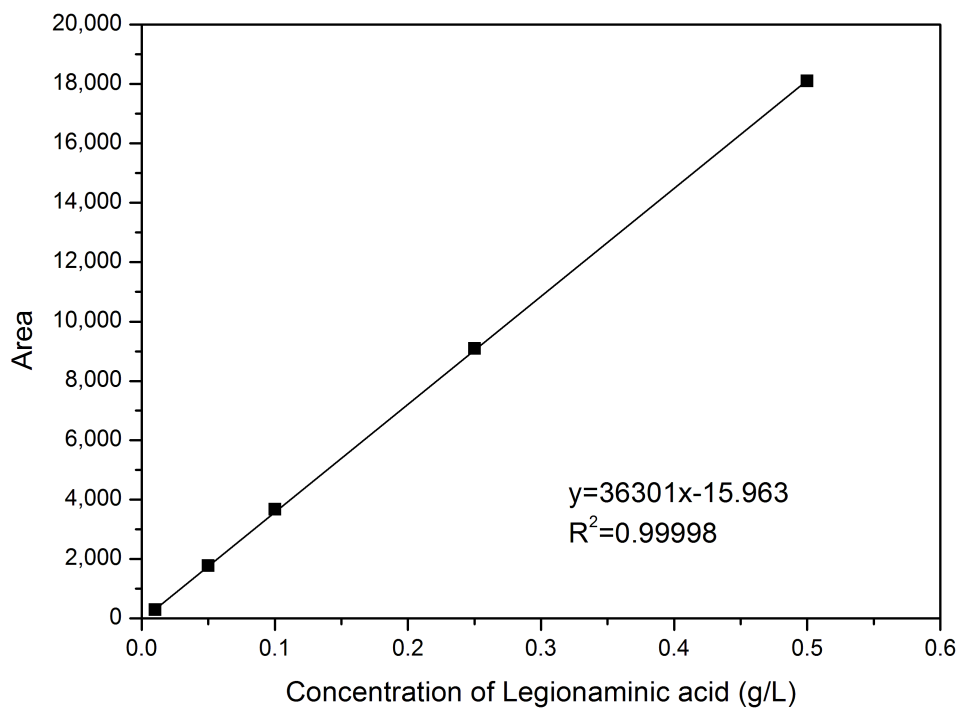


Figure A1 Correlation of area and concentration of legionaminic acid.

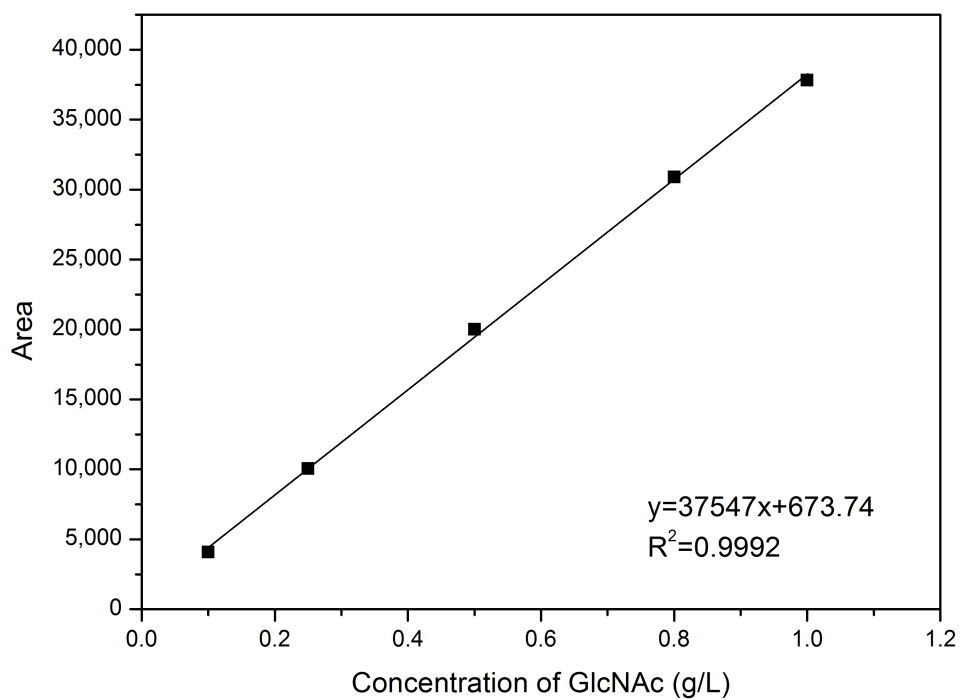


Figure A2 Correlation of area and GlcNAc concentration.

Appendix B: Correlation for dry cell weight and optical density

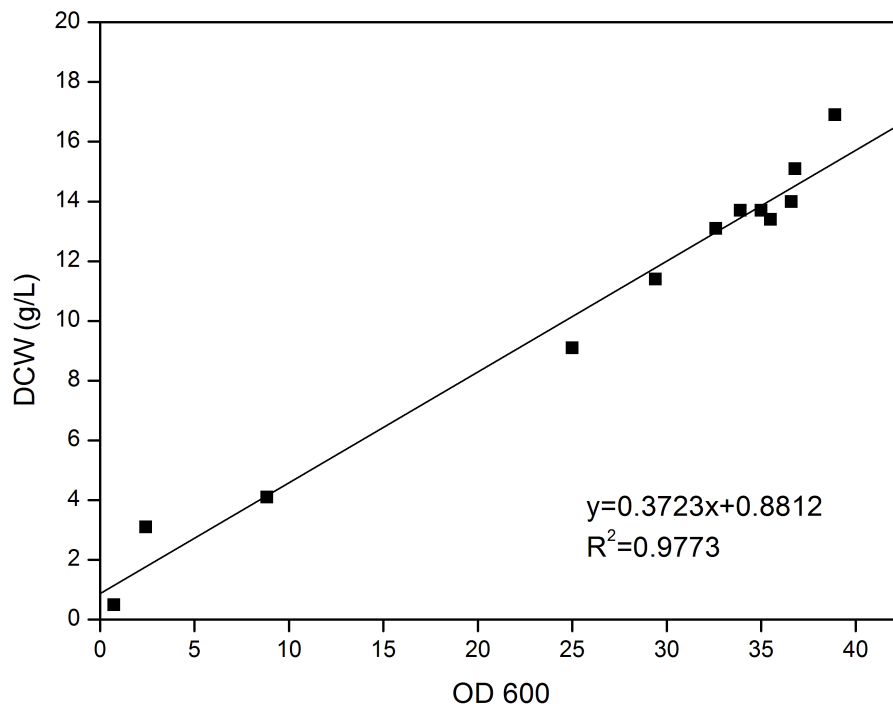


Figure A3 Correlation of dry cell weight and optical cell density