

***E. coli* fermentation for the production of sialic acid**

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Abstract

Sialic acid is the terminal sugar found on most glycoproteins and is crucial in determining serum half-life and immunogenicity on glycoproteins. The scarce supply of sialic acid hinders its advancement in basic research, diagnostic development and therapeutic production. In this work, the recombinant *E. coli* BRL04 (pBRL89) producing sialic acid was studied by some batch and fed batch runs of high cell density cultivation using a 3-L fermentor. Some cultivation conditions including carbon source, induction time, dissolved oxygen were optimized and different feeding strategies were compared to enhance sialic acid production. The results may be helpful to the further scale-up of sialic acid production and the production of other recombinant proteins by high cell density cultivation of *E. coli*.

Résumé

L'acide sialique, un groupe glucidique retrouvé à l'extrémité de la plupart des glycoprotéines, est utilisé pour déterminer leur demi-vie et leur immunogénicité. Sa disponibilité limitée, par contre, limite son utilité pour la recherche scientifique, le développement de tests diagnostiques et la production de thérapeutiques. Dans la présente recherche, la souche d'*E. coli* recombinante BRL04 (pBRL89), productrice d'acide sialique, fut étudiée lors de sa culture à haute densité dans un réacteur de 3L. La source de carbone, le temps d'induction, et la concentration d'oxygène dissout dans le réacteur furent optimisés, et différentes stratégies d'alimentation furent comparées afin de maximiser la production d'acide sialique. Les résultats obtenus seront utiles pour développer des systèmes de production d'acide sialique à plus grande échelle, ainsi que des systèmes pour la production d'autres produits recombinants à l'aide de systèmes de fermentation à haute densité d'*E. coli*.

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Nomenclature

Neu5Ac, N-acetylneuraminic acid

GlcNAc, N-acetylglucosamine

Neu5Gc, N-glycolylneuraminic acid

CMP, cytidine monophosphate

CTP, cytidine triphosphate

HCDC, high cell density cultivation

DCW, dry cell weight

IPTG, isopropyl- β -D-1-thiogalactopyranoside

ATP, adenosine triphosphate

NADH₂, nicotinamide adenine dinucleotides

ORI, origin of replication

MCS, multiple cloning sites

DO, dissolved oxygen

DMB, 4,5-methylenedioxy-1,2,-phenylenediamine dihydrochloride

Chapter 1: Introduction

Many cell surfaces, both prokaryotic and eukaryotic, are widely decorated with glycoconjugates that play significant roles in the maintenance of cell biological activities. Sialic acid encompasses a wide family of nine-carbon acidic sugars that are prominently found at terminal positions of many glycoconjugates on the surfaces of eukaryotic cells (1). In 1936, Blix isolated a crystalline reducing acid from bovine submandibular, which was thereafter named 'sialic acid' (2, 3). The most common sialic acid is N-acetylneuraminic acid (1, Neu5Ac), a carboxylated nine-carbon monosaccharide. Sialic acid is necessary in biological processes such as glycoprotein stabilization, cell signaling, solute transport, neuronal plasticity, cell adhesion and cellular immunity (4). For example, excess sialylation on malignant cells has been shown to promote invasiveness (5) and reduce intercellular interactions (6), which are both features of tumor metastasis.

Sialic acid is challenging to produce both chemically and by fermentation due to its unusual nine-carbon carbohydrate with six stereocenters, and is usually synthesized via an *in vitro* enzymatic process (7), which is costly and difficult to scale to the manufacturing level. The scarce supply of available sialic acid has hindered advancement in basic research, diagnostic development and therapeutic production. To provide a low cost and scalable process for sialic acid production, a metabolically and genetically engineered *Escherichia coli* BRL04 (pBRL89) was produced by removing the sialic acid catabolism pathway and expressing sialic acid biosynthesis

genes (8) and was kindly provided by Dr. Christopher N. Boddy from the Department of Chemistry at the University of Ottawa.

The work done in this thesis aimed to optimize some important cultivation parameters, including carbon source selection, glycerol to N-acetylglucosamine (GlcNAc) ratio, induction time, culture temperature, pH and dissolved oxygen. This was achieved by cultivating the recombinant *E. coli* through several batch culture runs. In addition, the effects of different feeding strategies were studied on the production of sialic acid from recombinant *E. coli* in fed-batch culture. Various strategies including pH-stat, exponential, constant rate, and linear decreasing feeding were studied, and two- and three-stage feeding methods were subsequently designed and investigated.

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Chapter 2: Literature Review

2.1 Sialic acid

2.1.1 Background

The surfaces of many bacteria are adorned with glyco-conjugates that play significant roles in certain biological processes, such as regulating the composition of the cell membrane, transmitting information between cell surfaces, and maintaining complicated structures by covalently binding to proteins (1).

In early related work, Ernst Klenk isolated a substance following the methanolysis of gangliosides, to which the name “neuraminic acid” was given, and which are now known as neuraminic acid- β -methyl glycosides (2). In 1936, Blix isolated a crystalline reducing acid from bovine submandibular mucin (3, 4), thereafter named “sialic acid” (4).

Sialic acid is composed of a large number of nine-carbon sugar neuraminic acids (5-amido-3, 5-dideoxy-D-glycero-D-galacto-nonulosonic acid) that are usually found at terminal positions of many glyco-conjugates on the surfaces of eukaryotic cells. It plays important roles in several crucial biological processes including glycoprotein stabilization, cell signaling, solute transport, neuronal plasticity, cell adhesion, cellular immunity and human diseases (5). For example, excess sialylation of malignant cells has been proven to increase invasiveness and reduce intercellular interactions, both characteristics of tumor cells (6, 7).

2.1.2 Structure

Sialic acids usually exist as α -glycosides which occupy the non-reductive

terminal of hetero-oligosaccharides in glyco-conjugates such as glycolipids and glycoproteins (8). Sialic acids consist of a family of more than 40 derivatives of the nine-carbon neuraminic acids including N- and O-substituted ones (9, 10). Their structural features are specialized due to the amino group at position 5 and the carboxyl group at position 1 that make it a strong organic acid with a negative charge under normal conditions (Figure 2-1). With no unsubstituted form of neuraminic acid found in nature, the most widely found sialic acid is N-acetylneuraminic acid (Neu5Ac), of which the amino group is usually acetylated. Another significant naturally occurring variation is N-glycolylneuraminic acid (Neu5Gc), which is formed by substituting one of the hydrogen atoms in the methyl moiety of the acetyl group by a hydroxyl group. Neu5Gc exists in a large number in animal species, especially in porcine tissue, but has not been detected in human tissue except in individuals afflicted by particular types of cancer (11). Although there is a wide variation of possible substitutions at the carbon 5 position, or covalent modifications of the sugar's hydroxyl domains (12), most work on sialic acid focuses on Neu5Ac, and so in most of the literature, the term 'sialic acid' refers specifically to this compound.

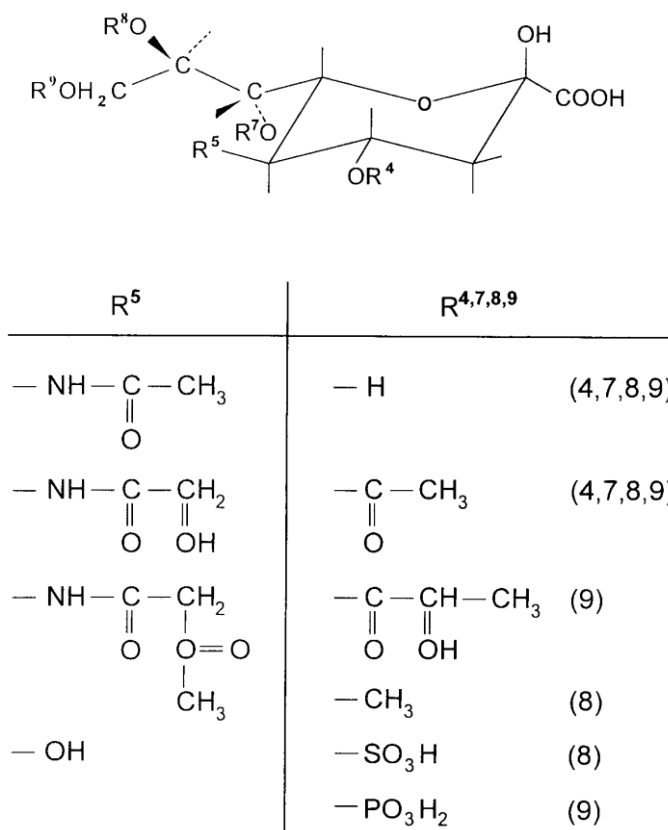


Figure 2-1 Chemical structure of the sialic acid molecule and a list of natural substituents, the positions of which are indicated in brackets (13).

2.1.3 Metabolism

Since sialic acid has so many important biological functions, it is necessary to discuss the metabolism of this sugar. The processes of sialic acid synthesis and degradation take place in different areas of the cell (Figure 2-2). The synthesis begins with N-acetylmannosamine-6-phosphate and phosphoenolpyruvate present in the cytosol. The product, Neu5Ac-9-phosphate, is dephosphorylated, and then activated in the nucleus by the transfer of a cytidine monophosphate (CMP) residue from cytidine triphosphate (CTP) mediated by CMP-Neu5Ac synthase. This sugar-nucleotide is the only known natural case of a β -linkage between sialic acid and another compound, as in glyco-conjugated form it is always α -linked. CMP-Neu5Ac

is then transported into the Golgi apparatus or the endoplasmic reticulum (10, 14), where the activated sialic acid is transferred by a sialyltransferase onto a corresponding acceptor molecule. The product constitutes the oligosaccharide chain of the initial glyco-conjugate, which can then be decorated by O-acetylation or O-methylation before being transported to the cell surface to serve as a functional glyco-conjugate (13).

Sialidase is the most important enzyme involved in sialic acid catabolism. Membrane-bound sialidases are responsible for the shift of sialic acid residues from the cell surface or serum sialoglyco-conjugates. In most cases, the glyco-conjugates are ready for degradation only once taken up by receptor-mediated endocytosis in higher animals. The terminal sialic acid residues are decomposed by lysosomal sialidases after fusion of the endosome with a lysosome.

In microorganisms, however, the enzymes are secreted to the surroundings to degrade their substrate prior to endocytosis. The removal of O-acetyl groups by sialate-O-acetyl esterases is necessary for the effective function of sialidases (5, 15). Free sialic acid molecules (Neu5Ac or Neu5Gc) are transported across the lysosomal membrane to the cytosol, where they can be activated and shifted to other nascent glyco-conjugate molecules in the Golgi. In other cases, sialic acid molecules can be degraded to acylmannosamine and pyruvate through the activity of a cytosolic acylneuraminate lyase, which is also commonly produced by microorganisms. Further details on the enzymes involved in sialic acid metabolism are introduced in subsequent sections.

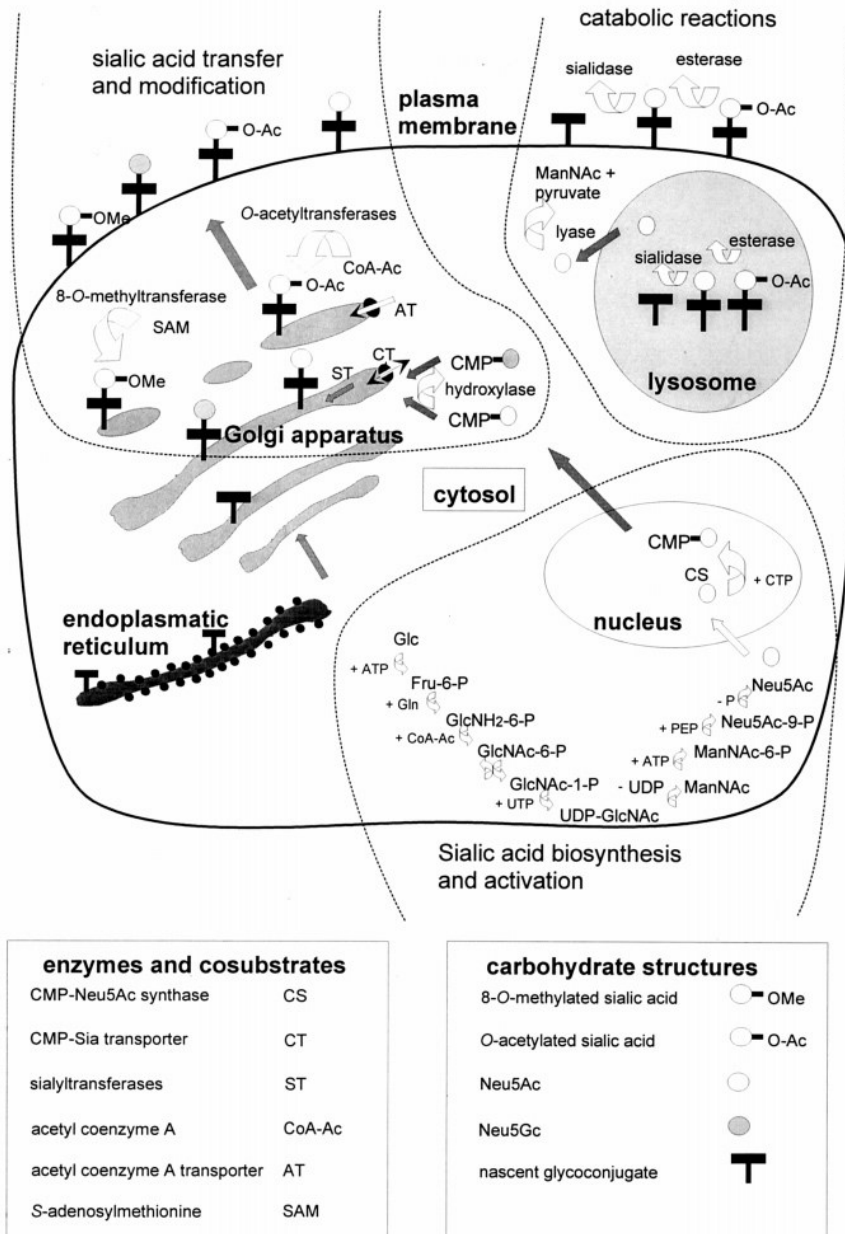


Figure 2-2 Metabolism of sialic acids. The enzymatic reactions involved in sialic acid biosynthesis, activation, transfer, modification and catabolism are shown with their intracellular localization (14).

2.1.4 Synthesis

Sialic acid's high cost is due to its scarce availability and supply. Traditionally, sialic acid has been separated from natural substances such as egg yolks, milk whey and edible bird nests. These methods, however, are time-consuming and burdensome

owing to the complicated purification process, which results in impure, low yields (less than 20%) (16). Chemical synthesis of sialic acid is not currently feasible, due to the complicated nine-carbon structures with a large number of stereocenters. *In vitro* enzymatic synthesis is currently the state-of-the-art method for sialic acid production (17), mainly due to its high yield (80%). This process, however, is expensive due to the high costs related to enzyme purification, chemicals, and scalability at the manufacturing level. To provide a cost-effective, scalable and manufacturer-friendly method for sialic acid production, an *Escherichia coli*-based fermentation method to produce sialic acid and N-acyl sialic acid analogs has been developed (18, 19), which is a feasible alternative to *in vitro* enzymatic synthesis as bacterial fermentation systems inherently contain target-molecule precursors, otherwise expensive cofactors, and the necessary enzymes required for sialic acid production.

The process of modifying *E. coli* to produce sialic acid involved the removal of its native sialic acid catabolism pathways and the expression of a foreign pathway for sialic acid biosynthesis (Figure 2-3). The endogenous NanT and NanA proteins were removed and the NeuB and NeuC enzymes from *N. meningitidis* group B were introduced. It is necessary to overexpress GlmS to produce high sialic acid yields from inexpensive carbon sources such as glucose, fructose and glycerol (20). The intracellular concentrations of UDP-N-acetylglucosamine (UDP-GlcNAc), the substrate of the sialic acid biosynthetic pathway, played an important part in the sialic acid yields, which supplied the backbones for sialic acid molecules. Various metabolic strategies have been used to increase carbon flow into UDP-GlcNAc biosynthesis and

thus increase sialic acid productivity, and multiple grams of sialic acid per liter of *E. coli* fermentation broth can be produced by this novel route.

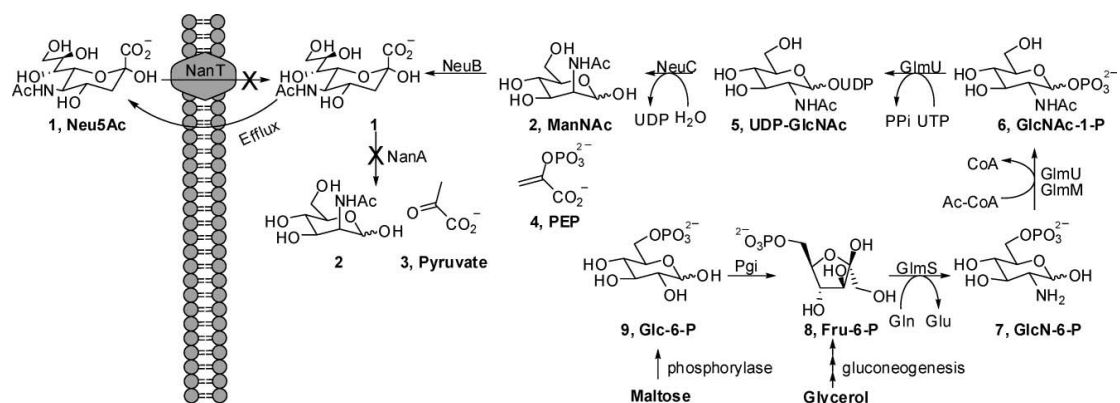


Figure 2-3 Sialic acid production by metabolically engineered *E. coli*. Abbreviations: GlmM: glucosamine mutase, GlmS: glucosamine synthase, GlmU: GlcNAc-1-P uridyltransferase/GlcN-1-P acetyltransferase, Pgi: phosphoglucose isomerase (20).

2.2 Engineering background

2.2.1 Introduction

Foods, plastics, fuels, medicines, and chemical reagents are produced at industrial levels using traditional methods such as chemical synthesis, extraction from native producers, and biotransformations including immobilized enzymes and cells. Chemical synthesis is a commonly used method for production, but it relies to a large extent on the petroleum industry and generates a great deal of waste. Many valuable products are also bio-based, which means that they are extracted from microbial primary or secondary metabolism. With recent improvements in fermentation technology, many of these bio-products are produced in large amounts through the

cultivation of their native producers. Nevertheless, some of these native producers are not able to provide yields high enough to constitute an effective production platform. Furthermore, in certain cases the native organisms might be pathogenic or fastidious which makes them more difficult to use in industrial settings. With the development of recombinant DNA approaches and modern biotechnology, many industrial processes have been re-engineered to focus on cleaner, more amenable bioprocesses, such as the use of microbial biocatalysts, *in vitro* enzymatic transformations, and high cell density cultivation (HCDC) of metabolically engineered microorganisms (21, 22), all of which can result in high yields. The usefulness of microbial biocatalysts and enzymatic transformations are, however, limited by the often large number of catalytic steps involved, or a requirement for expensive substrates and cofactors, both of which hinder their scalability for industrial application. In HCDC, microorganisms can provide all of the necessary enzymes, substrates and cofactors for the biosynthesis of the desired product. Furthermore, HCDC can be adjusted to utilize inexpensive carbon sources including glucose, glycerol, and sugars derived from renewable energy sources, like biodiesel waste and hemicellulose. Since HCDC technology provides such benefits, many industrial production processes are now using HCDC-based fermentation with industrially-friendly microorganisms such as *Saccharomyces cerevisiae* and *E. coli*.

2.2.2 High cell density cultivation of *E. coli*

E. coli has been the most widely used industrial microorganism and a pioneer organism in HCDC (23, 24). There is a great deal of information available on the

genetic, biochemical and physiological elements of *E. coli*, including an entire bioinformatics database that contains the detailed metabolism information of the *E. coli* K-12 strain MG1655 (25, 26), which has greatly facilitated the application of *E. coli* fermentation methods in a multitude of industrial processes, ranging from the production of therapeutic and catalytic proteins, to the production of chemicals and pharmaceuticals (27). With the increasing number of available strains, plasmids and engineering tools for *E. coli*-based technology, it is now possible to create bioprocesses for the production of simple compounds, such as threonine and pyruvate, or more complicated metabolites such as artemisinin and 6-deoxyerythronolide B (28, 29). HCDC is necessary for any *E. coli* bioprocess to be cost-effective, and is required not only for maximizing volumetric productivity ($\text{g L}^{-1} \text{h}^{-1}$) but also enhancing downstream processing and reducing overall production costs.

Cell concentrations from industrial HCDC typically fall within the range of 20-100 g dry cell weight (DCW) per liter of fermentation broth (23). A lot of processes, once optimized, have achieved biomass yields higher than 100 g L^{-1} for some organisms, while others can only achieve yields of a few grams per liter. *E. coli* has a relatively short doubling time, can grow in various medium compositions, and easily produce high cell densities, all of which make it an ideal organism for HCDC. For example, *E. coli* can accumulate to 15 g L^{-1} DCW in simple batch fermentation when supplied with a large variety of nutrients at high concentrations but below titers that may cause growth inhibition (30). A major drawback of HCDC with *E. coli* is growth inhibition caused by acetate generated as a byproduct in excess carbon or

oxygen limitation conditions (23). To solve this problem, acetate accumulation is usually minimized by controlling the rate of cell growth by limiting a nutrient. Other strategies used to reduce acetate concentrations and improve growth rate and biomass yields include cell-recycling methods, dialysis cultivation, genetic alterations that reduce acetate formation, and utilization of feedstocks that flow slowly into acetate metabolism, such as glycerol (31, 32). Eventually, the rate of oxygen consumption becomes the limiting factor, and acetate inevitably accumulates, resulting in a typical maximum DCW of $\sim 200 \text{ g L}^{-1}$ (23).

Fed-batch cultivation is commonly used to achieve high biomass concentrations in *E. coli* fermentation (Table 2-1). This method relies on the addition of a limiting nutrient according to a feeding schedule that maximizes culture growth. The limiting nutrient can be fed at either a predetermined rate (whether constant, decreasing or exponential) or in response to physiological parameters (feedback control), including pH- and DO-stat approaches. Some strains are auxotrophic for an essential nutrient, and this dependency has been exploited to control cell growth in a given fermentation broth. Although high cell concentration remains the primary objective of fed-batch cultivation, the modulation of certain metabolic processes are often also included, notably to increase carbon flow into essential metabolite pathways and to reduce byproduct formation (24), which is important for the over-production of metabolites that branch off from the primary metabolism pathways, such as amino and organic acids, isoprenoids and sialic acid.

Table 2-1 Cell mass yields from HCDC of common biotech/lab oriented *E. coli* strains. Typical DCWs range from 50-200 g L⁻¹ depending on both the strain and fermentation conditions used (medium, temperature, etc.) (20).

Host strain	Medium	Temperature (°C)	Culture method	Cultivation time (h)	Dry cell weight (g L ⁻¹)	Advantages
TG1	Defined (glycerol)	28	Exponential	44	148	Cloning strain
TG1	Defined	28	Exponential	24	128	
W	Defined (sucrose)	37	pH-stat	36	105	Can use sucrose as carbon source
W3110	Defined (glycerol)	37	Constant feeding with dialysis	23	174	Wild-type
MG1655	Complex	NA	Whey-fed	NA	80	Wild-type
B (OSU333)	Defined	32	Glucose-fed batch	9	65	Protease-deficient
BL21 (DE3)	Defined	37	pH-stat	30	183	Protease-deficient
JM109	Defined	37	Glucose-fed batch	15	75	DNA stability
MD42	Defined	37	Exponential	45	44	Reduced genome

2.2.3 Effects of parameters on cell growth and recombinant protein expression

Several strategies can be applied to enhance recombinant protein expression. For example, codon usage optimization in the recombinant protein-coding genetic sequence can enhance product expression in certain systems such as *E. coli* (Table 2-2), which, like virtually all living organisms, favours certain kinds of codons more than others (33, 34). Other options include adopting a strong promoter (Table 2-3) and using high copy number vectors, both of which can result in a higher protein yields. A

stronger promoter contributes to the production of more mRNA, in turn leading to more proteins. A larger number of expression plasmid copies will build up the protein production capacities of bacterial cells, thus resulting in higher yields. Furthermore, the selection of specific expression vectors (e.g. bacterial RNA polymerase or T7 bacteriophage RNA polymerase based vectors), and the choice of *E. coli* strain can also have a strong impact on the expression levels of proteins (34).

Table 2-2 Codons used by *E. coli* at a frequency of <1%. (Taken from (35), a cut-off of <1% was use to arbitrarily define rare codons)

Rare codons	Encoded amino acid	Frequency per 1,000 codons
AGG/ACG	Arg	1.4/2.1
CGA	Arg	3.1
CUA	Leu	3.2
AUA	Ile	4.1
CCC	Pro	4.3
CGG	Arg	4.6
UGU	Cys	4.7
UGC	Cys	6.1
ACA	Thr	6.5
CCU	Pro	6.6
UCA	Ser	6.8
GGA	Gly	7.0
AGU	Ser	7.2
UCG	Ser	8.0
CCA	Pro	8.2
UCC	Ser	9.4
GGG	Gly	9.7
CUC	Leu	9.9

Table 2-3 Commonly used promoters (27).

Promoter	Regulation	Induction	Problems
<i>lac</i> (<i>lacUV5</i>)	lacI, lacI ^q	IPTG, thermal	Low-level expression relative to other system; leaky expression
<i>trp</i>		Trp starvation IAA (indoleacrylic acid)	Leaky expression
<i>tac</i>	lacI, lacI ^q	IPTG, thermal	Leaky expression
λpL	λcIts 857	Thermal	Induction cannot be performed at low temperatures; partial induction cannot be achieved
T7	lacI, lacI ^q	IPTG, thermal	Leaky expression; difficult to achieve high cell densities
<i>PhoA</i>	phoB, phoR	Phosphate starvation	Not titratable; limited media options
<i>Ara</i>	araC	L-Arabinose	Few vectors available; catabolite repressed by glucose
<i>Cad</i>	cadR	pH	Limited characterization; few vectors available
<i>RecA</i>	lexA	Nalidixic acid	Not titratable

Engineers are able to control the growth and expression conditions of the constructed recombinant strain to optimize recombinant protein expression. Among the many factors that can affect recombinant proteins expression, the most significant ones are inducer selection and concentration, temperature at which growth and expression take place, fermentation medium pH and composition, cell growth rate, and production of metabolic by-products.

2.2.4 Inducer selection and concentration

The inducer is a chemical substance that is added to the fermentation broth to initiate expression at a desired time when induction conditions are favourable, in strains using expression vectors containing inducible promoters. The most commonly used inducer for recombinant protein expression is

isopropyl- β -D-1-thiogalactopyranoside (IPTG), which is used in expression systems regulated by a *lac* operon. IPTG, a lactose analog, initiates the transcription of sequences under the control of the *lac* promoter by halting the repression of gene expression by binding to a repressor sequence located downstream of the operon region. When bound to IPTG or another appropriate ligand, the repressor configuration is altered, allowing RNA polymerases access to promoter sites to initiate the transcription process. Once added to the fermentation medium, the concentration of IPTG remains constant, as its non-hydrolysable sulfur bond prevents its biodegradation.

Lactose itself can also be used as an inducer, as several studies have demonstrated (36-38). The use of lactose instead of IPTG as the inducer can greatly reduce the cost of fermentation as IPTG is relatively expensive, thus making it only suitable for small-scale operations. In larger-scale fermentation, cost-effective compounds are more preferable. In addition to its high price, the toxicity of large quantities of IPTG complicates its widespread use in industrial processes, especially in those producing therapeutics or other compounds destined for medical use (39, 40).

In most biological systems, process optimization requires multiple parameters to be adjusted for specific applications, and those related to the inducer are not excluded from these considerations (41). The inducer concentration plays an important role in recombinant protein expression. It has been reported that *E. coli* can be induced with IPTG concentrations varying from 0.1 mM to 2 mM (42), depending on the temperature at which the induction takes place, the induction timing (when the

inducer is added to the fermentation medium), and the duration of the induction period (43).

2.2.5 Temperature

Temperature affects both cell growth and recombinant protein expression in all bacterial strains. The effects of temperature on cell growth can be roughly described by the Arrhenius equation with an optimal temperature range from 23°C to 37°C, depending on the species and strains, outside of which growth rates decrease drastically (44).

Lower temperatures can greatly influence the expression of certain genes. It has been reported that the expression of the OmpA gene, responsible for the synthesis of transmembrane proteins, is less efficient at temperatures lower than 28°C compared to a temperature of 37°C (45). In another example, Bortolussi and Ferrieri demonstrated the absence of their target protein, an antigen, when cells were grown at a temperature lower than 30°C for a short period of time (46).

On the other hand, lower temperatures, although not amenable to high growth rates, may enhance protein solubility (47). The reduction in protein expression at lower temperatures results in a lower production of intracellular proteins which in turns decreases protein aggregation. Aggregated proteins tend to form insoluble inclusion bodies which remain enclosed in the bacterial debris after cell lysis, and further denaturing steps are required for the extraction of the recombinant products in a soluble form, which increases both processing time and costs.

2.2.6 pH

The pH of the fermentation broth is of great importance for well-balanced cell growth and protein expression, as it can have a great influence on the behaviour of the outer cell membrane (48, 49). Too high or too low of a pH can induce the gene transcription of stress response proteins which utilize metabolic resources. As a result, fewer of these resources are allocated to cell growth and the expression of other types of proteins, including recombinant ones (50). Furthermore, organic acids penetrate cell membranes and can affect the functioning of proton-driven pumps. When cells are grown at lower pH, organic acid accumulation in cells leads to lower intracellular pH, which can change the conformations of intracellular proteins, possibly interfering with their function.

2.2.7 Medium composition and carbon source

In *E. coli* fermentation, the optimal medium composition can change according to the particular strain used or the particular target protein to express. It may vary from a minimal medium, in which all elements are defined and precisely quantified, to complex medium whose composition remains undefined and unquantified, while still containing all necessary nutrients. Both of these types of medium have advantages and disadvantages. The minimal mineral medium is usually applied in laboratory experiments to select certain kinds of strains or to cultivate wild-type bacteria without providing other factors or supplements that may be found in complex medium which may be necessary to achieve the highest possible cell concentrations. Defined medium has advantages in being able to control each component in the media, allowing it to be used to investigate the influence of specific medium components on

cell growth or protein expression. Complex media cannot be used to determine the effect of different components, since the exact content of each component in this media is unclear. However, the complex media can greatly increase bacterial growth as it provides growth factors and other trace elements contained in yeast extract commonly used in such media. Additional carbon sources can be added to any of the above media, which may serve as an added energy source to enhance cell growth. The most commonly used carbon sources are glucose and glycerol, but other sources such as lactose, sucrose, fructose, mannose, xylose, or succinate may also be used (51-53). Different carbon sources have different effects on specific growth rate of *E. coli*, with glucose leading to the highest growth rate (54).

The carbon sources taken up by bacteria can be oxidized in the presence of oxygen and converted to ATP (adenosine triphosphate) and NADH₂ (nicotinamide adenine dinucleotides), which are used as direct energy sources by bacterial metabolism. When *E. coli* is grown aerobically, part of its energy comes from the Krebs cycle (also known as TCA cycle), a process shown in Figure 2-4. For one glucose molecule, this cycle is performed twice, as glucose is converted into two pyruvate molecules through glycolysis, each of which enters the Krebs cycle to produce a total yield of six ATP molecules. If no favourable carbon source, such as sugars or sugar alcohols, are available in the medium, bacteria are also able to utilize alternative carbon sources such as amino acids to support cell growth. This explains why when *E. coli* is grown in complex medium without enough sugar supplementation, the pH rises as a result of rising ammonia concentrations produced

as a byproduct of amino acid utilization.

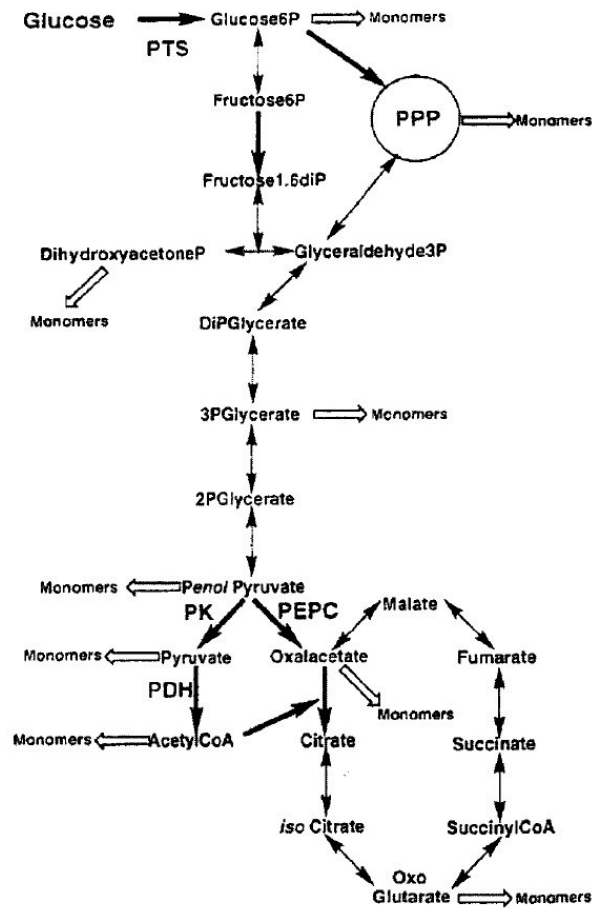


Figure 2-4 Metabolism pathway for glucose glycolysis and Krebs cycle (54).

2.2.7.1 Glucose catabolism

The aerobic catabolism of glucose can be divided into three stages: 1) the Embden-Meyerhof-Parnas pathway (EMP), 2) the Krebs cycle, and 3) the electron transport chain. During the EMP phase, a glucose molecule is converted into two pyruvate molecules, which enter the Krebs cycle and are converted to CO₂ and NADH. NADH serves as an electron carrier in many metabolic reactions. The Krebs cycle usually has three functions. First, supply electrons via NADH for respiratory pathways and biosynthesis. Second, provide a carbon backbone for anabolism and

finally, generate energy in the form of ATP which contain three phosphate groups and act as the "molecular unit of currency" of intracellular energy transfer, and this may be accomplished using the pool of NADH produced (55). Growth on glucose is fastest, with a specific growth rate of 0.94 h^{-1} . Due to glucose overflow in the TCA cycle (54), however, as can be seen in Figure 2-5, glucose-based fermentation also produces the most by-products.

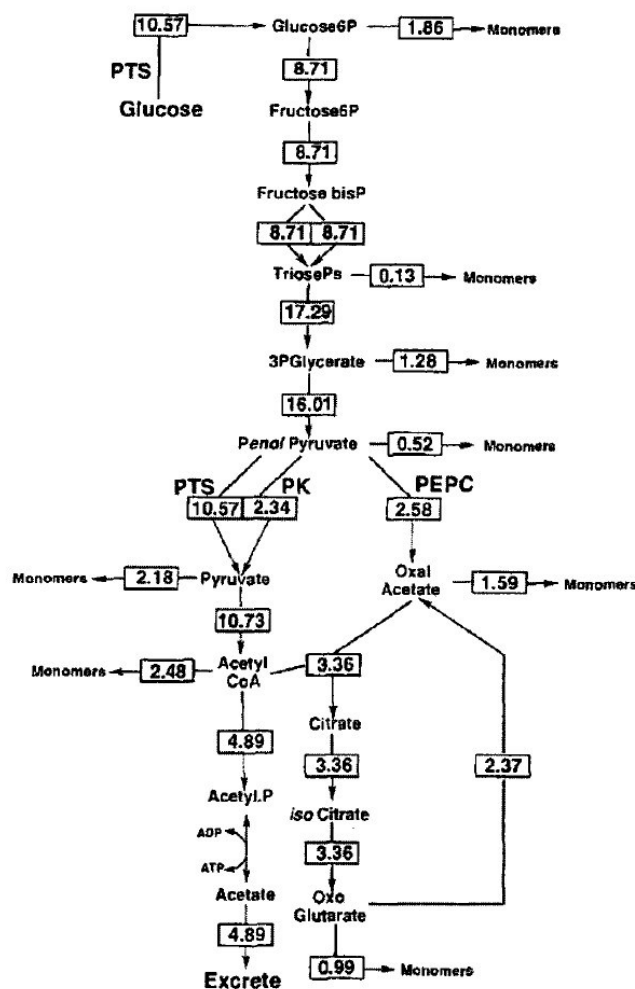


Figure 2-5 Flux analysis of *E.coli* grown on glucose (54).

2.2.7.2 Glycerol catabolism

E. coli can also utilize glycerol for cell growth and protein expression. The

growth rate on this carbon source is slower than that on glucose (0.70 h^{-1} compared to 0.94 h^{-1}), due to the fact that only 66% of glycerol's carbon atoms can enter the Krebs cycle. It should be noted that, as shown in Figure 2-6, glycerol catabolism produces almost no acetate and the only major products associated with glycerol utilization are biomass, water and CO_2 (54), which contributes to its better performance in the high cell density culture of *E. coli*.

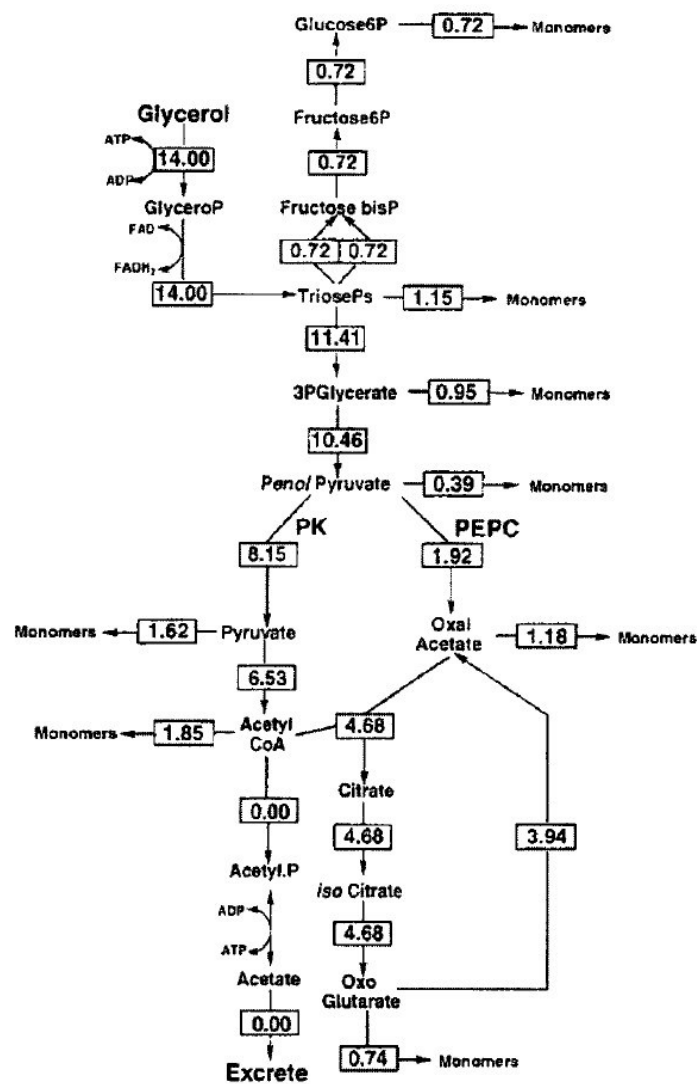


Figure 2-6 Flux analysis of *E. coli* grown on glycerol (54).

2.2.7.3 Acetate catabolism

Acetate can also serve as a carbon source for cell growth and is much less efficient with specific growth rates on acetate as low as 0.43 h^{-1} . As acetate is an anabolite of acetyl-CoA, catabolism of acetate requires that the reverse reactions be carried out, which consumes ATP in the process, as can be seen on Figure 2-7.

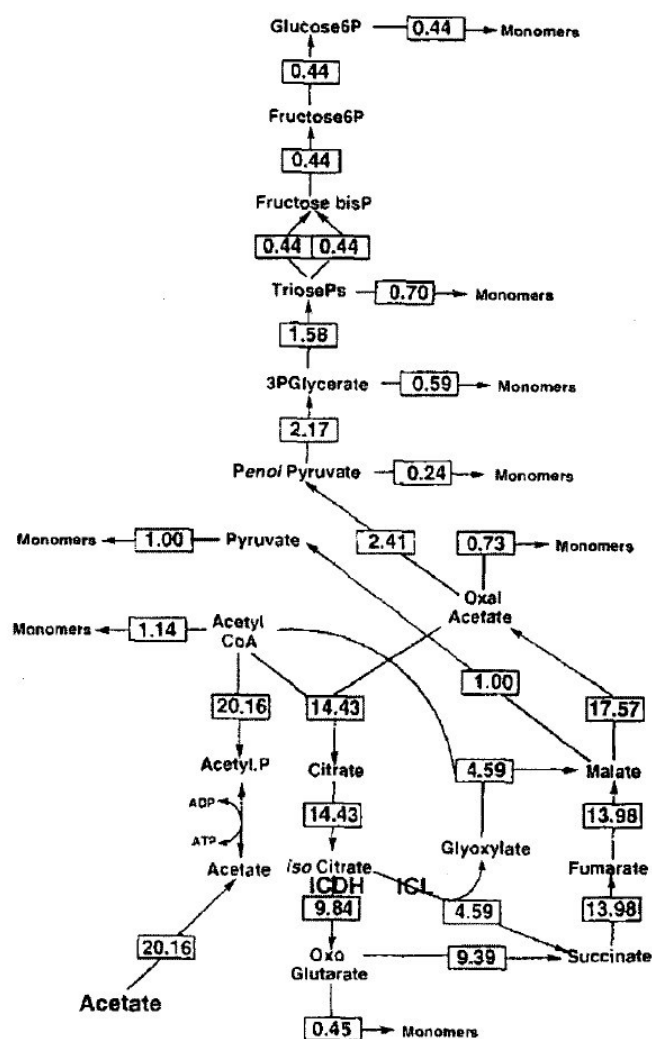


Figure 2-7 Flux analysis of *E. coli* grown on acetate (54).

2.2.8 Critical growth rate

There are several factors that can influence the growth rate of cells. First, one

kind of carbon sources is necessary to provide the carbon backbone of cell materials and the bio-energy for cell metabolism. Oxygen is also required for aerobic organisms, serving as the final electron acceptor. It has been reported that increasing the initial carbon source concentration increases the specific growth rate if the levels of other nutrients are adequate (56). It should be noted, however, that the maximum growth rate does not always result in the maximum cell concentration or that of target products. For example, when *E. coli* cells are in the presence of a high glucose concentration, an overflow of acetyl-CoA enters the Krebs cycle, which leads to higher concentrations of acetate (54, 57). It was reported that *E. coli*'s metabolism tends to maximize ATP production (58). As a result, acetate production is increased when glucose in the medium is excessive, since it is a secondary pathway for ATP production, and in fact produces the second largest amount of ATP and NADH₂ of any pathway (59). It was also demonstrated that *E. coli* strains grown in complex medium, such as yeast extract or tryptone, tend to accumulate acetate at lower growth rates (dilution rate of 0.2 h⁻¹) than those grown in a defined medium (dilution rate of 0.35 h⁻¹) (60). Acetate is a metabolic by-product that may inhibit both cell growth and recombinant protein expression, which is further discussed in a subsequent section. Since acetate formation has a large effect on cell growth rate, many strategies have been designed to decrease its production by limiting specific growth rates below a critical value (32, 61, 62). The critical growth rate is defined as the rate at which *E. coli* produces minimal amounts of acetate, and it can be achieved and maintained by controlling the uptake of the carbon source and the dissolved oxygen level. These

strategies are carried out by controlling the rate at which bacteria are growing, either by controlling the rate of carbon consumption, or by adding chemicals that can alter the consumption of carbon sources (57, 63).

2.2.9 Acetate and other by-product formation

As previously discussed, acetate formation correlates to the specific growth rate which is in turn affected by the glucose concentration in the medium (31). It has been reported that acetate can completely inhibit cell growth when its concentration exceeds 10 g L^{-1} (64, 65), but may exert inhibition at concentrations as low as 0.5 g L^{-1} (66). Furthermore, acetate specifically inhibits oxygen and carbon uptake, leading to the inhibition of recombinant proteins expression (67, 68). It is also suspected of weakening proton motive pumps by disturbing transmembrane pH gradients (61, 69). Cherrington *et al.* demonstrated that short-chain acids could impair the synthesis of RNA, DNA and other macromolecules (70). According to his conclusion, organic acids could lead to growth inhibition, as organic acids would dissociate upon diffusion into the cell and produce anions that could disturb the synthesis of these macromolecules. Lactate, the second preferred by-product excreted from an overflowing carbon chain, can also inhibit cell growth and protein expression, and is converted to lactic acid once protonated (54). Other inhibitory metabolic by-products include pyruvate and ethanol (32).

2.3 Molecular biology background

2.3.1 Recombinant protein expression

Recombinant DNA techniques have become popular since the 1960s and various expression and cloning vectors have been constructed that enable bacterial strains to produce recombinant proteins (Table 2-4). Consisting of a circular double stranded DNA, an expression vector or plasmid usually includes an origin of replication (ORI), a promoter sequence, a selection marker, and one or more multiple cloning sites (MCS). The ORI is necessary for the bacteria to replicate the plasmid DNA once it is present in the cytoplasm, leading to a large number of copies of the plasmid in each bacterial cell. RNA polymerase binds to the promoter sequence to initiate DNA transcription of the genes under the promoter's control, resulting in mRNA that will subsequently be translated into specific proteins. The selection marker is used to select bacteria bearing the vector. The MCS allows for the cloning and ligation of the desired genes into the expression plasmid, in the correct orientation. A gene to be transcribed into mRNA must be in the proper orientation relative to the promoter constructs, and in the same codon reading frame as the initiation codon, which is achieved by inserting flanking restriction enzyme cleaving sites to the desired gene construct during the polymerase chain reaction amplification step using specially designed primers (71).

Table 2-4 Chemical produced using genetically and metabolically engineered *E. coli*. Key metabolic strategies use to enhance productivity are given. Fermentations were typically done using fed-batch with defined media (exceptions are explicitly stated) (20).

Chemical	Remarks (strain; genetic alteration)	Cultivation	Titer (g L ⁻¹)
L-Threonine	KY10935; AHV ^r , AEC ^r , Asp ^r , Lys ^r , Hse ^r , Thr ^r , Met ^r , alterations in Thr transport	Glucose, 30 °C, 77 h	100
	W3110; plasmid-borne Thr-biosynthetic operon with ThrA ^{fb} , possesses LysA ^{fb} , Met-, lacks Thr catabolism and uptake, upregulation of Thr export (RhtABC)	Glucose, 31 °C, 50 h	80
L-Phenylalanine	K-12; Δ <i>pheA-aroF-tyrA</i> , plasmid borne <i>pheA^{fb}</i> and <i>aroFLB</i> genes	Glucose, 37 °C, 50 h, in situ product recovery	45
L-Tyrosine	N/A; <i>pheLA</i> , expression of <i>tyrA</i> by <i>trc</i> promoter	Glucose, 35 °C, 48 h	55
L-Tryptophan	N/A; Plasmid borne AroG ^{fb} , Trp ^{fb} , and the <i>trp</i> biosynthetic operon	Glucose, 52 h	45
Succinate	AFP111; Δ <i>pflAB</i> , Δ <i>ldhA</i> , Δ <i>pstG</i> , plasmid borne <i>pyc</i> gene under the control of <i>trc</i> promoter	Dual-phase, Glucose, 37 °C, 75 h	99
Lycopene	K-12 (Dupont); expression of <i>dxs</i> , <i>idi</i> and <i>ispFD</i> by T5 prompter, plasmid borne <i>crtEBI</i> operon, Δ <i>gdhA</i> , Δ <i>aceE</i> , Δ <i>yjiD</i>	Glucose, 37 °C, 24 h	0.22
	BL21; expression of yeast-derived melavonate pathway, plasmid borne <i>crtEBI</i> and <i>dxs</i> genes	LB with 5 mM MVA and 0.1% arabinose, 37 °C, 20 h	0.23
β -Carotene	DH5 α ; expression of bacterial-derived MVA pathway, plasmid borne <i>crtEBIY</i> and <i>dxs</i> genes	2YT with 2.5% (w/v) glycerol, 15 mM MVA and 0.2% arabinose, 29 °C, 144 h	0.5
Coenzyme Q10	BL21; plasmid borne <i>ddsA</i> gene, amplified expression of the <i>dxs</i> gene	Glucose, 37 °C, 49 h	0.05
Xylitol	PC09; possess cAMP-independent Crp, Δ <i>xylB</i> , plasmid borne xylose reductase from <i>Candida boidinii</i> XLY1	Glucose/xylose, 30 °C, 50 h	38
	PC07; Δ <i>xylB</i> , plasmid borne xylose reductase gene <i>Candida boidinii</i> XLY1, overexpression of xylose-transporter (Xy1FGH)	Glucose/xylose, 30 °C, 50 h	56

Mannitol	BL21; plasmid borne mannitol and formate dehydrogenases	Whole-cell biotransformation from fructose/formate, 30 °C, 8 h	66
N-acetylglucosamine	W3110; expression of GlmS ^{fbr} enzyme and yeast-derived Gnal, removal of amino sugar metabolism ($\Delta nagBA$, $\Delta nagE$, $\Delta manXYZ$)	Glucose, 37 °C, 72 h	110
Hyaluronic acid	JM109; plasmid-borne <i>kfiD</i> and the HA synthase gene (<i>pmHas</i>)	Glucose, 30 °C, 30 h	3.7
N-acetylneuraminic acid	DC; $\Delta nanATEK$, plasmid borne <i>neuB</i> and <i>neuC</i> genes controlled by T7-promoter	Glycerol, 34 °C, 84 h	22

Abbreviations: AHV, α -amino- β -hydroxyvaleric acid; AEC, amino ethyl-carbazole; MVA, mevalonate.

Once taken up by the bacteria, the vector is replicated multiple times, and when appropriate conditions are met, protein expression occurs. If an inducible promoter strategy is employed, as discussed previously, an inducer ligand is required to initiate protein expression. Such expression constructs consist of the gene(s) of interest, an upstream promoter/5'UTR region, and a 3'UTR region downstream of the expressed sequence. The transcription of DNA into RNA is initiated by the binding of RNA polymerase to an initiation sequence upstream of the initiation codon, within the promoter region. The most widely used inducible promoter is the *lac* promoter, isolated from wild-type strains in which it regulates the expression of a series of adjacent genes with the same orientation whose products are involved in lactose catabolism, collectively referred to as the *lac* operon. When no lactose is present, a regulatory protein binds to the *lac* operator sequence, located immediately downstream of the promoter, and prevents the binding of RNA polymerase to the promoter region, thus preventing the transcription of any gene under the *lac*

promoter's control. When lactose is available, allolactose, a metabolite of the lactose catabolic pathway, binds to the repressor protein, changing its conformation, and preventing its binding to the operator sequence, thus allowing RNA polymerase to bind to the promoter and to initiate transcription of the gene(s) of interest. Once transcribed, the mRNA is translated into the target protein product (71).

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Chapter 3: Optimization of cultivation conditions for sialic acid production by recombinant *Escherichia coli*

Abstract

A recombinant *Escherichia coli* BRL04 (pBRL89) producing sialic acid was studied in batch culture. The effect of various carbon sources on sialic acid production was investigated, and a higher sialic acid productivity was achievable using glycerol over that obtained with glucose. Additionally, various ratios of glycerol to GlcNAc were investigated, and a mixture of 40% v/v glycerol with 4g/100 mL GlcNAc was found to be preferable, considering both the productivity and cost effectiveness. The effect of initiation time of induction was studied, and an optimum was found at the middle exponential phase ($DCW \approx 4.5$ g/l), which resulted in the highest sialic acid production. The effects of culture temperature, pH and dissolved oxygen were studied, and a maximized production of sialic acid was observed at conditions of 33°C incubation temperature before induction and 37°C after induction, pH 7.4, and 20% dissolved oxygen.

Keywords

Sialic acid • *Escherichia coli* • batch culture

3.1 Introduction

Many cell surfaces, both prokaryotic and eukaryotic, are widely decorated with glycol-conjugates that play significant roles in the maintenance of cell biological activities (1). Sialic acid encompasses a large family of acidic sugars found on the surfaces of eukaryotic cells (2). In 1936, Blix isolated a crystalline reducing acid from bovine submandibular, which was thereafter named 'sialic acid' (3, 4). Most research to date on sialic acid focused on N-acetylneuraminic acid (Neu5Ac), and the term 'sialic acid' has been widely used in literature to refer to this particular chemical. Sialic acid plays an important role in several crucial biological processes including glycoprotein stabilization, cell signaling, solute transport, neuronal plasticity, cell adhesion and cellular immunity (5).

Sialic acid is challenging to produce both chemically and by fermentation, and is usually synthesized via an in vitro enzymatic process (6), which is costly and difficult to scale to the manufacturing level. The scarce supply of available sialic acid has hindered advancement in basic research, diagnostic development and therapeutic production. To provide a low cost and scalable process for sialic acid production, a metabolically and genetically engineered *Escherichia coli* BRL04 (pBRL89) was produced by removing the sialic acid catabolism pathway and expressing sialic acid biosynthesis genes (7).

To further characterize and study sialic acid production by this engineered *E. coli*, the effect of various fermentation conditions were investigated and optimized. The influence of various parameters such as carbon source selection (glycerol and

glucose), the ratio between glycerol and GlcNAc (N-acetylglucosamine, a substrate of the sialic acid biosynthetic pathway, which supplies backbones for sialic acid synthesis), cultivation temperature, dissolved oxygen (DO), and induction time on the sialic acid production from the recombinant *E. coli* were investigated. These results are crucial for the cost-effective and efficient scaling-up of sialic acid production via this novel route.

3.2 Materials and methods

3.2.1 Bacterial strain

The *E. coli* strain capable of producing sialic acid was kindly provided by Dr. Christopher N. Boddy from the Department of Chemistry at the University of Ottawa, and was prepared by disabling the native sialic acid catabolic pathway and adding an exogenous synthetic pathway, as previously detailed (6, 8, 9).

3.2.2 Culture medium and cultivation conditions

All chemicals were obtained from Fisher Scientific unless otherwise stated, and were reagent-grade or higher purity, and were used without further purification. Before starting fermentation, the plasmid pBRL89 (with the sialic acid synthetic gene) was transformed into *E. coli* BRL04 (with the sialic acid catabolic gene knocked out). The recombinant *E. coli* were streaked onto an LB agar (10 g/L tryptone, 5 g/L yeast extract, 10 g/L sodium chloride, 15 g/L agar) plate, where ampicillin (Sigma-Aldrich) and kanamycin (Sigma-Aldrich) were used at concentrations of 50 µg/mL and 40 µg/mL respectively, and incubated at 37°C for until colonies appeared (~12h).

The preculture was prepared by inoculating an individual colony into 200 mL LB medium and incubating at 37°C and 200 rpm on a rotary shaker for 12 h to create the inoculum for the bioreactor. Ampicillin (Sigma-Aldrich) and kanamycin (Sigma-Aldrich) were used at concentrations of 50 µg/mL and 40 µg/mL respectively.

The production of sialic acid in batch culture from fermentation of recombinant *E. coli* was performed in a 3-L fermentor (Bioflo 110 New Brunswick Scientific Co., INC. Edison, NJ, USA). The fermentation medium contained the following components: glycerol (20 g/L) or glucose (20 g/L), K₂HPO₄ (6.62 g/L), KH₂PO₄ (3.0 g/L), NH₄SO₄ (4.0 g/L), MgSO₄ (0.17 g/L), thiamine (4.5 mg/L), ampicillin (50 µg/mL), kanamycin (40 µg/mL), antifoam (0.1 mL/L) and trace metal solution (10 mL/L) (10). The prepared trace metal solution consisted of the following components: FeCl₃·6H₂O (15.0 g/L), ZnCl₂·4H₂O (2.0 g/L), CaCl₂·6H₂O (2 g/L), ZnSO₄·7H₂O (2.2 g/L), MnSO₄·4H₂O (0.5 g/L), CuSO₄·5H₂O (1.9 g/L), Na₂MoO₄·2H₂O (2 g/L), CoCl₂·6H₂O (4 g/L), H₃BO₃ (0.5 g/L), and concentrated HCl (100 mL/L) (11).

One hundred and fifty milliliters of preculture grown to mid-exponential phase (OD₆₀₀ of 10) was added into the 3-L fermentor containing 1.35 L of fermentation medium. The culture pH was adjusted and controlled at 7.4 by the addition of 15% (v/v) ammonium hydroxide solution using a computer-controlled pump. The temperature was controlled to 30°C (unless otherwise specified) by the heating jacket and cooling water in the system. The dissolved oxygen level was kept above 20% (unless otherwise specified) by cascading the agitation speed (200-1200 rpm). The air flow rate was maintained at 3 L/min for all batch studies. IPTG

(isopropyl- β -D-1-thiogalactopyranoside) (0.1 mM) was added at the mid-exponential phase, when the measured OD₆₀₀ was 10. In the first two runs comparing glycerol and glucose defined media, 10 g/L GlcNAc was added to the initial medium, while in other runs, 100 mL 40% (v/v) glycerol with 4 g GlcNAc (Sigma-Aldrich) (unless otherwise specified) were added to the medium when the initial glycerol was complete, as indicated by a sharp increase of dissolved oxygen concentration.

3.2.3 Analytical methods

Cell growth was monitored by measuring the optical density of the samples at 600 nm with a spectrophotometer (Ultraspec 60 DBL BEAM 2NM, Biochrom Ltd) after an appropriate dilution, where one OD₆₀₀ unit was previously found to be equivalent to 0.33 g/L dry cell weight (DCW) (12). The sialic acid concentration was determined by liquid chromatography–mass spectrometry (SHIMADZU, API 200 LC/MS/MS System) after derivatization with DMB (4,5-methylenedioxy-1,2,-phenylenediamine dihydrochloride) solution (20 μ L broth and 20 μ L of DMB solution). 1 mL of DMB solution was prepared and consisted of: 858 μ L H₂O, 56 μ L acetic acid, 42 μ L 2-mercaptoethanol, 1.8 mg sodium hydrosulfite, 2.4 mg DMB.

3.3 Results and discussion

3.3.1 Carbon source screening

The cell growth kinetics were investigated via batch culture in glycerol and glucose defined media and the results are shown in Figure 3-1. The maximum dry cell

weight in glycerol defined medium was found to be 8.53 g/L, and was 22.6% higher than that observed in glucose defined medium (6.96 g/L). The corresponding sialic acid production in the two mediums are shown in Figure 3-2, and a maximum sialic acid production of 6.50 g/L was observed in the glycerol defined medium, which was 20.1% higher than that in glucose-defined medium (5.41 g/L).

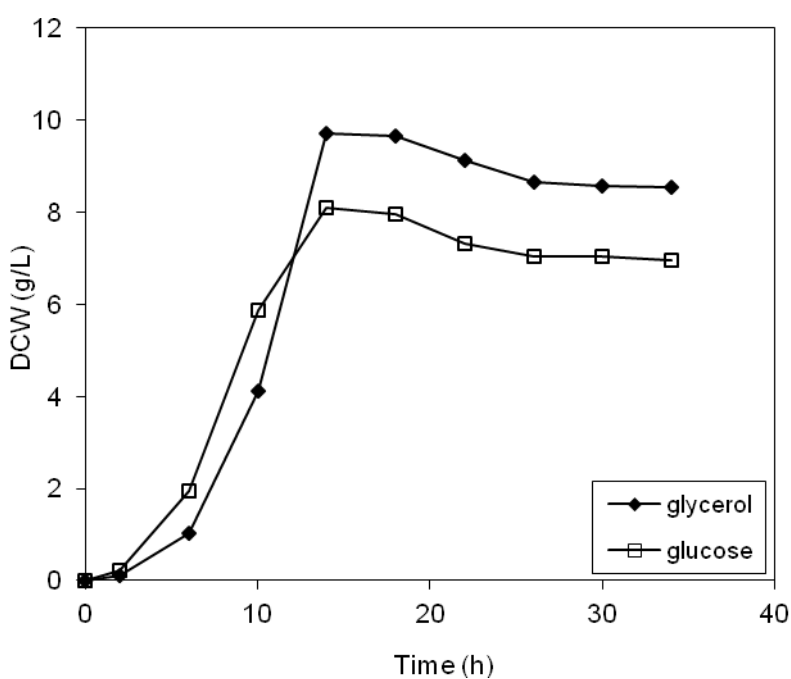


Figure 3-1 Effects of glycerol and glucose on cell growth.

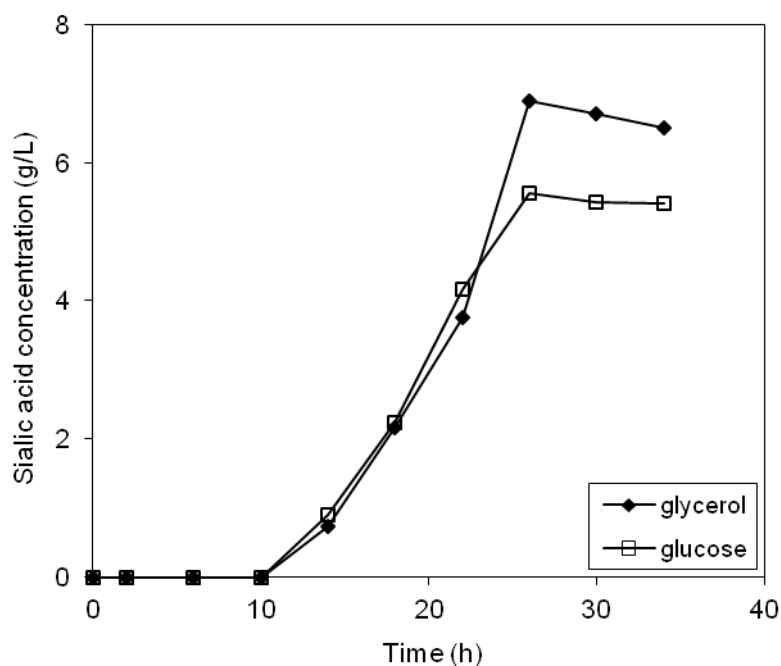


Figure 3-2 Effects of glycerol and glucose on sialic acid production.

The specific growth rate and specific sialic acid production rate in glycerol and glucose defined media are shown in Figure 3-3 and Figure 3-4, respectively. The average specific growth rate (μ) and the average specific sialic acid production rate for the glycerol defined medium (0.21 h^{-1} , 0.15 g/g cell/h , respectively) were 31.3% and 50.0% higher than those observed in the glucose defined medium (0.16 h^{-1} , 0.10 g/g cell/h , respectively), indicating that glycerol was more favorable for both cell growth and sialic acid production than glucose. Glycerol was therefore chosen for further study in the optimization experiments.

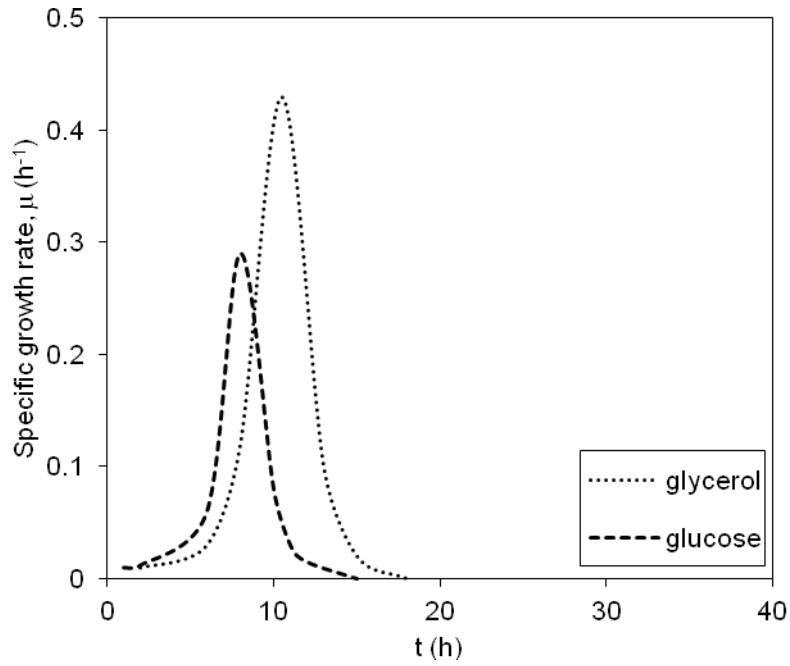


Figure 3-3 Effects of glycerol and glucose on specific growth rate.

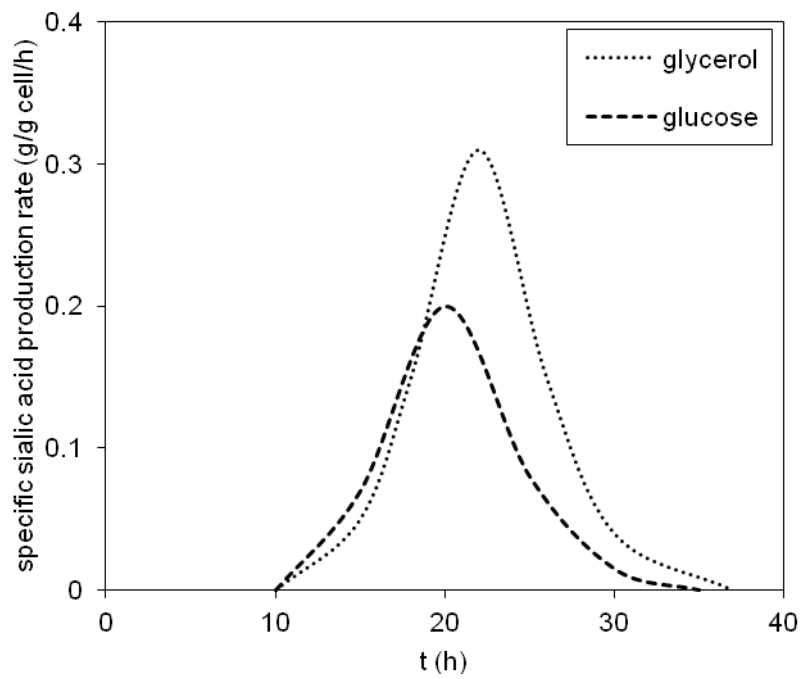


Figure 3-4 Effects of glycerol and glucose on specific sialic acid production rate.

3.3.2 Effect of glycerol to GlcNAc ratio

GlcNAc is a significant component necessary for the synthesis of sialic acid, supplying the backbone for the sialic acid molecule. However, its high price hinders large-scale sialic acid production. In order to reduce the required consumption of GlcNAc without significantly influencing sialic acid production, the growth medium was adjusted and the effect of the glycerol to GlcNAc ratio was studied. The results, in terms of dry cell weight and sialic acid production as a function of glycerol (mL/100 mL) to GlcNAc (g/100 mL) ratio, are given in Figure 3-5. It was observed that the sialic acid production decreased with increasing glycerol/GlcNAc ratio, although this increase can also enhance cell growth. When the glycerol/GlcNAc ratio was less than 10, the increase in sialic acid concentration was negligible. Considering this, and due to the high cost of GlcNAc, a glycerol (mL/100 mL) to GlcNAc (g/100 mL) ratio of 10 (100 mL 40% v/v glycerol with 4g GlcNAc) was considered to be optimal in practice.

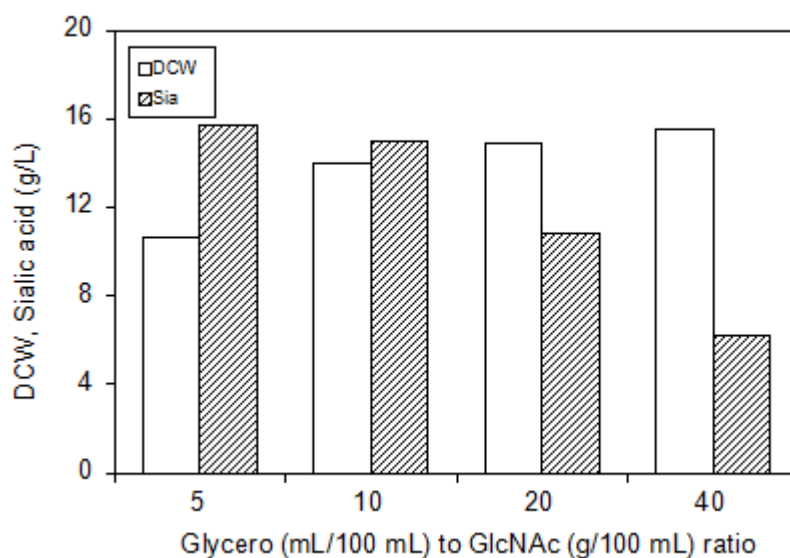


Figure 3-5 Effect of glycerol (mL/100 mL) to GlcNAc (g/100 mL) ratio on cell growth and sialic acid production.

3.3.3 Effect of induction time

The expression of the sialic acid production gene is induced by the addition of IPTG in this system. The effect of induction time on final cell growth and sialic acid production was studied, and the results are shown in Figure 3-6 when the IPTG was added at the early, middle and late exponential phases, respectively. These additions corresponded to dry cell weights of 1.5, 4.5 and 9.5 g/L, respectively, according to our previous experiments. The maximum cell concentration was achieved when the inducer was added in the later growth stages, which was likely attributable to the inhibition of IPTG on the cell growth. Among the three induction periods studied, induction at the middle exponential phase was found to result in a maximum sialic acid production of 15.02 g/L, which was 9.87% and 26.6% higher than the early (13.67 g/L) and late (11.86 g/L) exponential phases, respectively. These results

indicated that the middle exponential phase represented the optimal induction time for sialic acid production.

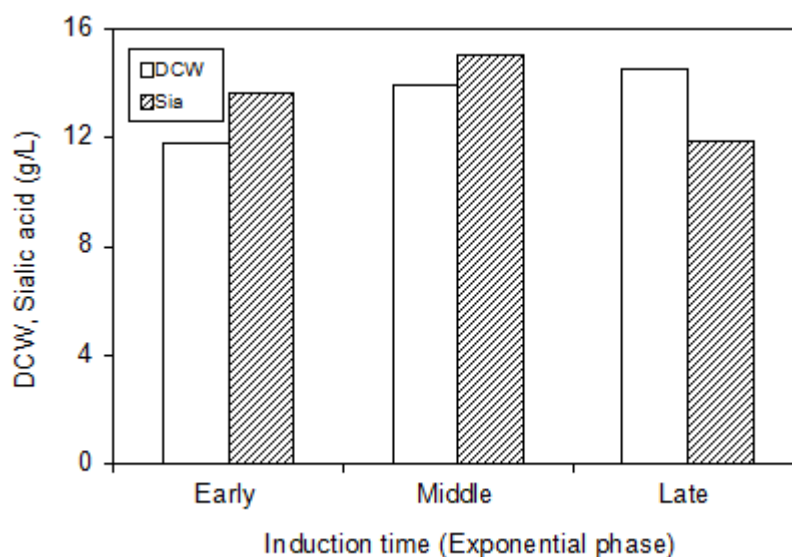


Figure 3-6 Effect of induction time on cell growth and sialic acid production.

3.3.4 Effect of temperature, pH and dissolved oxygen

The effect of temperature on cell growth and sialic acid production was studied, and the results are shown in Figure 3-7. The dry cell weight was found to decrease as the temperature increased from 30°C to 40°C, which was thought to be due to increased acetic acid accumulation at higher temperature. Despite this, the sialic acid production was observed to be highest at an optimal temperature of 37°C. In order to achieve an optimum between the sialic acid production and cell concentration, a strategy was used where the temperature was adjusted for different phases of the cell growth. A temperature adjustment strategy of 30°C before induction and 37°C after the induction was proposed, and these settings were confirmed to result in the highest

sialic acid production (18.56 g/L), as shown in Figure 3-7.

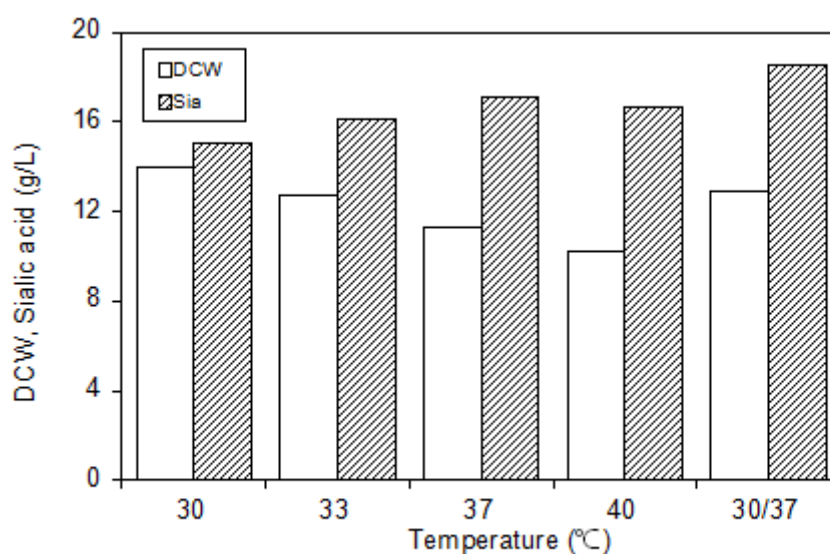


Figure 3-7 Effect of temperature on cell growth and sialic acid production.

Additionally, the effects of pH and dissolved oxygen on the cell growth and corresponding sialic acid production were also studied, and the results are shown in Figures 3-8 and 3-9 for pH and dissolved oxygen, respectively. From the results shown in Figure 3-8, the optimal pH was found to be 7.4 with both maximal cell concentration (13.97 g/L) and sialic acid production (15.02 g/L). As shown in Figure 3-9, when the dissolved oxygen was 10%, the cell growth and sialic acid production were greatly inhibited. The effects of various dissolved oxygen levels were not obvious above 20%. Considering the high cost of providing this oxygen supply in practical production, the dissolved oxygen level was thought to be optimal at 20% saturation.

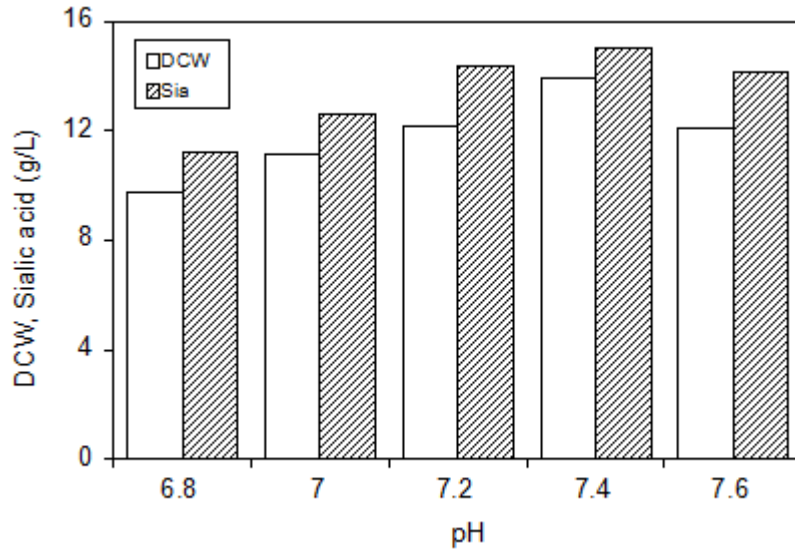


Figure 3-8 Effect of pH on cell growth and sialic acid production.

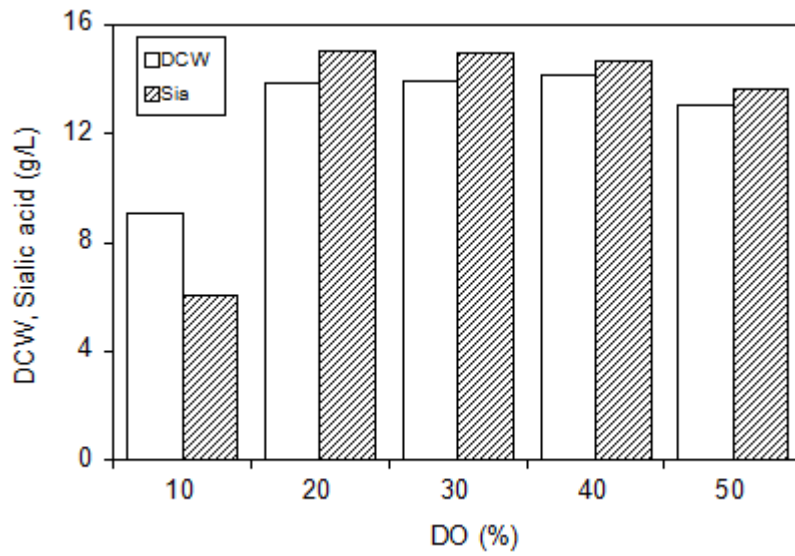


Figure 3-9 Effect of dissolved oxygen on cell growth and sialic acid production.

3.4 Conclusion

In this work, batch cultivation using a recombinant *E. coli* for the production of sialic acid were conducted, and the effects of various parameters on the cell growth and sialic acid production were studied. Glycerol was shown to be more efficient than glucose for both cell growth and sialic acid production. An optimal glycerol (mL/100 mL) to GlcNAc (g/100 mL) ratio of 10 was favorable considering both sialic acid productivity and cost-effectiveness. The optimal initiation time of induction was found to be the middle exponential phase ($DCW \approx 4.5$ g/L). Additionally, the effects of various important cultivation parameters such as temperature, pH, and dissolved oxygen were studied. A temperature adjustment strategy using 33°C before induction and 37°C after the induction was found to result in optimal cell growth and sialic acid production, while pH 7.4 and 20% dissolved oxygen level were also found to be beneficial. The study of growth conditions and their optimization is crucial for further studies, including growth in fed-batch culture, and may help guide future scale-up of sialic acid production via the fermentation route.

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Chapter 4: Effect of feeding strategies on sialic acid production by recombinant *Escherichia coli*

Abstract

In this work, the effects of various feeding strategies were studied on the production of sialic acid from recombinant *Escherichia coli* BRL04 (pBRL89) in fed-batch culture. Various strategies including pH-stat, exponential, constant rate, and linear decreasing feeding were studied, and two- and three-stage feeding methods were subsequently designed and investigated. A three-stage feeding strategy involving exponential feeding at $\mu_{\text{set}}=0.15 \text{ h}^{-1}$ before 22 h, constant rate feeding from 23 h to 32 h ($F=12 \text{ mL/h}$), followed by linear decreasing feeding [$F= (-0.174*t+17.435) \text{ mL/h}$] to the end of cultivation, was developed and found to result in optimized sialic acid production. The optimization of this fed-batch cultivation may help guide future design and scale up of sialic acid production via the fermentation route.

Keywords

Sialic acid • *Escherichia coli* • Fed batch culture • Feeding strategies

4.1 Introduction

Sialic acid encompasses a wide family of nine-carbon acidic sugars that are prominently found at terminal positions of many glycol-conjugates on the surfaces of eukaryotic cells (1). The most common sialic acid is N-acetylneuraminic acid (1, Neu5Ac), a carboxylated nine-carbon monosaccharide. Sialic acid is necessary in biological processes such as glycoprotein stabilization, cell signaling, solute transport, neuronal plasticity, cell adhesion and cellular immunity (2). For example, excess sialylation on malignant cells has been shown to promote invasiveness (3) and reduce intercellular interactions (4), which are both features of tumor metastasis. However, the scarce supply of sialic acid has hindered advancement in basic research, diagnostic development and therapeutic production.

A metabolically and genetically engineered *Escherichia coli* BRL04 (pBRL89) was produced by removing the sialic acid catabolism pathway and expressing sialic acid biosynthesis genes (5). This *in vivo* production process eliminates the requirements for costly substrates used in industrial synthesis of sialic acid.

In our previous study, the optimal conditions (dissolved oxygen, temperature, induction time) for sialic acid production by the recombinant *E. coli* were investigated through batch culture. *E. coli* excretes acetate as a major by-product, which can inhibit the cell growth and protein formation. To maintain sufficient carbon to support cell growth, while avoiding acetate accumulation, an appropriate feeding strategy must be determined (6). In this work, the effect of various substrate feeding strategies were examined for the production of sialic acid by the genetically engineered *E. coli*

BRL04 (pBRL89). Specifically, pH-stat, constant rate feeding, linear decreasing rate feeding and exponential feeding were investigated. Additionally, multiple-stage feeding strategies were designed and explored, and it was found that a three-stage feeding involving an initial exponential phase, constant feeding, followed by linear decreasing feeding resulted in an optimal sialic acid production.

4.2 Materials and methods

4.2.1 Bacterial strain

An *E. coli* strain capable of producing sialic acid was prepared by disabling the native sialic acid catabolic pathway and adding an exogenous synthetic pathway (7, 8), and was kindly provided by Dr. Christopher N. Boddy from the Department of Chemistry at the University of Ottawa.

4.2.2 Culture medium and cultivation conditions

All chemicals were obtained from Fisher Scientific (Canada) unless otherwise mentioned, and were of reagent-grade or higher purity. Before starting fermentation, the plasmid pBRL89 (with the sialic acid synthetic gene) was transformed into *E. coli* BRL04. The recombinant *E. coli* were streaked onto a LB agar plate (consisting of 10 g/L tryptone, 5 g/L yeast extract, 10 g/L sodium chloride, and 15 g/L agar). Ampicillin (Sigma-Aldrich) and kanamycin (Sigma-Aldrich) were used at concentrations of 50 µg/mL and 40 µg/mL, respectively. The plates were incubated at 37°C for until colonies appeared (~12h).

The preculture was prepared by inoculating an individual colony into 200 ml

LB medium and incubating at 37°C and 200 rpm on a rotary shaker for 12 h to create the inoculum for the bioreactor. Ampicillin (Sigma-Aldrich) and kanamycin (Sigma-Aldrich) were used at concentrations of 50 µg/mL and 40 µg/mL, respectively.

The production of sialic acid in fed-batch culture of the recombinant *E. coli* was studied by fermentation in a 3-L fermentor (BioFlo 115, New Brunswick Scientific Co., Edison, NJ, USA). The initial fermentation medium contained the following components: glycerol (20 g/L), K₂HPO₄ (6.62 g/L), KH₂PO₄ (3.0 g/L), NH₄SO₄ (4.0 g/L), MgSO₄ (0.17 g/L), thiamine (4.5 mg/L), ampicillion (50 µg/mL), kanamycin (40 µg/mL), antifoam 0.1 mL/L and trace metal solution (10 mL/L) (9). The trace metal solution was prepared with the following composition: FeCl₃·6H₂O (15.0 g/L), ZnCl₂·4H₂O (2.0 g/L), CaCl₂·6H₂O (2 g/L), ZnSO₄·7H₂O (2.2 g/L), MnSO₄·4H₂O (0.5 g/L), CuSO₄·5H₂O (1.9 g/L), Na₂MoO₄·2H₂O (2 g/L), CoCl₂·6H₂O (4 g/L), H₃BO₃ (0.5 g/L), and concentrated HCl (100 mL/L) (10). The feeding solution contained glycerol (40% v/v), N-acetylglucoseamine (GlcNAc, 4 g/100 mL, Sigma-Aldrich), MgSO₄·7H₂O (10 g/L), thiamine (0.1 g/L), and trace metal solution (20 mL/L).

One hundred and fifty milliliters of preculture grown to mid-exponential phase (OD₆₀₀=10) was added into a 3-L fermentor containing 1.35 L of fermentation medium. The pH of the culture was adjusted and controlled above 7.2 by the addition of 15% (v/v) ammonium hydroxide solution using a computer-controlled pump. The temperature was kept at 30°C during the cell growth phase and 37°C during the

induction phase by an external heating jacket and cooling water. The dissolved oxygen level was kept above 20% by cascading the agitation speed (200-1200 rpm). The air flow rate was maintained at 3 L/min throughout all runs. As an inducer, 0.1 mM IPTG (isopropyl- β -D-1-thiogalactopyranoside) was added when the OD₆₀₀ reached 10. When the initial batch growth was complete, as indicated by a sharp increase of dissolved oxygen concentration after approximately 14 h, the fed-batch phase was initiated by engaging various feeding strategies, as described in subsequent sections.

4.2.3 Feeding strategies in fed-batch culture

4.2.3.1 pH-stat feeding

A rapid rise in pH was observed when the glycerol was exhausted from the medium. Therefore, for the pH-stat strategy, the feed solution was automatically added using a computer-controlled pump when the pH rose above 7.4. The feeding was then stopped when the pH decreased to below 7.4.

4.2.3.2 Exponential feeding

For the exponential feeding strategy, the feeding solution was fed into the fermentor exponentially with a specific growth rate of $\mu_{set} = 0.15 \text{ h}^{-1}$. The feed was changed every hour using a computer-controlled pump at a predetermined rate, which was governed by the following equation (11):

$$F = \frac{\mu_{set} X_0 V_0 \exp(\mu_{set})}{Y S_0} \quad [1]$$

Where F is the feed rate (L/h), μ_{set} is the set specific growth rate (0.15 h^{-1}), X_0 is the initial cell concentration (g/L) and V_0 is the culture volume (L) at the beginning of

feeding, respectively, t is the cultivation time (h), Y is the theoretical cell yield on glycerol (0.45 g/g), and S_0 is glycerol concentration in the feeding solution (500 g/L).

4.2.3.3 Constant rate feeding

For constant rate feeding, the feed solution was added at a constant rate ($F=7$, 10, and 15 mL/h, respectively) into the fermentor using a computer-controlled pump.

4.2.3.4 Three-stage feeding (constant rates)

In the three-stage feeding strategy using constant rates, feed solution was added at $F=15$ mL/h from 14 h to 24 h, $F=10$ mL/h from 25 h to 42 h, and $F=7$ mL/h from 43 h to the end of cultivation (60 h).

4.2.3.5 Linear decreasing rate feeding

Using linear decreasing rate feeding, solution was fed at the following rate:

$$F = (-0.174 * t + 17.435) [2]$$

Where F is given in mL/h, by changing the feeding rates as a function of time (t) every hour.

4.2.3.6 Two-stage feeding

For two-stage feeding, solution was fed exponentially from the 14 h to 22 h of fermentation, followed by a linear decreasing rate from 23 h to the end of fermentation (60 h), according to the relationship shown in equation [2].

4.2.3.7 Three-stage feeding

For three-stage feeding, solution was fed exponentially from 14 h to 22 h, followed by a constant rate phase ($F=12$ mL/h) until 32 h, and a subsequent linear decreasing rate according to equation [2] from the 24 h hour to the end of the

fermentation run (60 h).

4.2.4 Analytical methods

Cell growth was monitored with time by measuring the optical density (OD) of the samples at $\lambda = 600$ nm using a spectrophotometer (Ultraspec 60 DBL BEAM 2NM, Biochrom Ltd.) and an appropriate dilution, where one absorbance unit measured at $\lambda = 600$ nm was previously determined to be equivalent to 0.33 g/L dry cell weight (DCW)(12). The sialic acid concentration was determined by liquid chromatography–mass spectrometry (SHIMADZU, API 200 LC/MS/MS System) after derivatization with DMB (4,5-methylenedioxy-1,2,-phenylenediamine dihydrochloride) solution (20uL broth and 20 μ L of DMB solution). 1 mL of DMB solution was prepared and consisted of: 858 μ L H₂O, 56 μ L acetic acid, 42 μ L 2-mercaptoethanol, 1.8 mg sodium hydrosulfite, 2.4 mg DMB. Glycerol and acetate concentrations were measured using an Agilent 1200 unit (Agilent Technologies, Foster City, CA) with a Shodex SH-1011 column (Showa Denko K.K., Kawasaki, Japan) and a Shodex SG-1011 guard column. Samples of 50 μ L were loaded and the mobile phase (H₂SO₄ 5 mM) was run at 0.6 mL/min. Glycerol were quantified using RI detector while acetate was quantified using UV Detector ($\lambda = 210$ nm).

4.3 Results and discussion

4.3.1 pH-stat feeding

The pH-stat feeding strategy represents a simple feedback control scheme that couples solution feeding with the change of pH, since pH increases were linked to the

excretion of ammonium ions when the principal carbon source was depleted (13, 14). The concentrations of various components in the culture during fermentation using pH-state feeding are shown in Figure 4-1. No acetate was detected using this feeding strategy, and the glycerol concentration was maintained at a relatively low level (below 3 g/L). The maximum dry cell weight and sialic acid production obtained were 12.49 g/L and 19.43 g/L, respectively. However, the pH-stat feeding mode was disadvantageous because the pH did not respond rapidly to glycerol starvation, leading to a long lag time (11). This may have caused substantial cell stress and also increased the required fermentation time.

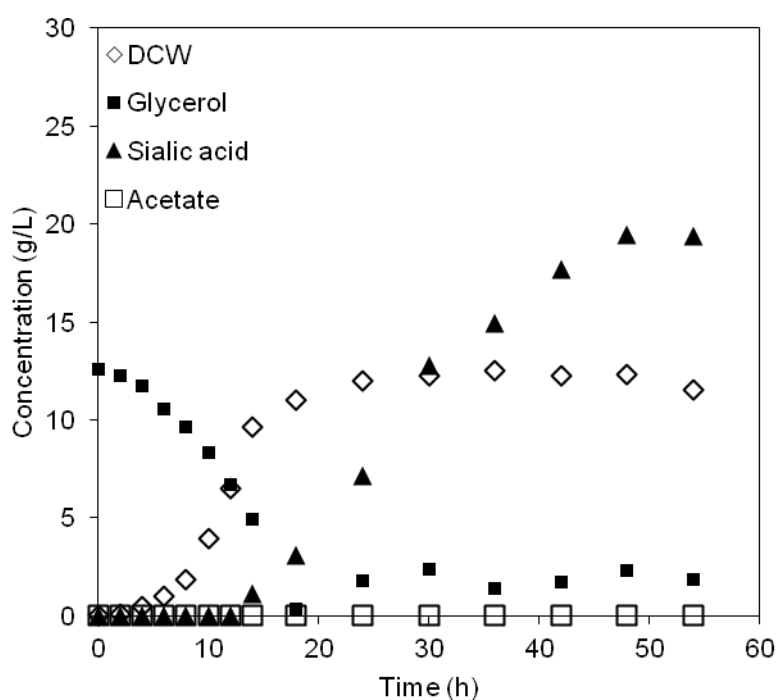


Figure 4-1 Components in *E. coli* fed-batch culture using pH-stat feeding mode.

4.3.2 Exponential feeding

In the exponential feeding mode, solution is added into the fermentor according to a pre-determined feeding rate. This rate ignores the actual glycerol concentration, cell concentration and physiological conditions in the fermentor and assumes that cells grow at a constant specific growth rate $\mu_{\text{set}}=0.15 \text{ h}^{-1}$, which was determined according to previous experimental data from various fed-batch cultures (15). Data from the exponentially-fed batch culture is shown in Figure 4-2. Using the exponential feeding mode, glycerol was found to significantly accumulate after 20 h, which consequently resulted in the accumulation of acetic acid, and limited cell growth and sialic acid production, achieving final concentrations of 11.27 g/L and 11.73g/L for dry cell weight and sialic acid, respectively.

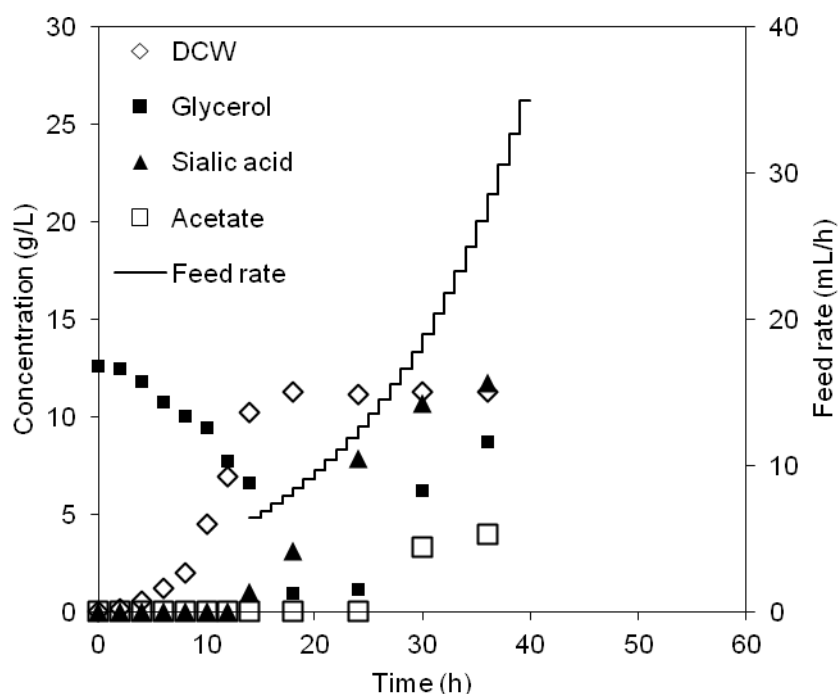


Figure 4-2 Components in *E. coli* fed-batch culture using exponential feeding mode.

4.3.3 Constant rate feeding

Three fermentation runs were performed using constant rate feeding ($F=7, 10,$ and 15 mL/h, respectively) and the results are presented in Figures 4-3 to 4-5. For a feed flow rate of $F=7$ mL/h, the glycerol concentration was observed to remain very low and almost no acetic acid was detected. The maximum dry cell weight and sialic acid concentrations were 13.54 g/L and 16.52 g/L, respectively. For a feed rate of $F=10$ mL/h, some glycerol and acetic acid accumulation was detected after 48 h, resulting in the inhibition of cell growth and sialic acid production. A maximum of 13.58 g/L for dry cell weight and 20.08 g/L for sialic acid were observed in this run. For a feed rate of $F=15$ mL/h, glycerol was found to be in excess at 30 h, and the accumulation of acetic acid was observed, which led to an inhibited sialic acid concentration (14.56 g/L), despite the high dry cell weight obtained (15.10 g/L).

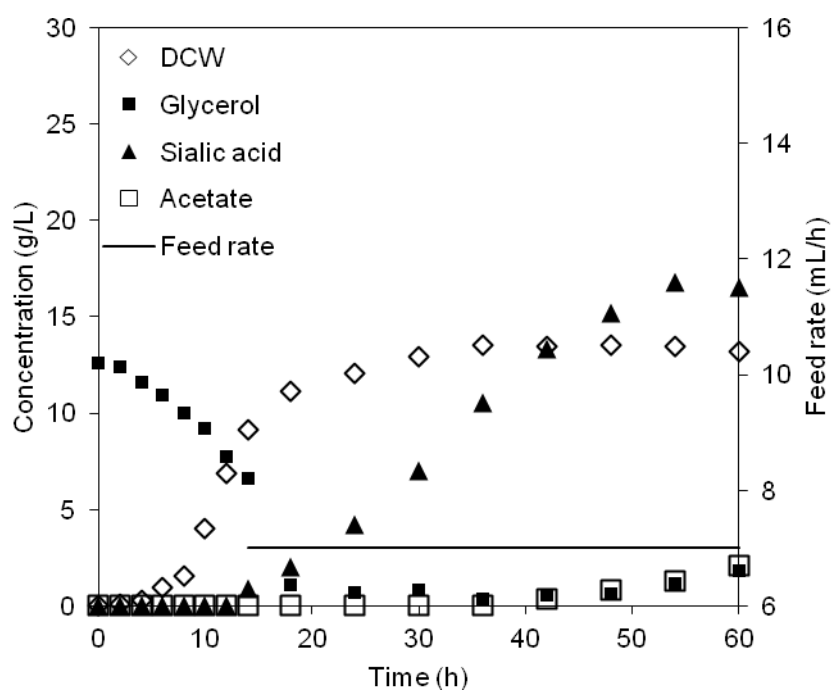


Figure 4-3 Components in *E. coli* fed-batch culture using constant rate feeding;

$F=7$ mL/h.

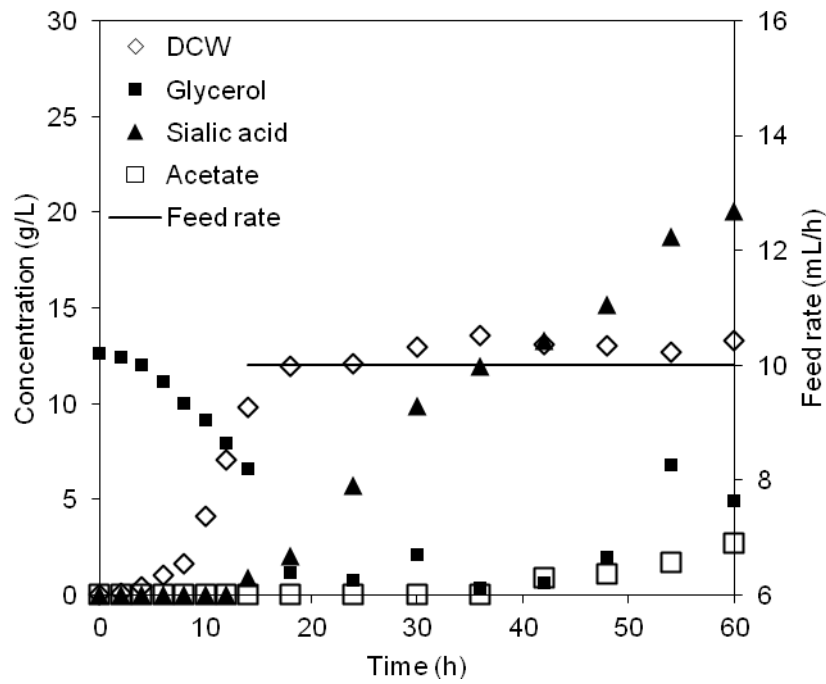


Figure 4-4 Components in *E. coli* fed-batch culture using constant rate feeding;

F=10 mL/h.

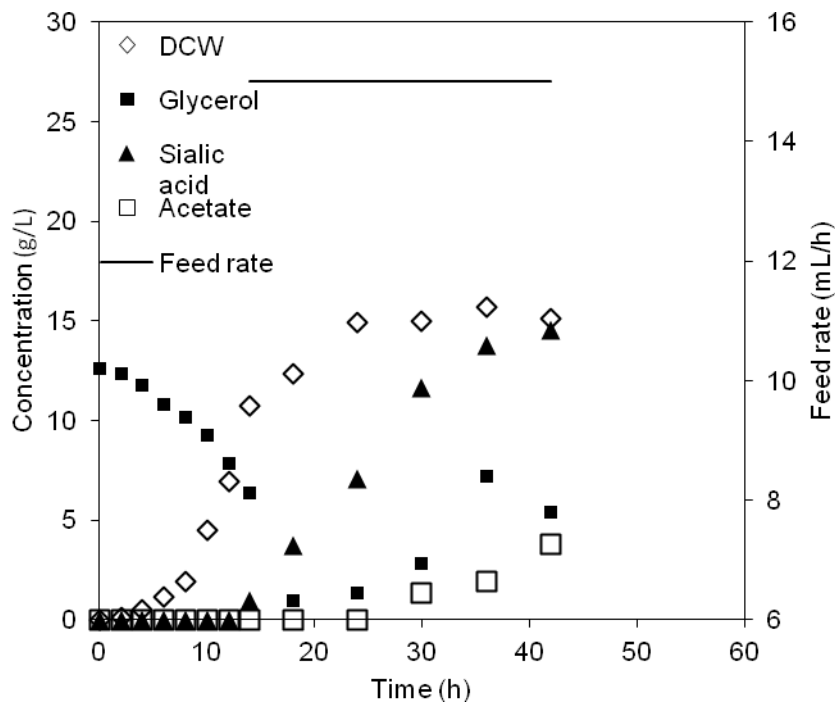


Figure 4-5 Components in *E. coli* fed-batch culture using constant rate feeding;

F=15 mL/h.

4.3.4 Three-stage feeding (constant rates)

Since the three fed-batch trials performed using constant rate feeding could not produce satisfactory results throughout the whole fermentation due to the required trade-off between carbon demand for cell growth and product production, a three-stage constant rates feeding strategy was developed based on the analysis of these three constant modes. In this multiple-stage strategy, the feed rates used were $F=15$ mL/h from 14 h to 24 h, $F=10$ mL/h from 25 h to 42 h, and $F=7$ mL/h from 43 h to end of the fermentation run (60 h). The results from the fed-batch culture are shown in Figure 4-6. Another multiple-stage linear decreasing feeding strategy was also studied, using the relationship for feeding rate as defined in equation (2). This mode was similar to the previously described three-stage strategy, although there was a more frequent change of feeding rate, and the results are shown in Figure 4-7. Comparison of the results obtained using both feeding methods indicate that the sialic acid production was significantly increased, achieving final concentrations of 22.17 g/L and 22.89 g/L for the multiple-stage constant and linear decreasing feeds, respectively. However, a relatively high amount of glycerol (above 3.5 g/L) was detected in both cultures after 30 h, and this unused carbon indicated that further optimization of the fermentation was possible.

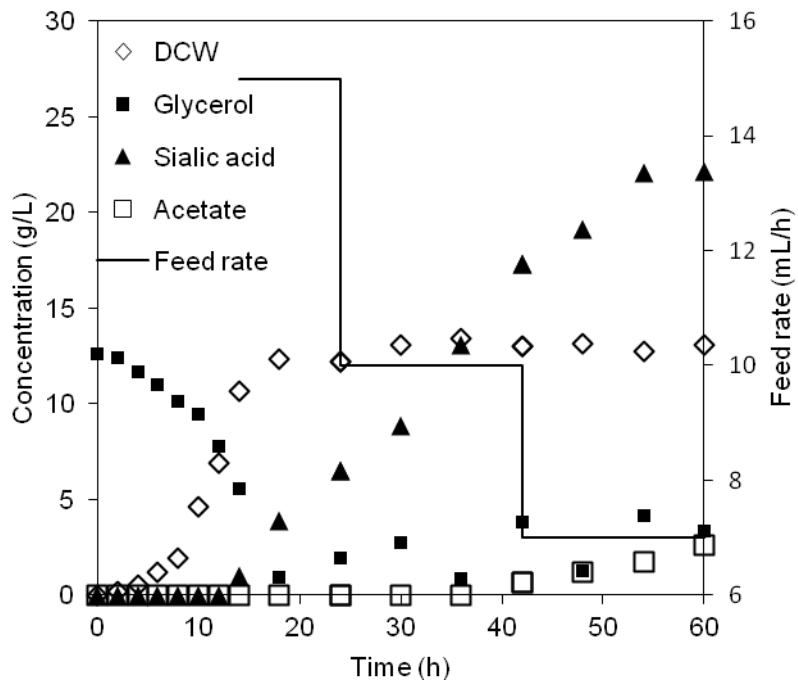


Figure 4-6 Components in *E. coli* fed-batch culture using three-stage constant rate feeding.

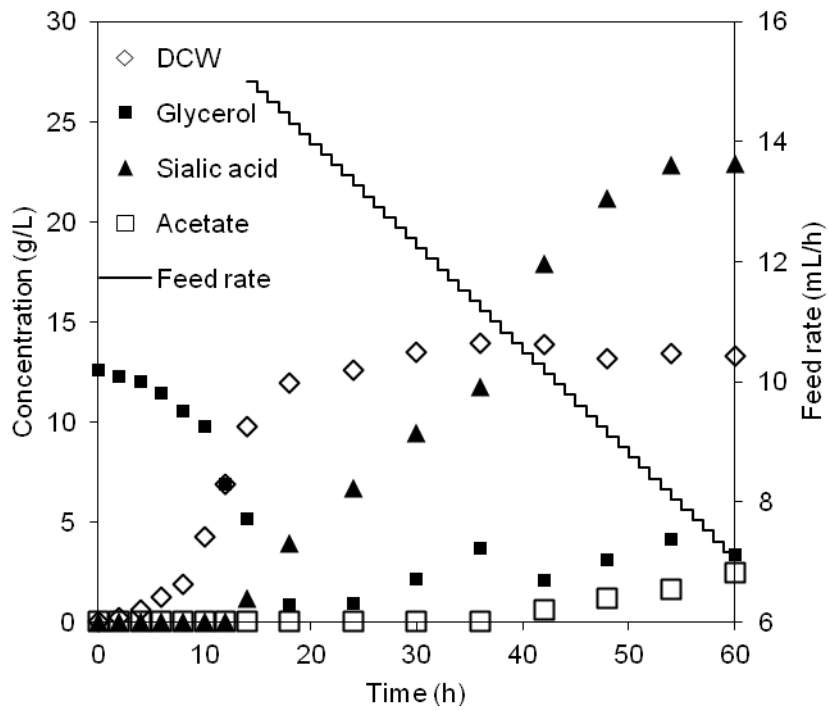


Figure 4-7 Components in *E. coli* fed-batch culture using linear decreasing feeding.

4.3.5 Comparison of two- and three- stage feeding

Various parameters from trials using different feeding modes were compared as shown in Table 4-1. It was found that exponential feeding was able to produce a high average specific sialic acid production rate (0.051 h^{-1}) than the constant-rate and linear decreasing modes (16), thus two- and three- stage feeding strategies with exponential feeding at the first phase were proposed and compared in the subsequent studies. The two-stage feeding strategy consisted of exponential feeding at $\mu_{\text{set}}=0.15 \text{ h}^{-1}$ before 22 h, followed by linear decreasing feeding using the relationship for feeding rate as defined in equation (2), while the three-stage strategy involved exponential feeding at $\mu_{\text{set}}=0.15 \text{ h}^{-1}$ before 22 h and constant rate feeding $F=12 \text{ ml/h}$ from 23 h to 32 h, followed by linear decreasing feeding according to equation (2). The results from the two-stage and three-stage strategies are shown in Figures 4-7 and 4-8, respectively. For both feeding methods, the glycerol concentration was found to remain low (below 2 g/L) during almost the entire feeding process (18-50 h), with less than 2 g/L acetic acid formation. The three-stage mode performed a little better than the two-stage process in controlling the glycerol concentration.

The three-stage feeding strategy was found to produce the highest sialic acid concentration (26.01 g/L), compared to the two- stage feeding (23.94 g/L), and linear decreasing feeding (22.89 g/L), respectively. This result was also higher than the highest sialic acid production (22.0 g/L) to date through recombinant *E. coli* fermentation (Eric Samain 2007).

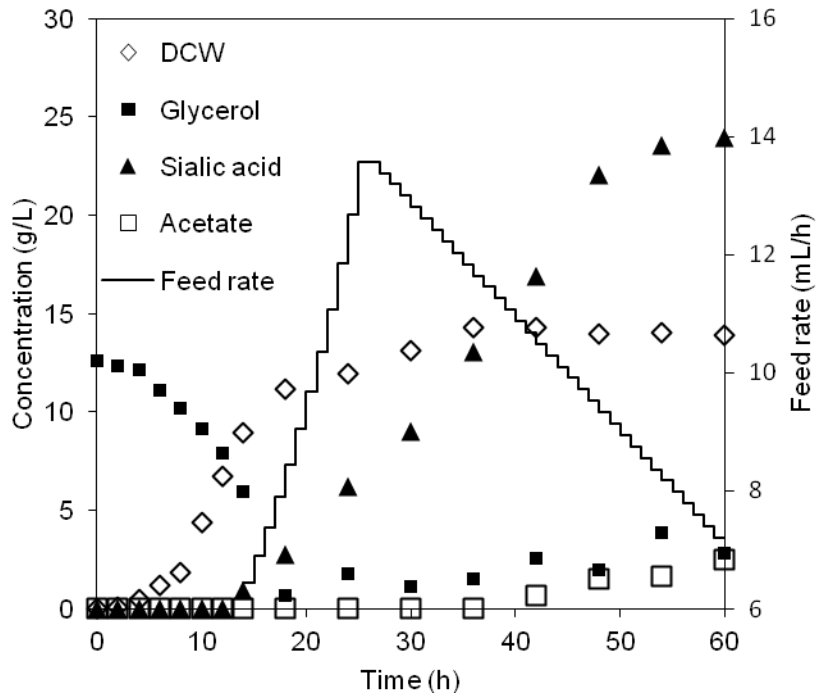


Figure 4-8 Components in *E. coli* fed-batch culture using two-stage feeding.

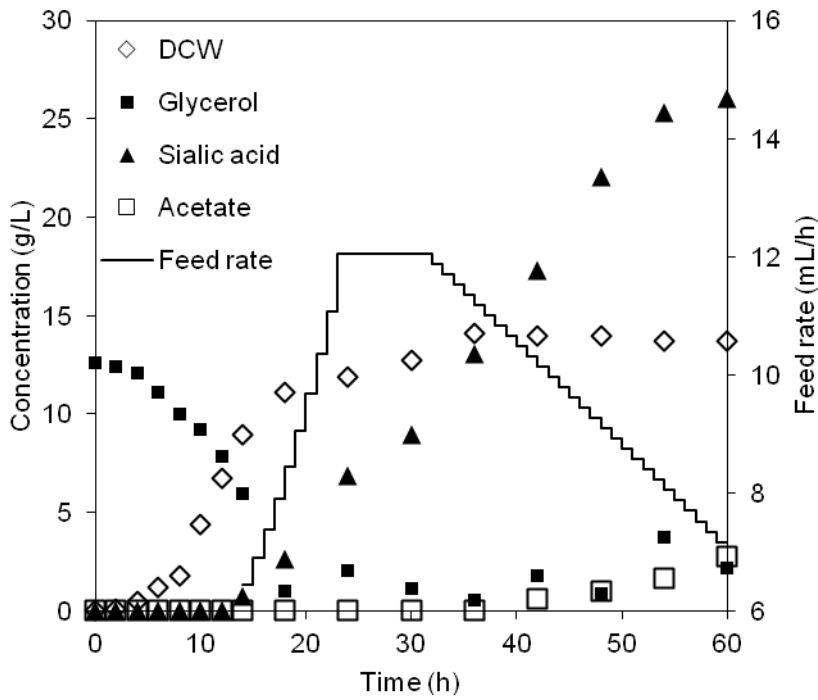


Figure 4-9 Components in *E. coli* fed-batch culture using three-stage feeding.

The various feeding strategies studied were compared as shown in Table 4-1. In addition to producing a maximum sialic acid concentration, the three stage feeding strategy also resulted in higher average specific sialic acid production rate (0.59 h^{-1}), sialic acid productivity (0.434 g/L/h) and sialic acid yield on glycerol (0.169 g/g) than any of the other strategies investigated, and it was therefore thought to be well-suited to sialic acid production by high cell density cultivation of recombinant *E. coli*.

Table 4-1 Comparison of different feeding strategies for sialic acid production.

Parameters	pH-stat	Exponential	Constant rate feeding (mL/h)			3 constant stages	Linear decreasing	2-stage	3-stage
			F=7	F=10	F=15				
Maximum DCW (g/L)	12.49	11.30	13.54	13.58	15.70	13.38	13.95	14.28	14.09
Maximum sialic acid concentration (g/L)	19.41	11.73	16.52	20.08	14.56	22.17	22.89	23.94	26.01
Culture time (h)	54	36	60	60	42	60	60	60	60
Glycerol consumption (g/L)	149.15	129.08	108.30	154.72	141.27	153.37	172.84	160.87	154.16
Average specific growth rate (h ⁻¹)	0.103	0.147	0.071	0.077	0.136	0.104	0.093	0.099	0.092
Average specific sialic acid production rate (h ⁻¹)	0.038	0.051	0.042	0.039	0.031	0.046	0.044	0.055	0.059
Cell productivity (g/L/h)	0.230	0.314	0.226	0.226	0.374	0.223	0.233	0.238	0.235
Sialic acid productivity (g/L/h)	0.359	0.326	0.275	0.335	0.347	0.370	0.380	0.399	0.434
Cell yield on glycerol (g/g)	0.084	0.088	0.125	0.088	0.111	0.087	0.081	0.089	0.091
Sialic acid yield on glycerol (g/g)	0.130	0.091	0.153	0.130	0.103	0.145	0.132	0.149	0.169

4.4 Conclusion

Several different feeding strategies were applied to the fed-batch culture of recombinant *E. coli* for the production of sialic acid, and the effect of feeding strategies on cell growth and sialic acid production were studied. The highest sialic acid production (26.01 g/L) and sialic acid productivity (0.434 g/L/h) were obtained using a three-stage feeding strategy incorporating exponential feeding at $\mu_{\text{set}}=0.15 \text{ h}^{-1}$ before 22 h and constant rate feeding $F=12 \text{ mL/h}$ from 23 h to 32 h, followed by linear decreasing feeding [according to the relationship $F= (-0.174*t+17.435) \text{ mL/h}$] to the end of fermentation. This optimal feeding mode may be helpful to the development and further scale-up of sialic acid production and the production of other recombinant proteins through high cell density cultivation of *E. coli*.

4.5 References

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Chapter 5: Conclusion and Recommendations

5.1 Conclusion

Batch cultivation using a recombinant *E. coli* for the production of sialic acid was conducted, and the effects of various parameters on the cell growth and sialic acid production were studied. Glycerol was shown to be more efficient than glucose for both cell growth and sialic acid production, and , a glycerol (mL/100 mL) to GlcNAc (g/100 mL) ratio of 10 was favorable considering both sialic acid productivity and cost-effectiveness. The optimal initiation time of induction was found to be in the middle exponential phase (when DCW \approx 4.5 g/L). The effects of various important cultivation parameters such as temperature, pH, and dissolved oxygen were also studied. A temperature adjustment strategy using 33°C before the induction and 37°C after induction was found to result in optimal cell growth and sialic acid production, while pH 7.4 and a 20% dissolved oxygen level were also found to be beneficial. Additionally, several different feeding strategies were applied to the fed-batch culture of recombinant *E. coli* for the production of sialic acid, and the effect of feeding strategies on cell growth and sialic acid production were studied. The highest sialic acid production (26.01 g/L) and sialic acid productivity (0.434 g/L/h) were obtained using a three-stage feeding strategy incorporating exponential feeding at $\mu_{\text{set}}=0.15 \text{ h}^{-1}$ before 22 h and constant rate feeding $F=12 \text{ mL/h}$ from 23 h to 32 h, followed by linear decreasing feeding (according to the relationship $F= (-0.174*t+17.435) \text{ mL/h}$) to the end of fermentation. This result was also higher than the highest sialic acid

production (22.0 g/L) to date through recombinant *E. coli* fermentation (Eric Samain 2007). The study of optimal growth conditions and feeding modes may be helpful to the development and further scale-up of sialic acid production and the production of other recombinant proteins through high cell density cultivation of *E. coli*.

5.2 Recommendations

In the light of the results obtained, some suggestions for further improvement and study can be made. In the first part of the research, the various parameters affecting cultivation conditions, such as pH, temperature, dissolved oxygen and induction time, were studied and optimized individually, ignoring any interaction effects between them. For example, when induction time was studied, other conditions including pH, temperature and dissolved oxygen were fixed at certain values while the parameter of interest (induction time) was varied. However, this one-variable approach to optimization only explored one dimension of the response surface at a time, missing entirely the interaction between the different dimensions. While this approach was sufficient to observe the main effect a factor had on a response, it was not sufficient to observe any effects of the interactions between factors, and their influences on the response. To observe these interactions between experimental factors, they must be varied in a simultaneous fashion using a more rigorous statistical design experimentation methodology. Future works should include the statistical design of such experiments and their associated statistical models, including the studied parameters and their interaction factors.

HPLC was used to measure the concentration of different substances, including

sialic acid, acetic acid and glycerol in the fermentation medium, however, this analysis was slow and did not allow for real-time quantification of fermentation results. In the future, implementation of an on-line detecting method is suggested in order to allow for easier analysis of the fermentation components, especially acetic acid and glycerol. The feeding rate could be adjusted instantly in response to these component concentrations, and the cell growth and sialic acid expression may be further optimized and enhanced based on this. In addition, the 3-L bioreactor used for fermentation is much smaller than the reactors used in industry. The change in size related to future scale-up of this process will also have an effect on important process parameters and their operation, such as heating and oxygen transfer. Therefore, further investigation of these phenomena is recommended in order to adapt this process beyond the bench-scale.