Master Thesis

On-line control of glucose feeding in an *Escherichia coli* fed-batch cultivation expressing a recombinant protein.

Robert Gustavsson

September 2011

On-line control of glucose feeding in an *Escherichia coli* fed-batch cultivation expressing a recombinant protein.

Soft sensors have been suggested as potent tools for on-line estimations of critical bioprocess variables to be able to control the biological process in an as high extent as possible. The formation of inhibitory by-products in the form of organic acids, caused by an overflow of glucose, is a problem in most bioprocesses expressing recombinant proteins.

In this project a new method of controlling the glucose feeding in an *Escherichia coli* fed-batch cultivation expressing the green fluorescent protein (GFP) was investigated. The new controller system implemented in the software controlled the feed rate based on on-line HPLC measurements of the concentration of organic acids.

The results showed that the controller managed to down-regulate the inhibitory organic acids to a low level as it tried to keep the glucose uptake rate at an optimum for maximum cell growth. The results suggested that the controller could be a powerful tool to create a more secure reproducibility and to generate high product yields in recombinant protein productions.

Soft sensors, *Escherichia coli*, Green fluorescence protein, Overflow metabolism and mixed acid fermentation, Glucose regulation
On-line control of glucose feeding in an *Escherichia coli* fed-batch cultivation expressing a recombinant protein.

Robert Gustavsson

September 2011

Handledare
Carl-Fredrik Mandenius

Examinator
Carl-Fredrik Mandenius
Abstract

Soft sensors have been suggested as potent tools for on-line estimations of critical bioprocess variables to be able to control the biological process in an as high extent as possible. The formation of inhibitory by-products in the form of organic acids, caused by an overflow of glucose, is a problem in most bioprocesses expressing recombinant proteins.

In this project a new method of controlling the glucose feeding in an *Escherichia coli* fed-batch cultivation expressing the green fluorescent protein (GFP) was investigated. The new controller system implemented in the software controlled the feed rate based on on-line HPLC measurements of the concentration of organic acids.

The results showed that the controller managed to down-regulate the inhibitory organic acids to a low level as it tried to keep the glucose uptake rate at an optimum for maximum cell growth. The results suggested that the controller could be a powerful tool to create a more secure reproducibility and to generate high product yields in recombinant protein productions.
Contents

1. Introduction ........................................................................................................1
   1.1 Goals of thesis .........................................................................................1
   1.2 Approach ..................................................................................................1

2. Background ......................................................................................................3
   2.1 Protein production ..................................................................................3
      2.1.1 Escherichia coli ...............................................................................3
      2.1.2 Green fluorescent protein ..............................................................4
      2.1.3 Vector system ................................................................................5
   2.2 Control of cultivation with the use of soft sensors .................................6
   2.3 Metabolic effects in E. coli .................................................................8

3. Material and Methods ..................................................................................11
   3.1 Strain ......................................................................................................11
   3.2 Medium ..................................................................................................11
   3.3 Cultivation condition ............................................................................12
   3.4 Analyses ..................................................................................................13
      3.4.1 On-line measurements ..................................................................13
      3.4.2 Off-line measurements ..................................................................14

4. Models and soft sensors .............................................................................17
   4.1 Estimation of biomass ............................................................................17
   4.2 HPLC-data .............................................................................................17
   4.3 Fixed feed profile ..................................................................................18
   4.4 Feedback controller ...............................................................................19

5. Results and Discussion ..............................................................................23
   5.1 Test setup ................................................................................................23
   5.2 Biomass and correlation to the NIR signal ............................................25
   5.3 Carbon dioxide level as start signal for feeding ...................................26
   5.4 Fixed profile cultivations ......................................................................28
   5.5 Difference in organic acid production ..................................................30
   5.6 Feedback controlled cultivations ..........................................................31
   5.7 Reference cultivation ............................................................................35

6. Conclusion ....................................................................................................37

7. Acknowledgements ......................................................................................39

8. References ....................................................................................................41
1. Introduction

1.1 Goals of thesis

The objective with this master thesis has been to develop an on-line based control system for monitoring and controlling the glucose feeding of a fed-batch cultivation expressing a recombinant protein. The purpose of the controller is to enable an as high glucose uptake rate possible for the cells and avoid the formation of inhibitory by-products caused by metabolite overflow model in the reactor.

1.2 Approach

A developed controller system was programmed and applied in an operating software and tested in fed-batch cultivations. By on-line measurements by a high performance liquid chromatography (HPLC) system, the current concentration of organic acids were measured and used as a control parameter for the controller.

The organism used was *Escherichia coli* expressing the green fluorescent protein under control of the strong *T7* vector. On-line monitoring was carried out by standard bioreactor measurements devices, on-line HPLC, gas analyzer, NIR-monitoring and off-line determination of fluorescence, optical density (OD) and biomass dry weight.
2. Background

2.1 Protein production

2.1.1 Escherichia coli

Today many of the recombinant therapeutic proteins are produced by using recombinant microorganisms. Their efficiency can strongly be influenced by changes in the process and the cells need to be cultivated under stable conditions. A microorganism often used is the well studied gram negative bacterium *Escherichia coli* (*E. coli*) (Fig. 1), first described by the German bacteriologist Escherich (1885). The knowledge about this bacterium is highly extended and its whole genome is sequenced (Blattner et al, 1997). *E. coli* growth is fast and uncomplicated and its genetics is relatively simple and easy to manipulate which makes it an excellent bacterium to use for protein production (Madigan et al, 2000; Miesfeld, 1999; Schwartz, 2001). The organism used in this project was the *Escherichia coli* K12 derivate HMS 174 (DE3).

![Fig. 1](image-url)  
*Fig. 1.* The gram negative bacterium *Escherichia coli.*
2.1.2 Green fluorescent protein

In this project the green fluorescent protein (GFP) was used as an example of a typical recombinant protein production in *E. coli*. The well known protein with its β-barrel structure, shown in Fig. 2, was first described by Shimomura et al (1962) and was originally isolated from the jellyfish *Aequorea victoria*. Wild type GFP has a major excitation peak at wavelength 395 nm and a minor one at 475 nm, with its emission maximum at 509 nm which is in the lower green portion of visible light. Other mutants of the protein have been made that emit in the blue, yellow and at other wavelength regions (Pakhomov, 2008). The protein is mostly used as a reporter for gene expression (Phillips 2001). An advantage of using GFP as a reporter protein is that it does not need any cofactors, substrates or additional stabilization to give a detectable signal (Chalfie et al, 1994; Cha et al, 1999). Reischer et al (2003) investigated the expression of GFP in *E. coli* to develop new ways of in-line bioprocess monitoring. The GFPmut3.1 variant used in this project has a high fluorescence yield, a good signal-to-noise ratio and a low detection limit. The well known detection of the protein can be performed on-line (Reischer et al, 2003) but no such equipment was available in this project.

![Fig. 2](image.png)

**Fig. 2.** β-barrel structure of the green fluorescent protein. The protein was first derived from the jellyfish *Aequorea victoria*. 
2.1.3 Vector system

The vector expression system used was the well characterized lac operon, which has been used to develop and illustrate molecular genetically methods and the operon model was the first proposed control of prokaryotic gene expression (Walker & Rapley, 2000). The lac operon encodes for three of the enzymes, lacZ, lacY and lacA, which are part of the lactose metabolism in E. coli. The operon is regulated by a 38kDa protein, the lac repressor, which binds specifically to the lac operator site and inhibits the transcription. The inducer molecule isopropylthiogalactoside (IPTG), which is an artificial non-metabolizable allolactose analogue, binds to the repressor molecule and initiates the transcription of the operon as shown in Fig. 3. The expression plasmid pET30aGFPmut3.1 described by Nemecek et al (2008) was used in all experiments. The reporter gene in the vector, GFPmut3.1, was controlled by the T7/lac promoter, containing a 25bp lac operator sequence.

Recombinant proteins in E. coli are often produced through this kind of over-expression with a strong promoter which is an effective way of generating high product yields. The drawback of this strong vector system is that it may overburden the metabolism of the host cells and cause the cells to collapse.

Fig. 3. The inducible expression system in E. coli. The repressor protein blocks the transcription of the operon but when IPTG is added, the repressor protein is inactivated and transcription is initiated.
2.2 Control of cultivation with the use of soft sensors

During recombinant protein production it is crucial to get information about the current conditions in the bioreactor. A successful bioprocess in microbial or mammalian cell cultivation requires both good process monitoring and control. Nemecek et al (2008) describes the lack of appropriate sensors and methods which makes the protein production processes still far from optimized. The existing on-line sensors are not robust enough for the tough conditions in the bioreactor. Monitoring and control systems for bioreactors require further development in combination with better evaluation techniques in order to reach an understanding of the organism in the production process (Mandenius, 2004). One step towards these developments is the Process Analytical Technology (PAT) initiative by the food and drug administration (FDA) which moves into the direction of a more knowledge-based process supervision and control to improve the quality of both the process and the product (Gnoth et al, 2007). Feedback control from on-line sensors and other analytical sensors are efficient tools for achieving good product quality and process performance by providing information to maintain favorable conditions in the bioreactor (Clementschitsch and Bayer, 2006).

Dochain (2003) describes a combination of using reliable online-devices and use the information in mathematical models to get an estimation of variables in the biological system. The combination of using hardware sensors with estimation algorithms applied in a software to get estimations of variables have by several authors (Chéruy, 1997; Sundström and Enfors, 2008; Kiviharju et al, 2008; Warth et al 2010) been known as software sensors. Other authors (Tham et al 1991; Assis and Filho, 2000) describes this as soft sensors which is a common term used in the process industry (Kadlec et al, 2009), and the trend is that soft sensors becomes a more commonly accepted term in the field of biotechnology. Different architectures of the soft sensors are showed in Fig. 4.
Fig. 4. Soft sensor architectures showing (A) linear signal transformation, (B) cascade signal transformation, and (C) circuit soft sensor signal transformation. (Warth et al, 2010)
2.3 Metabolic effects in E. coli

Recombinant protein production in *E. coli* is often produced with a fed-batch feeding strategy, using glucose-based media. One challenge in achieving a high product yield is the avoidance of accumulation of by-products such as acetate and other organic acids. The formation of acetate is undesirable because it delays cell growth (Han et al, 1992; Luli and Strohl, 1990) and it has been shown to inhibit recombinant protein synthesis (Jensen and Carlsen, 1990; Koh et al, 1992; Turner et al, 1994). The accumulation of acetate is caused by overflow metabolism and mixed acid fermentation (Xu et al, 1999), shown in Fig. 5. One explanation for the acetate formation is the overflow metabolism where the flux of acetyl-CoA is elevated from high glucose levels, and is directed to form acetate instead of entering the TCA cycle (Eiteman and Altman, 2006). Acetate can also be formed along with the formation of other by-products such as organic acids (Fig.4), in the mixed acid fermentation in *E. coli* which is caused by anaerobic conditions (Lara et al, 2006). This mixed acid fermentation can also be seen in aerobic cultivations where local dissolved oxygen tension gradients can occur (Amanullah et al, 2004) and could be caused by insufficient mixing in large scale reactors (Bylund et al, 1998).

Due to this problem, the feeding of glucose plays a crucial part in the aim to get a high product yield. There are different kinds of methods and strategies for a controlled carbon feeding. One strategy is to use a pre-determined feed rate calculated to be beneath the cells threshold rate (DeLisa et al, 1999). The drawback with this method is that these complex biological systems are difficult to predict and the glucose uptake rate must be kept below optimum for maximum cell growth and is therefore limiting the productivity. Other strategies are feedback control by keeping the glucose levels low through control from glucose measurements (Kleman et al, 1991) or by keeping the acetate levels low by controlling the feed rate with a dissolved oxygen sensor (Åkesson et al, 2000). Instead of regulating the feed rate, strategies to remove inhibitory acetate via dialysis (Fuchs et al, 2002) or by engineering the metabolic pathways in *E. coli* to reduce by-product formation (Lara et al, 2006) have been investigated.
Fig. 5. Metabolic pathways showing the overflow metabolism and the mixed-acid fermentation in Escherichia coli. The aerobic overflow metabolism is a route branching off the central metabolic pathways at the level of acetyl-CoA after the decarboxylation of pyruvate by pyruvate dehydrogenase (PDH) before entering the tricarboxylic acid cycle (TCA). The mixed acid fermentation operates anaerobically and all the products shown are derived from pyruvate. ACK acetate kinase, ADH alcohol dehydrogenase, FHL formate hydrogen-lyase, LDH lactate dehydrogenase, PHL pyruvate formate-lyase, PTA phosphotransacetylase.
3. Materials and Methods

3.1 Strain

During all the experiments, an *Escherichia coli* strain HMS 174(DE3) (Novagen, Madison, WI, USA) transformed with plasmid pET30a (Novagen) containing GFPmut3.1 (Clontech, US) was used. The plasmid was under control of the T7/lac promoter, which contained a 25bp lac operator sequence. The transformed strain was obtained from the Department of Biotechnology, University of Natural Resources and Life Sciences, Vienna.

3.2 Medium

The semi-synthetic medium described in Nemecek et al (2008) was used for the overnight culture while a modified medium was used in the batch and fed-batch cultivation. All reagents and chemicals were purchased from Merck, if not otherwise stated. All components were added in relation to the working volume. The composition of the medium for the pre-culture cultivation is shown in Table 1 and the trace element solution used can be seen in Table 2. The modified medium used for the batch and feed solution is shown in Table 3.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>KH$_2$PO$_4$</td>
<td>3.0 g/L</td>
</tr>
<tr>
<td>K$_2$HPO$_4$·3 H$_2$O</td>
<td>4.5 g/L</td>
</tr>
<tr>
<td>C$_6$H$_5$Na$_3$O$_7$·2H$_2$O</td>
<td>2.5 g/L</td>
</tr>
<tr>
<td>MgSO$_4$·7H$_2$O</td>
<td>1.0 g/L</td>
</tr>
<tr>
<td>(NH$_4$)$_2$SO$_4$</td>
<td>4.5 g/L</td>
</tr>
<tr>
<td>NH$_4$Cl</td>
<td>3.7 g/L</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>1.5 g/L</td>
</tr>
<tr>
<td>Glucose</td>
<td>6.0 g/L</td>
</tr>
<tr>
<td>Trace element solution</td>
<td>0.5 mL/L</td>
</tr>
</tbody>
</table>

**Table 1. Composition of the pre-culture medium**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>FeSO$_4$·7 H$_2$O</td>
<td>40.0 g/L</td>
</tr>
<tr>
<td>MnSO$_4$·H$_2$O</td>
<td>10.0 g/L</td>
</tr>
<tr>
<td>AlCl$_3$·6 H$_2$O</td>
<td>10.0 g/L</td>
</tr>
<tr>
<td>CoCl$_2$</td>
<td>4.0 g/L</td>
</tr>
<tr>
<td>ZnSO$_4$·7 H$_2$O</td>
<td>2.0 g/L</td>
</tr>
<tr>
<td>Na$_2$MoO$_4$·2 H$_2$O</td>
<td>2.0 g/L</td>
</tr>
<tr>
<td>CuCl$_2$·2 H$_2$O</td>
<td>1.0 g/L</td>
</tr>
<tr>
<td>H$_3$BO$_3$</td>
<td>0.50 g/L</td>
</tr>
</tbody>
</table>

**Table 2. Composition of the trace element solution**
3.3 Cultivation Condition

Preparation of working cell bank
The *E. coli* strain [HMS 174(DE3) (pET30aGFPmut3.1)] was already prepared as bank vials according to Warth et al (2010).

Fed-batch cultivation
For all the experiments, a 10 L computer controlled bioreactor (Model LMS 2002, Belach, Sweden) was applied. The used software BioPhantom was adapted and custom-made to satisfy the extra requirements with the software sensors and the controller system. At first a shake flask cultivation was grown through aseptically inoculation of two vials of WCB into a 500 mL flask containing 200 mL pre-sterilized semi-synthetic media. The aeration rate was set to 200 rpm and the culture was grown at 37°C overnight. In the morning the overnight culture had reached an OD-value between 8 and 11. The batch medium was added to the reactor and sterilized in situ. Due to a loss of about 10% of water during sterilization, a higher volume was first added to compensate this loss. After sterilization the overnight culture was inoculated and the working volume at the start of the batch phase was now 4 L. During feeding 2 L of feed solution was added. The pH range was set between 6.9 and 7.1 and regulated by addition of 1 M sulfuric acid and 20% ammonia. The aeration rate was 5 L per min and the temperature was set to 37°C. Dissolved oxygen level (DO) was set to 30% and was controlled by the stirrer speed, 300-1200 rpm. Foaming was decreased by addition of antifoam solution (Glanapon, Bussetti, Vienna, Austria).

The batch phase lasted for about 5 h and when all the initial substrate was consumed, which could be seen by a sudden decrease in the carbon dioxide levels, exponential feeding was started according to a calculated feed profile. The specific growth rate was set to 0.3 h⁻¹ by the feed profile. The feeding was monitored by a PID-regulation system which controlled a peristaltic pump (P4 U1-MXV, Alieta, Sweden) by reading the consumed weight of the feed with a scale (XL-3100, Denver Instrument) to confirm the right pump speed.

Table 3. Composition of the batch medium and feed solution.

<table>
<thead>
<tr>
<th>Components</th>
<th>Batch Medium</th>
<th>Feed Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>5.0 g/L</td>
<td>100 g/L</td>
</tr>
<tr>
<td>K₂HPO₄</td>
<td>6.665 g/L</td>
<td></td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>0.25 g/L</td>
<td></td>
</tr>
<tr>
<td>NaCl</td>
<td>1.2 g/L</td>
<td></td>
</tr>
<tr>
<td>K₂SO₄</td>
<td>1.1 g/L</td>
<td></td>
</tr>
<tr>
<td>Yeast extract</td>
<td>0.5 g/L</td>
<td></td>
</tr>
<tr>
<td>(NH₄)₂SO₄</td>
<td>10 g/L</td>
<td>27.5 g/L</td>
</tr>
<tr>
<td>MgSO₄ · 7 H₂O</td>
<td>0.15 g/L</td>
<td>1.5 g/L</td>
</tr>
<tr>
<td>CaCl₂ · 2 H₂O</td>
<td>0.013 g/L</td>
<td>0.026 g/L</td>
</tr>
<tr>
<td>Trace element solution</td>
<td>0.125 mL/L</td>
<td>250 mL/L</td>
</tr>
</tbody>
</table>
The induction with 0.119 g IPTG (Sigma) was added via inoculation of the filtered inducer through a membrane about 4-5 h after feed-start when the OD-value had reached a value between 20 and 25. The concentration of IPTG in the reactor depended on how much feed had been added, but the final concentration was always around 0.03 g/L which has been showed to be sufficient (Vostiar et al, 2004).

3.4 Analyses

3.4.1 Online-measurements

Standard bioreactor measurements
The reactor was equipped with standard electrodes for monitoring dissolved oxygen (DO) and pH as well as probes for temperature, level and pressure measurements. These measurements were performed every second.

NIR-monitoring
The cell-density was measured online with a near infrared probe (Cell Growth Monitor Model 650; Wedgewood Technology, CA) using a path length of 5 mm. This sterilizable probe provides in-situ measurements of the OD-value at 560 nm. To achieve a linear correlation, a first order linearization of the instrument was accomplished by selecting the second linear power set position. The selected range was 0-1 and the aeration suppression, which suspends noise mainly caused by bubbles, was switched on. The probe was implemented to the reactor from the side next to the stirrer. To get the correlation between the output reading and the current biomass concentration, a factor needed to be established by measuring known cell concentrations. This was calculated by comparing measured readings with biomass dry matter samples taken at different stages of test fed-batch cultivations.

Gas analysis
The outlet gas generated from the reactor was measured by a gas analyzer (CP460-O2/CO2; Belach, Sweden) which analyzed oxygen- and carbon dioxide levels.

On-line HPLC
An in-situ filtration membrane probe (FISP-sampling probe, Flownamics, Madison, US) taking continuous samples from the reactor to the analytical devices of the HPLC system. In this study the HPLC system was used to detect changes in concentrations of glucose, acetate, formic acid, lactate and ethanol throughout the process. The concentration of the organic acids was then used to control the feeding of the process. The membrane of the probe was a 0.2 µm ceramic membrane (Flownamics, Madison, US). The probe and the membrane was washed with first a 0.5 M NaOH solution and then with distilled water after every run. The HPLC system in which the samples were analyzed was of the brand Shimadzu. It consisted of a pump unit LC-20AD, a column oven CTO-20A and a refractive index
detector RID-10A. Measurements were taken every 20 minutes through sampling from the bioreactor with the membrane probe. A peristaltic pump (Alitea, C8/2-XV, Sweden) as well as a six-port two-position automatic injection valve (Rheodyne, US) with a 20 µL loop (TPMV, Rheodyne, US) were controlled by the software LC Solution (Shimadzu, Japan).

An ion-exclusion column (Aminex® HPX-87H, 300x7.8mm; BioRad, US) works on a resin-based principle and separates organic acids, alcohols and carbohydrates. A guard column (Guard Cartridge 125-0129, BioRad, US) protects the analytical column from impurities and carries out a pre-separation.

The validity of the system was proved by standard solutions of glucose, acetate, formic acid, lactic acid and ethanol. The column was applied at 60°C with degassed 5mM sulfuric acid as mobile phase and a flowrate of 0.8 ml/min. Detection glucose and the other organic acids were carried out by the refractive index detector after their set retention times.

### 3.4.2 Off-line measurements

Samples for off-line measurements were taken every second hour from the fermentation broth through a steam sterilizable pipe. The optical density was measured for every sample and dry matter of biomass was measured less frequent. For determination of recombinant protein production, samples for fluorescence measurements were taken and rapidly frozen at -20°C directly before IPTG induction and then every hour.

**OD_{600}**

For determination of the optical density, the fermentation broth was diluted with deionized water to reach an absorbance within the linear region (≤0.6). In a UV-spectrometer (Ultraspec 1000, Pharmacia Biotech, UK), the absorbance at λ=600 nm was measured with a path length of 1 cm.

**Biomass dry matter (BDM)**

For determination of dry matter, 1 mL fermentation broth were filtered through a pre-weighted 0.2 µm mixed cellulose ester membrane filter (Whatman, Dassel, Germany) followed by 3 mL deionised water. The filter was dried to constant weight for two hours at 105°C.
Fluorescence

Albano et al. (1996) demonstrated that the fluorescence of GFP can be used as quantitative reporter of the protein concentration in *E. coli* since the ratio of fluorescence signal to protein concentration correlates. To measure the fluorescence, 1 mL fermentation broth was taken and frozen at -20°C. Samples were taken regularly after IPTG induction. One sample was taken as blank directly before induction to obtain the culture fluorescence, which is mainly due to NAD(P)H. After unfreezing, the samples were diluted 1:1000 in two steps and measured in a spectro-fluorometer (Fluostar Galaxy, MTX lab systems, VI, US). Fluorescence was measured with excitation/emission wavelengths of 470/515 nm.
4. Models and soft sensors

4.1 Estimation of biomass

This model is based on the well-known correlation between the optical density of a cell suspension and the biomass, described by Lambert Beers law. The computer system reads the NIR probe and converts the signal to an $A_{NIR}$ value [0-100%]. The system then uses a separately calculated factor ($K_{NIR}$) to convert the $A_{NIR}$ signal to biomass ($X_{NIR}$). The adjusted factor was calculated and described in Section 5.2.

$$X_{NIR} = K_{NIR} \cdot A_{NIR} \quad (I)$$

4.2 HPLC data

The concentration of glucose and organic acids was analyzed every 20 min by the HPLC system described in 2.4.1, and directly transferred to the operating software. The system was programmed to read data files generated by LCSolution HPLC software by a shared folder network in a certain time schedule. Files were generated every 20 min and the file data included Conc [g/L] values for glucose and organic acids. When a new file was generated, the values were transferred straight away and the concentrations were read by the BioPhantom software. Consequently, rates and specific rates were calculated compared to the “last” file, read 20 min earlier. Due to that, the steps were “stepwise”. The mechanism of the file transfer and rate calculation was carried out as following:

The generated file names of the HPLC software LC Solution were:

HPLC_XXXX_001.TXT
HPLC_XXXX_002.TXT
HPLC_XXXX_003.TXT

The files were stored in a shared folder with the BioPhantom software and once the operator initiated the “import” function, files were read in a synchronized order. A new file were generated every 20 min from LC Solution, with the BioPhantom software checking for updates in the shared folder every 10 s to find fresh data. When read, the new data replaced the previous and stored as e.g. for glucose as $c_{glu,n}$ while the previous data was stored as $c_{glu,n-1}$. As for the concentration, the time of the new data was stored as $t_n$ while the previous was stored as $t_{n-1}$.
**Sum mixed acids**

The concentrations for the organic acids were summarized as a new parameter, Sum Mixed Acids:

\[
c_{\text{Sum Mixed Acid}} = c_{\text{ac}} + c_{\text{lactate}} + c_{\text{formic acid}} + c_{\text{acetone}}
\]  
(2)

The parameter Sum Mixed Acid was used as set point for the controller, and the feed-rate was regulated by the value of the Sum Mixed Acid.

### 4.3 Fixed feed profile

During the fed-batch phase, the demand of substrate increases over time along with the growth of the cells. The rate of the feed needs to be adjusted to match the demand of substrate in different stages of the cultivation. Therefore a profile, that increased the feed rate over time, was used to try to resemble the exponential growth of the cells. The profile was calculated with following equation:

\[
y(t) = (y_2 - y_1) \cdot \left( e^{(\mu \cdot t)} - 1 \right) / \left( e^{\mu \cdot t_2} - 1 \right) + y_1 
\]  
(3)

- \(t_1\) = starting time (0)
- \(t_2\) = end time
- \(y_1\) = Initial feed rate
- \(y_2\) = Final feed rate
- \(\mu\) = Specific growth rate

The parameters are set in the BioPhantom software seen in Fig. 6A and in this case the initial feed rate is set to 100 g/h and reaches its final feed rate of 400 g/h in 8 hours. The specific growth factor is chosen and the result of this profile is illustrated in Fig. 6B.
4.4 Feedback controller

The problem with just using the profile is that it is just a prediction on how the system should behave, but this complex biological system makes it difficult since the variations between cultivations are very high. It would be more useful to know the actual conditions of the cultivations than a calculated prediction. As described in Section 2.3 a high production of organic acids is caused by the overflow of glucose. These organic acids could be a good indicator to measure for monitoring current conditions in the reactor. A feedback controller system was constructed as shown in Fig. 7. The HPLC data was transferred to the BioPhantom software in which a new controller was installed. This controller, shown in Fig. 8, regulated the feed rate of the profile according to the current Process value \( (P_v) \) of the \( c_{\text{SumMixedAcid}} \). Other parameters could be used for regulation but in this project the concentration of Sum Mixed Acids was chosen. All the parameters for the controller are listed in Table 4.

Fig. 6. (A) Setup for the profile in the BioPhantom software. Initial and final feed rate is set and the duration time of the increasing feed is set. Specific growth factor, here called \( b \), is also set. (B) graphic view of the result of how the feed rate increases over time with the setups used in (A).
Fig. 7. Schematic diagram of the feedback controller system. Samples from the reactor is analyzed by the HPLC system and the software LC Solutions calculates the concentrations of glucose and organic acids, which are transferred to the BioPhantom software. In the BioPhantom software, feed rate is run by the profile and adjusted by the controller based on the data received from LC Solutions. The feed is added by a pump and a scale controls, with a PID-regulation system implemented in the software, so that the right amount of feed is added.
Table 4. List of symbols, description and units for the parameters used in the feedback controller model. Symbols marked with * are parameters or constants set by the process operator.

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
<th>Unit</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>$P_v$</td>
<td>Process value, $c_{\text{SumMixedAcid}}$</td>
<td>g/L</td>
<td>0-10</td>
</tr>
<tr>
<td>$\text{Sp}^*$</td>
<td>Set point value for the controller</td>
<td>g/L</td>
<td>0-10</td>
</tr>
<tr>
<td>$K_c^*$</td>
<td>Amplification factor, increases the controller output</td>
<td></td>
<td>0-200</td>
</tr>
<tr>
<td>$e(t)$</td>
<td>Err%, error between $\text{Sp}$ and $P_v$</td>
<td>%</td>
<td>(-)50-(-)50</td>
</tr>
<tr>
<td>$u(t)$</td>
<td>Out%, output percent</td>
<td>%</td>
<td>(-)50-(-)50</td>
</tr>
<tr>
<td>$F_r$</td>
<td>HiRange – LoRange (+400 to -400)</td>
<td>g/h</td>
<td>800</td>
</tr>
<tr>
<td>$Y_1(s)$</td>
<td>Profile output</td>
<td>g/h</td>
<td>0-400</td>
</tr>
<tr>
<td>$Y_2(s)$</td>
<td>Controller output</td>
<td>g/h</td>
<td>(-400-(-)400)</td>
</tr>
<tr>
<td>$Y(s)$</td>
<td>Feed output</td>
<td>g/h</td>
<td>0-400</td>
</tr>
</tbody>
</table>

Fig. 8. Setup view of the controller in the BioPhantom software. Parameter of regulation is chosen, in this project the Sum Mixed Acids were chosen.
The feedback controller worked according to the following equations:

\[ e(t) = \frac{(Sp - Pv)}{300} \cdot 100\% \quad (4) \]

\[ u(t) = Kc \cdot e(t) \quad (5) \]

\[ Y_2(s) = u(t) \cdot Fr \quad (6) \]

\[ Y(s) = Y_1(s) + Y_2(s) \quad (7) \]

Once the Process value \((Pv)\) variable is received and the Set point \((Sp)\) and \(Kc\) is entered, the controller can be activated. Once active the controller calculates the scaled error \((e(t))\). The \(u(t)\) is calculated proportional to the \(e(t)\). When \(e(t)\) is 0 the \(u(t)\) is set to 0. Otherwise depending on the \(e(t)\) the \(u(t)\) is set to (-50% to +50%) of the feed pump range (-400 to +400 g/h). The resulting \(Y_2(s)\) shall be added to the actual output of the profile \((Y_1(s))\).
5. Results and Discussion

5.1 Test setup

In the project, a total of 13 fed-batch cultivations were performed. In order to test the equipment and to find suitable parameters for the fixed feed profile and the controller, six test cultivations were performed. Only two of these six cultivations were performed after installing the new controller system. Another seven qualification experiments were performed and five of these were successfully done while two failed due to technical problems. The five successful experiments are listed and described in Table 5.

Table 5. The five successful experiments named and described. Two cultivations were performed according to a fixed feed profile, two cultivations with the feedback controller and one cultivation was performed without induction of IPTG.

<table>
<thead>
<tr>
<th>Experiment (name)</th>
<th>Feeding strategy used in the experiment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fixed feed profile experiments</td>
<td></td>
</tr>
<tr>
<td>Profile A1</td>
<td>fixed feed profile</td>
</tr>
<tr>
<td>Profile A2</td>
<td>fixed feed profile</td>
</tr>
<tr>
<td>Feedback controlled profile experiments</td>
<td></td>
</tr>
<tr>
<td>Controller B1</td>
<td>fixed feed profile + feedback controller</td>
</tr>
<tr>
<td>Controller B2</td>
<td>fixed feed profile + feedback controller</td>
</tr>
<tr>
<td>Reference experiment</td>
<td></td>
</tr>
<tr>
<td>Reference C</td>
<td>fixed feed profile without IPTG</td>
</tr>
</tbody>
</table>
During the test cultivations, variation in the results could be seen between different runs. Although all experiments were performed with the same composition of medium and amounts of substrate added were the same for all the cultivations significant variation could be seen. In Fig. 9 the results from the experiment with the lowest amount of GFP produced (A) can be compared with the experiment with the highest amount of GFP produced (B). The amount of GFP is more than twice as much in B than in A. As mentioned, the same amount of substrate was added but the feeding rate was different in the two experiments. This shows the importance of having a feeding rate that matches the current physiological conditions in the bioreactor.

![Diagram](image)

**Fig. 9.** During the test cultivations a high variation of results were obtained. In (A) the lowest result and in (B) the highest result are presented.
5.2 Biomass correlation to the NIR signal

To estimate the factor $K_{NIR}$ in Eq. 1 for the correlation between the signal from the NIR-probe and actual biomass concentration, the output of the NIR-probe was plotted against dry weight measurements. After the first test cultivation the $K_{NIR}$ factor was estimated to 0.25 which was used in all the following experiments during the project. In Fig. 10, the dry weight measurements from four different test cultivations are presented plotted against the NIR-probe output. Due to low precision with dry weight measurements at low biomass concentration, no data for biomass dry matter is presented during the batch phase. Since the NIR-probe was adjusted to zero before inoculation, the linearization was aligned to cross origo. As seen in Fig. 10, no perfect fitting was found although the correlation was estimated to 0.2638. A $K_{NIR}$ factor of 0.25 was used in all the test cultivations as well as in all the qualification experiments.

Fig. 10. The biomass dry weight measurements (■) plotted against the linearized output signal of the NIR-probe.

Slope = 0.2638
5.3 Carbon dioxide level as start signal for feeding

The feeding started when all the initial glucose in the batch-phase was consumed. The starting time varied in the experiments due to varying duration of the batch-phase of the cultivations. To determine when the glucose was consumed for knowing when to start the feeding, could have been achieved by observing the glucose concentration given by the HPLC analysis. But since chromatograms were only run every 20 minutes and the sample transport between the filtration probe and the HPLC detector took additional time, the readings showed the current condition in the bioreactor with a delay of 20-25 minutes. This delay could cause the cells growing in an exponential phase to enter into a stationary phase due to low glucose level. It was noted that the longer time the cells remained in a stationary phase the longer it took to return to exponential growth. This severely hindered the culture to be as time efficient as possible. A more rapid indication on when the glucose levels in the reactor gets low would be preferred. During the test cultivations, it was observed that the carbon dioxide levels followed the growth of the cells, since the cells produces carbon dioxide during growth, in a reproducible pattern. A sudden decrease in the carbon dioxide levels occurred which marked that the growth of the cells was slowing down. Since this occurred about 20-25 minutes before the HPLC system showed that the glucose levels were low, this suggested that the sudden decrease in carbon dioxide level could give a close to real-time response on when the glucose levels were low. This was a well-defined starting point which could easily be reproduced in all the cultivations. The start of feeding for the qualification experiments are shown in Fig. 11 and the sudden drop in carbon dioxide levels is shown as the starting point.
Fig 11. Feedstart according to starting point with sudden decrease of carbon dioxide levels, which can be compared with decrease in glucose concentrations. (A) shows a cultivation with a fixed profile (Profile A1), (B) another cultivation with the same fixed profile (Profile A2), (C) a cultivation with a feedback controlled profile (Controller B1) and (D) another cultivation with a feedback controlled profile (Controller B2). The HPLC system was started a few hours after the batch start which explains the missing data of glucose levels for the first hours.
5.4 Fixed profile cultivations

During the test cultivations different fixed profiles for the feeding were tried out to find a suitable one for the qualification experiments. In the first set of cultivations performed a profile from previous work was tried out. Both the starting feed rate as well as the final feed rate was experienced to be too low so alternative profiles were tested. The profile selected, shown in fig 6B, had a starting feed rate of 100 g/h and a final rate of 400 g/h. By this, 2 L of feed media was added in approximately 8 hours. The previously used profile with a starting rate of 20 g/h and a final rate of 220 g/h, required 13 hours for adding 2 L of media. In the previous work no pre-culture cultivation was carried out - instead two 1 mL vials of the WCB were added directly to the reactor which resulted in a batch phase of approximately 12 hours. In this project a pre-culture cultivation was used which shortened the batch phase to about 5 hours. Consequently, this new approach shortened the reactor fed-batch time with up to 12 hours while the amount of GFP produced was approximately still the same as in previous work. This time saving approach made it possible to perform this kind of fed-batch cultivation during a course for Master students. The parameters used in the new profile are shown in Table 6.

Table 6. Parameters selected for the profile used in all the qualification cultivations

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>( t_1 )</td>
<td>0</td>
</tr>
<tr>
<td>( t_2 )</td>
<td>8</td>
</tr>
<tr>
<td>( y_1 )</td>
<td>100</td>
</tr>
<tr>
<td>( y_2 )</td>
<td>400</td>
</tr>
<tr>
<td>( \mu )</td>
<td>0.3</td>
</tr>
</tbody>
</table>

The results from the two cultivations using this profile can be seen in Fig. 12 and Fig. 13. These two cultivations were selected since their optical density (OD\(_{600}\)) and GFP production values matched each other. The feed was started at 5 hours after the start of the batch in both cultivations and the induction by IPTG was launched when the OD\(_{600}\) reached a value above 20. In both cultivations the OD\(_{600}\) reached a maximum value of 50 which corresponded to a dry weight biomass concentration of about 11 g/L. The production of GFP reached a final level just below 4000 fluorescence units. These results were experienced to be the highest ones you could expect, when using an OD\(_{600}\) value of 20 as starting point for induction, when a cultivation run by the profile was successful. During the test cultivations with this profile, not all resulted in as high results. This showed that there was a considerable variability in the cultivation process even when it was run by exactly the same profile. These two experiments (Profile A1 and Profile A2) were used as benchmarks of successful runs with which the subsequent feedback controlled cultivations could be compared.
Fig. 12. Results from a cultivation using selected fixed feed profile (Profile A1).

Fig. 13. Results from a comparative cultivation using selected fixed profile (Profile A2).
5.5 Difference in organic acid production

The amount of organic acids produced during the batch phase varied between batches and this variation was also seen in the ensuing feed-phase. The concentration of the Sum Mixed Acids for the two cultivations with the fixed profile and the feed rate can be seen in Fig. 14. The two fixed-profile cultivations had completely different production of organic acids even though they were run by the same profile. Profile A1 (Fig. 14A) had very low production of organic acids during the batch-phase and thereby the concentrations of organic acids were low during the feed-phase as well, except in the later part when organic acids started to accumulate. Profile A2 (Fig.14B) had a higher initial organic acid concentration from the batch and organic acids continued to be produced in a high rate. In Profile A1 the feed rate could have been increased from the start and should have been lowered in the later part to avoid to high glucose concentration. In Profile A2 the feed rate should instead have been lowered from the start to avoid the high accumulation of organic acids which did occur. This showed how hard it is to predict the behavior of such a complex biological system and try to find the right feeding rate from calculations and models made for the system in advance.

Fig. 14. Feeding rates and mixed acids concentrations for the two fixed-profile cultivations (A) Profile A1 and (B) Profile A2. The feed rate is run according to the fixed profile. The set-point value of the mixed acid concentration in the feedback control cultivations are presented as dashed lines, however no feedback control was used in these cultivations.
5.6 Feedback controlled cultivations

During the feedback controlled cultivations, the controller regulates the feed rate as described in Section 4.4. The original rate from the profile is adjusted depending on the current concentration of the Sum Mixed Acids. The set point value for mixed acid concentration was set to 0.5 g/L and in Fig 14 it can be observed how the feed rate was adjusted for the two feedback controlled cultivations depending on whether the mixed acid concentration was above or below the set-point value. The adjustment of the profile looks different for the two experiments since the amount of organic acids produced is differed in every batch and therefore the conditions at the start of feeding were unique in each run. If a high accumulation of organic acids had started during the batch-phase, the accumulation continued at a high rate if the substrate was added in an uncontrolled manner. In Fig. 15A the concentration of organic acids were just above the set-point and could easily be kept in control by small adjustments of the feed profile. In Fig. 15B there was a higher accumulation of organic acids short after feed start and the feed rate was lowered to decrease the high accumulation of organic acids. This was successfully done as the organic acid concentrations stopped to increase and was regulated down below the set-point value in a short time. If this regulation would not have been used the accumulation of organic acids would probably continued and lowered the final results.

Fig. 15. Regulatory effect on the feeding rates in (A) Controller B1 (B) Controller B2 cultivations due to changed mixed acids concentrations. The original profile and set-point value for the controller are presented as dashed lines.
The results of the regulation using Controller B1 are shown in Fig. 16. Both results for $\text{OD}_{600}$ and GFP were slightly higher but still equal to the results for the successful cultivations in the fixed-profile experiments (Profile A1 and Profile A2). The concentrations of organic acids during this run were not very high which makes it likely to expect the same results as for a successful cultivation run by the profile. The $\text{OD}_{600}$ value reached a high peak of 53 and then dropped rapidly below 40, which could not easily be explained.

![Graph showing results from a feedback controlled cultivation experiment (Controller B1).](image)

**Fig. 16.** Results from a feedback controlled cultivation experiment (Controller B1).
In another feedback controlled cultivation (Controller B2), the accumulations of organic acids were very high at the start of the feeding-phase which probably would have resulted in lower results if the feed was added according to the profile. Instead the controller lowered the feed rate until the mixed acid concentrations were below the set-point and as seen in Fig. 17, the results were higher than the results for both cultivations using a fixed profile (experiment Profile A1 and Profile A2). Fluorescence measurements reached a value above 4500 which was distinctly higher than the fixed profile cultivations.

The objective with this regulation system is not primarily to get as high productivity or as much GFP as possible. You always strive optimal or at least good process performance but with this complex biological system it might be a priority achieve reproducible results every time. To achieve this with this kind of system it is not sufficient to perform the cultivations in exactly the same way since each cultivation performs differently due to the variability in the complex biological system. With regulation system demonstrated in this report, each cultivation is run in a unique way based on the actually conditions in the bioreactor.

This new strategy of regulating the feed rate based on the organic acid levels to get a proper glucose concentration for the cells, have with these first experiments shown to be one potent tool in reproducing the cultivations with good results. The strategy used by DeLisa et al (1999) to regulate the feed rate according to predictions of the glucose uptake rate is the same strategy used in the fixed profile cultivations experiments reported.

Fig. 17. Results from a second feedback controlled cultivation (Controller B2). (Data for biomass concentration from NIR measurements is not available).
...here (Profile A1 and Profile A2). Even if the calculations used for these fixed profile cultivations are not that precisely and accurately calculated as the one in the literature, an obvious problem is seen with the high variations between different runs. As mentioned in Section 2.1, the growth of *E. coli* is fast and rather uncomplicated but it is still a rather complex biological system. This makes it more difficult to predict and control these kinds of systems than e.g. chemical production systems with rather simple chemical reactions which have a much lower level of variation. Therefore it would be more useful to have a feeding strategy based on the current conditions of the cells and their environment in the bioreactor, as the one used in this project.

Other ways of controlling the feed rate by on-line measurements is described by Åkesson et al (2000) where the feed rate was controlled with a dissolved oxygen sensor in an attempt to keep the acetate levels low. In their experiment the DO-level was regulated by the stirrer speed and the feed was down-regulated when the stirrer speed reached its maximum. The advantage with this strategy is the quick response time. The DO level is measured every second while the organic acid concentrations used in this project are only measured every 20 minutes by the HPLC system. This makes the DO measurement a more potent tool in detecting changes in the cultivations and to be able to give a quick regulation of the feed rate. The drawback is that the DO-measurement is just an indication of the current acetate and glucose levels in the reactor and it would be more useful to measure the actual concentrations to get a more veracious view over the actual conditions. The biological system is described as more complex than other production systems and it is also much more robust and slower than other systems. It takes time for the cells to adapt to a new environment and eventually start to grow, which makes it possible to have a slower regulation system since the cells do not need the same kind of quick response regulations as many other production systems. The 20 minutes it takes for the HPLC system to get a new measurement could be rapid enough to be able to have a good control over the feed rate.

A further development with this strategy of controlling the feed rate with measurements from the HPLC system would be to use the formation rate of the organic acids instead of just the concentration. These kinds of calculations are already implemented in the BioPhantom software and the formation rates of the organic acids are available. Warth et al (2010) showed that the NIR-probe used in this project can give a good estimation of the biomass concentration and thereby the specific formation rate for the organic acids can also be calculated. With the specific formation rates available the controller could predict the accumulation of organic acids and down regulate the feed before the organic acid levels are too high. So far, the feed is down-regulated when the concentration of the organic acids already is high, when just controlling the $c_{\text{SumMixedAcid}}$ parameter. Since this is a new developed control system the first step was to see if the controller was able to regulate the feed to get reproducible results with the concentration of the organic acids as a control parameter. The next step is to continue using the $c_{\text{SumMixedAcid}}$ as a control parameter and to evaluate different kinds of settings for the controller. A further step would then be to try to use the specific formation rates as a control parameter in the future, which is a more advanced parameter with more advantages but also with more difficulties.
5.7 Reference cultivation

To investigate if the production of GFP had an effect on the growth of the cells and if there were components other than GFP who contributed to fluorescence, a cultivation with no induction by IPTG was performed. This cultivation was performed when there were troubles with the HPLC system, so data for organic acids and glucose concentrations were not available so the feed followed the fixed feed profile. The results in Fig. 18 shows that no GFP was produced and no other component had any effect on the result since the fluorescence measurement showed low values through the whole process. The OD$_{600}$ reached a value of 56 which is the best result during the whole project. Also, the dry weight measurements suggested that GFP had a negative effect on the growth of the cells since the biomass concentration reached a value of more than 17 g/L, which was distinct higher than the biomass concentration in the other cultivations. This showed that after induction the cells’ metabolism was more focused on producing GFP and less focused on their own growth.

![Fig. 18. Results for the Reference cultivation. No induction with IPTG resulting in no production of GFP. A higher value of both OD$_{600}$ and dry weight measurements was obtained in this cultivation compared with all previous cultivations with IPTG induction.](image)
If we compare the results for OD$_{600}$ and biomass dry weight for the non-induced reference cultivation with one of the other cultivations, e.g. Controller B2, it was possible to see a remarkable difference in the results. The OD$_{600}$ results did not differ so much, 56 in the Reference and 52 in the Controller B2, but the dry weight measurements were distinctly different. The Reference reached a biomass concentration of 17 g/L while the Controller B2 reached only 13 g/L. These 4 units in optical measurements should not be equal to 4 g/L of biomass. In the other cultivations the biomass concentration also reached a biomass concentration of maximum 13 g/L and also reached OD$_{600}$ values above 50. The difference is that this Reference cultivation had no induction of IPTG and as shown in Fig 17 there were no production of GFP. This suggests that GFP could have an effect on the optical density measurements. As described in Section 2.1.2 GFP has its emission peak at 509 nm and you would not expect it to interfere with the optical density measurements at 600 nm. This association has not been reported earlier and more tests need to be done before any conclusions can be made.

An observation made during the project was that in all cultivations the OD$_{600}$ value reached a maximum level in the end of the feed-phase and was much lower in the samples taken after the growth was indicated to have stopped e.g. rising pH, decreased carbon dioxide levels. The biomass was decreasing but the production of GFP continued since the fluorescence levels were higher in the last samples in the end of the cultivation. This decrease in OD$_{600}$ levels and increase in GFP contradict the thesis that the amount of GFP could have an effect on the optical density measurements. It would be interesting to investigate the effect GFP could have on the OD measurements by measuring the optical density at different wavelengths.
5. Conclusion

The objective of the current study was to develop a control system for glucose feeding to avoid formation of inhibitory by-products in an *E. coli* fed-batch cultivation expressing green fluorescent protein. The controller was successfully developed and applied in the software system and tested during fed-batch cultivations. A HPLC system was used to do on-line measurements of the current conditions in the reactor by serving data of glucose and organic acid concentrations. The concentration of the organic acids was selected as control parameter and the glucose feed rate was automatically adjusted depending on the acid concentrations in the bioreactor.

The controller managed to keep a high glucose uptake rate for the cells as it constantly kept the inhibitory organic acid levels at a low concentration. This shows the potential this kind of on-line control of the feed rate has, to create a more secure reproducibility and to generate high product yields the cultivation.

With further development, this kind of control system could be very useful in large scale production of recombinant proteins. A more cost efficient soft sensor than the relatively costly HPLC system would be preferred, but the cost for the HPLC system is a low cost in order to secure high product yields in large scale productions.
6. Acknowledgements

First I would like to thank Professor Carl-Fredrik Mandenius for giving me the opportunity to perform my thesis at the department of Biotechnology at Linköping University. I am grateful for his big commitment to my work and all the help he gave me during the whole project.

A big thank you to Maria Carlsson for her help with all the laboratory parts of the project and for the friendly reception when I started to work here.

I also want to thank Sara Sjölund for being my opponent and to György Rajkai from Belach Biotechnology for the programming and implementation of all our new updates in the BioPhantom software.

My acknowledgements also go to Benedikt Warth, whose previous work gave a big contribution to my project.
7. References


Figures

Fig. 1: http://www.sciencedaily.com/releases/2006/11/061110092234.htm; Retrieved on 130911

Fig. 2: http://www.tsienlab.ucsd.edu/Images.htm; Retrieved on 300911

Fig.3: http://www.biochem.arizona.edu/classes/bioc471/pages/Lecture4/Lecture4.html; Retrieved on 130911